



Review

Challenges and Considerations during In Vitro Production of Porcine Embryos

Paula R. Chen ¹, Bethany K. Redel ², Karl C. Kerns ³, Lee D. Spate ^{1,4} and Randall S. Prather ^{1,4,*}

¹ Division of Animal Sciences, University of Missouri, Columbia, MO 65211, USA

² USDA-ARS, Plant Genetics Research Unit, Columbia, MO 65211, USA

³ Department of Animal Science, Iowa State University, Ames, IA 50011, USA

⁴ National Swine Resource and Research Center, University of Missouri, Columbia, MO 65211, USA

* Correspondence: PratherR@missouri.edu

Abstract: Genetically modified pigs have become valuable tools for generating advances in animal agriculture and human medicine. Importantly, in vitro production and manipulation of embryos is an essential step in the process of creating porcine models. As the in vitro environment is still suboptimal, it is imperative to examine the porcine embryo culture system from several angles to identify methods for improvement. Understanding metabolic characteristics of porcine embryos and considering comparisons with other mammalian species is useful for optimizing culture media formulations. Furthermore, stressors arising from the environment and maternal or paternal factors must be taken into consideration to produce healthy embryos in vitro. In this review, we progress stepwise through in vitro oocyte maturation, fertilization, and embryo culture in pigs to assess the status of current culture systems and address points where improvements can be made.

Keywords: porcine embryo culture; in vitro maturation; in vitro fertilization



Citation: Chen, P.R.; Redel, B.K.; Kerns, K.C.; Spate, L.D.; Prather, R.S. Challenges and Considerations during In Vitro Production of Porcine Embryos. *Cells* **2021**, *10*, 2770. <https://doi.org/10.3390/cells10102770>

Academic Editor: Lon J. van Winkle

Received: 22 September 2021

Accepted: 13 October 2021

Published: 15 October 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

In vitro production of embryos has several advantages over in vivo-derived embryo production, including efficient selection of superior genetics for transfer or genetic modification to rapidly obtain animals with desirable traits. Genetic engineering provides a powerful tool to aid in understanding basic mechanisms regulating animal physiology. Several studies using genetic engineering approaches have been conducted to ascertain the function of signaling molecules and enzymes during early pregnancy in pigs [1–4]. Importantly, interleukin 1 beta 2 (IL1B2) was shown to be required for conceptus elongation to proceed [1]. For production agriculture, embryos can be modified to produce animals with better carcass traits, such as improved fatty acid profiles with increased levels of omega-3 fatty acids [5], and resistance to diseases, such as porcine reproductive and respiratory syndrome (PRRS), that result in millions of dollars of losses per year [6,7]. Moreover, genetically modified swine have become powerful tools for studying genetic diseases in humans, such as cystic fibrosis [8] and phenylketonuria [9], and for xenotransplantation [10]. Therefore, production and manipulation of porcine embryos in vitro is crucial for advancements in agriculture and human medicine.

Each step of the culture system, from oocyte maturation to embryo culture, has been continuously optimized and adjusted based on the requirements of porcine oocytes and embryos. Our current system, from collection of cumulus-oocyte complexes (COCs) to surgical transfer into surrogates, is outlined in Figure 1 with media formulations described in the subsequent sections. However, in vitro culture of porcine embryos is still suboptimal as only about 40% of presumptive fertilized oocytes develop to the blastocyst stage for embryo transfer [11], and the number of cells in the blastocyst-stage embryos is decreased compared to those that developed in vivo [12]. Clues from other species have provided insight for culture of porcine embryos, but embryos from every species exhibit unique

metabolic characteristics that need to be considered for media formulations. Even with advances in porcine embryo culture media formulations, stress from the environment and maternal or paternal factors can impact the developmental trajectory of the embryos. Thus, a significant amount of information has yet to be learned regarding the interaction of embryos with the culture environment, the ways in which the culture system can be modified to improve developmental viability, and important metabolic features that can be used to distinguish healthy embryos.

