CURRENT STATUS OF PORCINE EMBRYO *IN VITRO* PRODUCTION

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ABSTRACT

Systems for the in vitro production (IVP) of porcine embryos have advanced to the stage where large numbers of embryos can be reliably generated from the oocytes of abattoir-sourced ovaries in a single production run. These systems are extremely useful for studying various aspects of porcine gamete and embryo biology, developing associated reproductive technologies, such as embryo cryopreservation and somatic cell nuclear transfer (SCNT), and generating pigs that have been genetically engineered (GE) for agricultural or biomedical research purposes. However, while the efficiencies of these systems are generally considered to be acceptable, a number of issues remain. Following oocyte in vitro maturation, polyspermic fertilization continues to be a problem and the viability of embryos is poor compared with in vivo derived embryos. This is especially the case for oocytes recovered from small antral follicles. The molecular determinants of oocyte quality are still poorly understood, although studies examining the differences between oocytes from small and large follicles, and their corresponding follicular environments, are revealing interesting insights into the acquisition of oocyte quality. Also, despite recent advances in the in vitro culture of pig embryos, the conditions used are still considered to be sub-optimal. The use of completely defined media that support the development of viable embryos has been critical to further improvements in this area. However, the effectiveness of culture medium refinements may differ with embryo type (i.e. IVP embryo, parthenote, SCNT embryo), complicating the findings of such studies. The detailed annotation of the pig genome heralds the next era of GE pig production. In addition, new gene editing tools, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, have facilitated precise manipulation of the pig genome. The need for high quality porcine embryos for the generation of GE pigs will continue to drive improvements in the efficiency of in vitro production systems, as well as the procedures used to transfer embryos into recipient sows. Coupled with the improvements in embryo cryopreservation, the in vitro production of porcine embryos for breeding purposes may become commercially feasible in the not too distant future.

Keywords**:** Sperm, Seminal Plasma, Cryopreservation, Pig

INTRODUCTION

Porcine embryo in vitro production (IVP) is an integral technology of the systems used to produce genetically engineered (GE) pigs for agricultural and biomedical research purposes. This technology is also widely used by researchers to study various aspects of gamete and embryo physiology and by biotechnologists to develop associated techniques and manipulations. 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 GE pigs have now been generated for xenotransplantation studies and as models of human disease. The use of GE pigs that benefit animal production also holds great promise; however, these benefits are yet to be realized. 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.

Currently, large numbers of porcine embryos can be reliably generated from the oocytes of abattoir-sourced ovaries

in a single in vitro production run. While the efficiencies of the procedures used to mature and fertilize oocytes in vitro, and the in vitro conditions used to culture the resulting embryos, are generally considered to be acceptable, there are still a number of problems that need to be addressed. Polyspermic fertilization continues to be a major issue, and the embryo culture conditions remain sub-optimal. Studies that attempt to overcome the polyspermy problem and increase our understanding of the requirements of the developing embryo continue. Numerous studies have revealed that the developmental competence of oocytes recovered from small antral follicles is inferior to those recovered from large antral follicles. Likewise, the developmental potential of oocytes from prepubertal gilts has been shown to be lower than that of oocytes from adult sows. Comparative analyses of these different oocyte populations have provided useful insights into the acquisition of oocyte quality, but this process is still poorly understood. The advances and knowledge gained from recent studies in these areas are discussed in this review.

OOCYTE IN VITRO MATURATION

Oocyte quality

It is clear that the quality of the immature oocytes selected for in vitro maturation (IVM) is associated with the follicular environment from which they are recovered. Consistent with studies in other species, porcine oocyte quality increases with increasing antral follicle size (Bagg *et al.* 2007, Marchal *et al.* 2002) and oocytes from adults are superior in quality compared with those from prepubertal animals (Bagg *et al.* 2007). Prepubertal porcine oocytes isolated from antral follicles 3, 4 and 5-8 mm in diameter formed blastocysts in vitro at rates of 17%, 36% and 55% respectively (Bagg *et al.* 2007). Recently, Bertoldo *et al.* (2010) demonstrated that porcine oocyte quality is also affected by season. The ability of oocytes from large follicles to form blastocysts in vitro was significantly lower in summer (i.e. during the period of seasonal infertility) than in winter (21% vs 55%) (Bertoldo *et al.* 2010). As pigs are generally slaughtered at 6-7 months of age to meet market demands, the vast majority of abattoir-sourced ovaries are from prepubertal gilts. Furthermore, prepubertal ovaries predominantly contain small (3 mm) follicles and have very few large (5-8 mm) follicles. Therefore, the supply of oocytes that are of intrinsically good quality is very limited.

One of the first cytoplasmic indicators of oocyte quality was the intra-oocyte concentration of glutathione (GSH). Maturing oocytes in the presence of cysteine, a precursor of GSH, increased the concentration of GSH in IVM oocytes to the same level as that in in vivo-derived oocytes and increased the rate of male pronuclear formation following in vitro fertilization (IVF) (Yoshida *et al.* 1993). Furthermore, supplementation of IVM medium with cysteamine, which is a potent antioxidant that reduces cystine to cysteine, increased the proportion of monospermic oocytes undergoing synchronous pronuclear formation 4.5-fold, and increased the percentage of cleaving embryos developing to the blastocyst stage (Grupen *et al.* 1995). Hence, the addition of cysteamine and similarly acting thiols to IVM medium is now common place.

Recent markers of oocyte quality

The pig is a particularly useful model species in which to study the acquisition of oocyte developmental competence because of the clear difference in oocyte quality between small and large follicles. The concentration of cAMP, a key regulator of meiotic progression, was found to be three times greater in oocytes from large follicles, compared with oocytes from small follicles, at 11 h of IVM (Bagg *et al.* 2009). Concomitantly, cumulus cell expansion and the disruption of gap junctional communication were reduced in cumulus-oocyte complexes (COCs) from small follicles, compared with COCs from large follicles (Bagg *et al.* 2009). Supplementation of maturation medium with dibutyryl cAMP, a membrane permeable analogue of cAMP, increased the concentration of cAMP in oocytes from small follicles (Bagg *et al.* 2009) and enhanced their capacity to form blastocysts (Bagg *et al.* 2007). Therefore, a key determinant of oocyte quality appears to be the ability of the oocyte to accumulate cAMP during the initial period of IVM.

Recent analyses of mRNA transcripts and proteins in porcine oocytes have revealed a number of changes as the size of the follicle increases, identifying some molecular markers of oocyte quality. A number of genes that encode glycoproteins involved in conferring the oocyte with fertilizing ability, including pZP1, pZP3 and integrins beta 1 and beta 2, were found to be expressed at greater levels with increasing follicle size (Antosik *et al.* 2009). The same researchers showed that expression of the inhibin beta-a gene, which has been proposed to have a role in oogenesis,

was greater in oocytes from large follicles than in oocytes from small follicles, both before and after IVM (Kempisty *et al.* 2012). Also, Kohata *et al.* (2013) found that the transcript level of the Moloney sarcoma oncogene, the product of which is involved in regulating meiotic arrest at metaphase II, was greater in immature oocytes from large (3-6 mm) follicles, than in immature oocytes from small (<2 mm) follicles (Kohata *et al.* 2013). Corresponding analyses showed that the level of pZP3 protein differed between oocytes from small and large follicles (Antosik *et al.* 2009), while the distribution, but not the level, of inhibin beta-a protein differed in oocytes due to follicle size (Kempisty *et al.* 2013). Microarray transcriptomic analysis of porcine oocytes derived from prepubertal and cyclic females has revealed additional insights into the acquisition of oocyte quality. In oocytes of cyclic females, the abundance of transcripts were of genes involved in metabolism and the regulation of biological processes, whereas in oocytes of prepubertal females, the expression of genes involved in translation was elevated (Paczkowski *et al.* 2011).

Information about the metabolic processes occurring in small and large follicles of oestrous sows was recently obtained by analyzing the composition of collected follicular fluid. This metabolomic analysis, which used high resolution proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, found that the concentrations of numerous metabolites, predominantly organic acids and amino acids, differed significantly between the follicular fluids from small and large follicles (Bertoldo *et al.* 2013). The majority of the metabolites that changed in concentration were linked to the tricarboxylic acid (TCA) cycle, and, consistent with findings in other species, there appeared to be a distinct shift in follicular glucose metabolism as the diameter of the follicle increased (Bertoldo *et al.* 2013). Additionally, strong correlations with the concentrations of steroids present in follicular fluid were found for nearly all of the metabolites shown to differ in concentration due to follicle size (Bertoldo *et al.* 2013). This was not surprising, as the concentrations of progesterone, oestradiol and androstenedione in porcine follicular fluid were previously shown to increase markedly with increasing follicle size (Bertoldo *et al.* 2011). Further studies are needed to clarify whether the changes in follicular steroid levels alter the metabolism of follicular cells, or whether the changing metabolic environment alters the steroidogenic activity of follicular cells.

Improving the in vitro maturation conditions

One of the features of porcine oocyte IVM systems has been the inclusion of follicular fluid (FF) to improve oocyte quality. A number of early studies demonstrated the benefits of supplementing the medium with FF to oocyte nuclear and cytoplasmic maturation, indicating a requirement for factors present in FF. Recent evidence shows that the requirement for these factors is temporal. Exposing oocytes to FF during the first half of IVM enhanced their capacity to complete nuclear maturation, while exposing oocytes to FF during the second half of IVM enhanced their capacity to form blastocysts after IVF (Grupen and Armstrong 2010). The later exposure to FF was also of great benefit to cumulus cell viability and function (Grupen and Armstrong 2010), suggesting that the protective effects of FF are particularly important at this stage. Tatemoto *et al.* (2004) also found that porcine oocyte developmental competence was enhanced concomitantly with reductions in cumulus cell apoptosis and proposed a role for the radical scavenging activity of superoxide dismutase isoenzymes in FF (Tatemoto *et al.* 2004). Furthermore, oocytes exposed to greater oxidative stress had reduced intra-oocyte levels of GSH, and a decreased capacity to form male pronuclei (Tatemoto *et al.* 2004). Clearly it is essential that IVM systems provide the oocytes with adequate protection against oxidative stress.

Over the years, numerous studies have attempted to improve the quality of IVM oocytes by supplementing the maturation medium with growth factors. Of the factors studied, epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) have consistently provided some benefit to oocyte nuclear and cytoplasmic maturation, even in the presence of FF (Abeydeera *et al.* 1998, Grupen *et al.* 1997, Illera *et al.* 1998). Oberlender *et al.* (2013) showed that the addition of IGF-I to IVM medium supplemented with FF from small follicles improved oocyte maturation to the same extent as when IVM medium was supplemented with FF from large follicles alone, implicating a role for IGF-I during follicle development (Oberlender *et al.* 2013). Interestingly, Mao *et al.* (2012) showed that the EGF-like growth factor, neuregulin, stimulated mitochondrial replication in porcine oocytes during IVM, whereas FF at doses typically used in IVM media inhibited mitochondrial replication (Mao *et al.* 2012). Furthermore, the increase in mitochondrial DNA copy number was positively correlated with the developmental potential of oocytes (Mao *et al.* 2012). In the case of EGF and EGF-like ligands, it is clear that their stimulatory effects are mediated by the FSH-enhanced ability of the cumulus cells to activate the EGF receptor (EGFR) signalling pathway (Prochazka *et al.* 2012, Prochazka *et al.* 2011, Yamashita *et al.* 2011). Recent studies have shed light on the mechanisms by which the EGFR signalling pathway is activated; however, a detailed discussion of these findings is beyond the scope of this review.

Unfortunately, the supplementation of IVM media with FF, which has an undefined composition that varies between prepared batches, may give rise to inconsistent results between studies. Additionally, groups working in this area use a variety of IVM base media, including Medium 199, North Carolina State University (NCSU)-23 medium, and NCSU-37 medium, further complicating the interpretation of results from different studies. Hence, the development of Porcine Oocyte Medium (POM), a chemically-defined maturation medium that enables porcine oocytes to acquire full developmental competence, has been an important advance (Yoshioka *et al.* 2008). Not only does the use of a chemically-defined medium improve the reliability of the IVM system and the reproducibility of results, it also eliminates concerns over the potential for contamination by pathogens in FF. Using POM, which contains the macromolecule polyvinyl alcohol (PVA) and a total of 22 amino acids, in a gonadotrophin-free step-wise IVM system that incorporated dibutyryl cAMP and the EGF-family peptides, amphiregulin and betacellulin, Akaki *et al.* (2009) successfully produced piglets from IVM oocytes (Akaki *et al.* 2009). The efficacy of using POM to assess the effects of growth factors on oocyte maturation has been demonstrated in investigations of transforming growth factor-α (TGF-α) and leukemia inhibitory factor (LIF). In the presence of gonadotrophins, both TGF-α and LIF significantly enhanced nuclear maturation (Dang-Nguyen *et al.* 2014, Mito *et al.* 2013), and improved cytoplasmic maturation, as measured by the ability of fertilized oocytes to form blastocysts (Dang-Nguyen *et al.* 2014, Mito *et al.* 2009).

The oocyte secreted factors, bone morphogenetic factor-15 (BMP-15) and growth differentiation factor-9 (GDF-9), have been found to be pivotal to follicular growth in mice and sheep (Galloway *et al.* 2000, Yan *et al.* 2001), and cumulus cell function in a number of species, including pigs (Lin *et al.* 2014). Future studies into the effects of BMP-15 and GDF-9 on porcine oocyte maturation will undoubtedly benefit through the use of a chemically-defined medium.

IN VITRO FERTILIZATION

A major inefficiency of porcine embryo in vitro production systems is the decreased capacity of IVM oocytes to effectively induce the “block to polyspermy”, a key event occurring upon initial gamete fusion that ensures normal monospermic fertilization is achieved. The ability of polyploid zygotes to form blastocysts at a rate similar to that of diploid zygotes further compounds the polyspermy problem (Han *et al.* 1999). Surprisingly, polyploid zygotes may form diploid embryos that are able to develop to term after transfer to recipient females, but the vast majority of polyploid zygotes form aneuploid embryos that are non-viable (Han *et al.* 1999). Therefore, blastocyst formation rate is an inaccurate measure of viable embryo IVP efficiency. To determine which IVP zygotes have been fertilized normally, their pronuclei must be visualized microscopically. This can be performed without the use of damaging stains by centrifuging the zygotes to polarize the abundant cytoplasmic lipid droplets (Gil *et al.* 2013, Han *et al.* 1999, Somfai *et al.* 2008). Fortunately, centrifuging the zygotes does not appear to exert any detrimental effects on their subsequent development (Gil *et al.* 2013).

At the in vivo site of fertilization, the dynamic and complex environment of the oviduct interacts with the sperm to control their function and transit to the oocyte. In contrast, IVF conditions are static and relatively simple and oocytes are typically co-incubated with a vast excess of sperm. The incidence of polyspermy using traditional porcine IVF systems is closely associated with the number of sperm per oocyte (Rath 1992) and the duration of gamete co-incubation (Funahashi *et al.* 2000). However, simply reducing the number of sperm co-incubated with the oocytes is not an effective approach to reduce the incidence of polyspermy, because this also decreases the proportion of oocytes that are penetrated.

Strategies used to reduce the incidence of polyspermy

Numerous studies have attempted to reduce the incidence of polyspermy by mimicking the oviductal environment more closely in vitro. Exposing the gametes, before or during IVF, to undefined oviductal components, including follicular fluid (Funahashi and Day 1993), oviductal fluid (Kim *et al.* 1997) and oviductal cells (Nagai and Moor 1990), has been found to improve the rate of monospermic fertilization to various, but still limited degrees. Supplementing IVF media with isolated oviductal factors, such as oviduct-specific glycoproteins (Kouba *et al.* 2000), hyaluronan (Suzuki *et al.* 2000) and osteopontin (Hao *et al.* 2006), has also been shown to reduce sperm penetration. The findings of Coy *et al.* (2008a) indicate that exposure to oviductal fluid makes the zona pellucida (ZP) resistant to sperm penetration, first through the binding of an oviduct-specific glycoprotein to ZP glycoproteins,

then through the stabilization of this ZP complex by the binding of glycosaminoglycans, such as heparin (Coy *et al.* 2008a). A comparison of oviductal fluid fractions that had different ZP hardening capacities revealed that oviductal glycoprotein 1 (OVGP1), and members of the heat shock protein (HSP) and protein disulfide isomerase (PDI) families may be involved in this process (Mondejar *et al.* 2013).

Pre-fertilization hardening of the ZP, as indicated by an increased resistance of the ZP to proteolytic digestion, is one approach that has been used to decrease sperm penetration in porcine oocytes. Treatment of oocytes prior to IVF with di-(N-succinimidyl)-3,30-dithiodipropionate (DSP), an amine-reactive agent that cross-links proteins, effectively increased the incidence of monospermic fertilization (Coy *et al.* 2008b) and improved the efficiency of embryo production (Canovas *et al.* 2009). The results of studies by Lay and colleagues (2011a, 2011b) indicate that acidification, by sialylation and sulfation, and N-glycosylation of ZP glycoproteins do not contribute to ZP hardening during oocyte maturation and enhance the capacity of oocytes to bind sperm (Lay *et al.* 2011a, Lay *et al.* 2011b).

Another strategy used to reduce the incidence of polyspermic fertilization involves restricting the number of sperm reaching the oocytes within the insemination dish. Various systems have been described, including the climbing-over-a-wall (COW) method (Funahashi and Nagai 2000), biomimetric microchannel IVF system (Clark *et al.* 2005), straw IVF (Li *et al.* 2003), modified swim-up method (Park *et al.* 2009) and microfluidic sperm sorter (Sano *et al.* 2010), which all present some sort of obstacle to the sperm, thereby ensuring that only highly motile sperm are able to interact with the oocytes. Most of these systems require specialized insemination dishes or additional sperm handling and preparation compared with traditional IVF systems, but they still do not avert the polyspermy problem completely.

The use of a simple 2-step IVF procedure, in which oocytes are briefly co-incubated with sperm before transfer to fresh insemination droplets, also reduces the number of sperm interacting with the oocytes. Several studies have shown that the sperm that bind to the ZP within 10 minutes of co-incubation effectively penetrate a high proportion of oocytes (Alminana *et al.* 2008b, Gil *et al.* 2004, Grupen and Nottle 2000). In fact, sperm penetration and the development of the resulting embryos were found to be improved by using a 2-step IVF method (Grupen and Nottle 2000). This improvement was attributed to the removal of the oocytes and bound sperm from excess sperm, which may exert a detrimental effect on the environment if they undergo cell death. The presence of caffeine during the brief co-incubation period, followed by an absence of caffeine in the subsequent insemination period, also reduced the rate of polyspermic fertilization (Funahashi and Romar 2004). Other sperm capacitating agents may be superior to caffeine, as adenosine and fertilization-promoting peptide, were found to stimulate the fertilizing capacity of boar sperm without significantly increasing the incidence of polyspermic fertilization (Funahashi *et al.* 2000). Unfortunately, the effectiveness of the 2-step IVF procedure is still dependent on the number of sperm per oocyte in the initial co-incubation droplets (Gil *et al.* 2007). The strategy of combining methods that limit sperm-oocyte interaction has some merit, because Alminana *et al.* (2008a) demonstrated that using a straw IVF system together with a short gamete co-incubation period improved the efficiency of fertilization and the quality of the blastocysts produced (Alminana *et al.* 2008a).

Identification of the proteins involved in the sperm-oocyte interaction may provide insights that lead to the development of new strategies to overcome the polyspermy problem. Pre-incubation of porcine oocytes with recombinant deleted in malignant brain tumor 1 (DMBT1), which is involved in sperm selection in the oviduct (Teijeiro *et al.* 2008), induced an increase in monospermic fertilization (Ambruosi *et al.* 2013). Having shown that DMBT1 is localized to the ZP and ooplasm, Ambruosi *et al.* (2013) used surface plasmon resonance studies to demonstrate that DMBT1 interacts with boar sperm. Recently, a member of the folate-receptor family, Folr4, which spans the oocyte plasma membrane, was found to be the sole receptor for the protein Izumo1, which is tethered to the membrane of sperm (Bianchi *et al.* 2014). When Folr4, also referred to as Juno, was blocked using an antibody, fertilization was prevented in vitro in mice. Furthermore, when fertilization was allowed to proceed, Juno was lost from the oocyte plasma membrane 30 to 40 minutes after sperm-oocyte fusion, suggesting this receptor protein plays a role in the block to polyspermy (Bianchi *et al.* 2014).

EMBRYO CULTURE

During the establishment of porcine embryo culture systems, a number of media formulations, including Whitten's medium (Menino and Wright 1982), modified Kreb's Ringer bicarbonate (mKRB) medium (Krisher *et al.* 1989), NCSU-23 medium (Petters and Wells 1993) and Beltsville Embryo Culture Medium (BECM)-3 (Dobrinsky *et al.* 1996), were found to successfully support development to the blastocyst stage. All of these media needed to be supplemented with a protein source that was either undefined or semi-defined in order to be effective. Early studies that compared the capacities of different media to support the formation of blastocysts consistently showed that NCSU-23 medium, which contains 4 mg/ml bovine serum albumin (BSA), was superior (Long *et al.* 1999, Petters and Wells 1993). On the basis of these findings, many research groups adopted the use of NCSU-23 medium for porcine embryo culture, and numerous studies that assessed refinements to this medium ensued.

However, the creation of a chemically-defined culture medium, called Porcine Zygote Medium (PZM), that sustains the development of high quality porcine embryos, has been essential to better understand the efficacy of media modifications. The composition of PZM is based on the concentrations of inorganic elements and energy substrates found in the porcine oviduct (Iritani *et al.* 1974, Nichol *et al.* 1992). A number of studies have now shown that in vivo-derived zygotes and IVP embryos cultured in PZM-4 and PZM-5 for 5 days have the capacity to develop to term after transfer to recipient females (Yoshioka *et al.* 2012, Yoshioka *et al.* 2003, Yoshioka *et al.* 2002). Also, the superiority of PZM over other embryo culture media, including NCSU-based media, has been confirmed repeatedly using IVP, parthenogenetic and SCNT porcine embryos (Im *et al.* 2004, Nanassy *et al.* 2008, Wang *et al.* 2009, Yoshioka *et al.* 2002).

Modifications to carbohydrate composition

Analysis of IVP porcine embryo metabolism has revealed that glucose utilization increases from the one-cell to the blastocyst stage (Gandhi *et al.* 2001). This change in the requirement for glucose is similar to that observed in the embryos of other species, and reflects the changing peri-ovulatory glucose concentration in porcine oviductal fluid (Nichol *et al.* 1992, Nichol *et al.* 1998). The results of numerous studies in other species consistently showed that pyruvate and lactate were the preferred carbohydrates at the early cleavage stages. The fact that NCSU-based media contain high levels of glucose, and no pyruvate or lactate, suggested that porcine embryos do not require pyruvate and lactate. However, culture of porcine embryos in NCSU-37 medium, first lacking glucose and containing low concentrations of pyruvate (0.17 mM) and lactate (2.73 mM) for 48 h, and then containing a high concentration of glucose (5.55 mM), significantly improved blastocyst quality compared with the high glucose only group (Kikuchi *et al.* 2002). A similar alteration to the energy substrate concentrations of NCSU-23 medium was also found to be beneficial to the development of parthenogenetic porcine embryos (Beebe *et al.* 2007). Interestingly, PZM-3 contains pyruvate and lactate, but no glucose. Yoshioka et al (2002) proposed that the pyruvate:lactate ratio in PZM-3 provides a more suitable condition for cellular oxidation-reduction equilibrium than glucose-containing NCSU-23 medium. However, the addition of glucose to PZM-5 from days 5 to 7 of culture was shown to be beneficial to blastocyst development, demonstrating that glucose is an important energy substrate at the later stages of preimplantation embryo development in vitro (Mito *et al.* 2012).

Modifications to amino acid composition

Oviductal and uterine fluids contain significant amounts of free amino acids, and embryo culture studies in numerous species have demonstrated beneficial effects of amino acid supplementation on embryo development (Gardner 1998). The superiority of PZM over NCSU-23 medium may be attributed to the presence of essential and non-essential amino acid mixtures in PZM. However, the addition of commercial solutions of amino acids to NCSU-23 has had varying effects on embryo development, with high concentrations being detrimental (Long *et al.* 1999) and low concentrations being marginally beneficial (Beebe *et al.* 2009). The effects of premixed solutions of amino acids on blastocyst formation rates were also found to be concentration dependent in PZM (Suzuki and Yoshioka 2006). As amino acids are known to degrade during culture to form ammonia, the beneficial effects of amino acids may be abolished at higher concentrations due to ammonia buildup. Kim *et al.* (2013) showed that replacing glutamine, one of the more potent amino acids, with alanine-glutamine (GlutaMAX), a dipeptide that does not spontaneously degrade, significantly increased blastocyst formation rates and blastocyst quality, presumably by reducing the buildup of ammonia during in vitro culture (IVC) (Kim *et al.* 2013). A study that examined the effect of adding glycine, which is present at high concentrations in porcine uterine fluid 3-5 days after oestrus (Li *et al.* 2007), from days 5 to 7 of culture, improved the total cell number of blastocysts and the blastocyst hatching rate

(Mito *et al.* 2012). The beneficial effect of glycine was only observed in the presence of glucose (also only added from days 5 to 7 of culture), suggesting that these substrates act synergistically to better meet the metabolic needs of blastocyst stage embryos (Mito *et al.* 2012).

Media supplements

An extensive array of culture medium supplements has been tested for their ability to improve the development of IVP, parthenogenetic and SCNT porcine embryos. A comprehensive discussion of how the various additives are understood to act is beyond the scope of this review, but the majority of the supplements can generally be classified as antioxidants that provide cellular protection against oxidative damage, or growth factors and cytokines that stimulate the proliferation of embryonic cells. Agents with antioxidant properties that have been added to culture medium and found to enhance porcine embryo development include L-ascorbic acid (Hu *et al.* 2012, Huang *et al.* 2011, Kere *et al.* 2013), α-tocopherol (Jeong *et al.* 2006), anthocyanin (You *et al.* 2010), 3-hydroxyflavone (Uhm *et al.* 2011) and selenomethionine (Tareq *et al.* 2012). By reducing the levels of reactive oxygen species (ROS) in embryonic cells, the intracellular level of glutathione is increased and the proportion of cells that become apoptotic is decreased, thereby improving the formation and quality of blastocysts. Growth factors and cytokines that have been added to culture medium and found to enhance porcine embryo development include epidermal growth factor (EGF) (Lee *et al.* 2005), insulin-like growth factor-I (IGF-I) (Kim *et al.* 2006), sonic hedgehog (SHH) (Nguyen *et al.* 2011) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Kwak *et al.* 2012, Lee *et al.* 2013). The observed increases in the blastocyst formation rates and/or the total number of blastocyst cells may be attributed to the growth-promoting actions of these factors; however, IGF-I, SHH and GM-CSF also appear to act as survival factors (Kim *et al.* 2006, Lee *et al.* 2013, Nguyen *et al.* 2011). A number of these media supplements were also reported to alter the expression of various genes in porcine embryos (L-ascorbic acid: *Nanog*, *Oct4*, *Sox2* and *Klf4*; IGF-I: *Bcl-2* and *Bax*; SHH: *ZPF42* (*REX01*); GM-CSF: *POU5F1*, *Cdx2*, *Bcl-2* and *PCNA*) (Hu *et al.* 2012, Huang *et al.* 2011, Kim *et al.* 2006, Kwak *et al.* 2012, Nguyen *et al.* 2011).

CONCLUSIONS

Porcine embryo in vitro production systems have advanced to the point where large numbers of embryos can be reliably generated from the oocytes of abattoir-sourced ovaries in a single production run. Additionally, following transfer of embryos to recipient females, it is now possible to routinely obtain piglets derived from embryos that have been generated using chemically-defined media. The quality of the embryos produced is largely reliant on the quality of the immature oocytes selected for IVM. Collection of ovaries from sexually mature females, recovery of immature oocytes from large antral follicles (>5 mm in diameter), and stringent selection of the recovered oocytes based on morphological criteria, maximizes the efficiency of porcine embryo IVP systems. However, pigs are normally slaughtered before attaining sexual maturity and the majority of follicles on the ovaries of prepubertal gilts are small (3-4 mm in diameter). Therefore, improving the quality of porcine IVM oocytes, especially those from small follicles, will remain a focus of research. Polyspermic fertilization continues to be a problem following IVF of porcine IVM oocytes despite some recent minor improvements. The identification of proteins involved in the sperm-oocyte interaction, such as Juno and Izumo1, offers new approaches to increase the efficiency of porcine IVF. Finally, numerous refinements have been made to culture media, but the development of porcine embryos in vitro is still poor compared with that in vivo. Transcriptomic, proteomic and metabolomic analyses are providing insights into the cellular and molecular processes involved in the acquisition of oocyte quality and the requirements of the developing embryo, which will undoubtedly lead to future improvements to the efficiencies of porcine embryo IVP systems.

Porcine embryo in vitro production is a critical technology for the generation of genetically engineered pigs. The list of GE pigs that have been produced for enhancing the efficiency of animal production and for the study of human diseases and xenotransplantation is growing rapidly. Alzheimer's disease, atherosclerosis, retinitis pigmentosa, osteoporosis, breast cancer, diabetes and cystic fibrosis are among the diseases for which transgenic pig models have now been produced (Luo *et al.* 2012). The detailed annotation of the swine genome (Groenen *et al.* 2012) has opened up new possibilities and potential uses for GE pigs. In addition, new gene editing tools, such as zinc finger nucleases (ZFNs) (Hauschild *et al.* 2011), transcription activator-like effector nucleases (TALENs) (Carlson *et al.* 2012) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas (Cong *et al.* 2013), have facilitated precise manipulation of the pig genome. Researchers at the Roslin Institute recently announced the birth

of “Pig 26”, a male pig in which a single base pair deletion was created using the CRISPR/Cas system. The modification, which was performed on fertilized oocytes, confers immunity to African swine fever. Generating GE pigs by this method, rather than by SCNT, promises to revolutionize the field, but clearly still needs good quality porcine embryos to be produced. Advances in associated techniques, specifically embryo cryopreservation and non-surgical embryo transfer, are also still needed to allow piglets derived from IVP embryos to be obtained more readily. Such developments, together with further improvements in the efficiencies of oocyte IVM, IVF and embryo IVC, may soon make the use of IVP embryos a commercially viable option in pig breeding programs.

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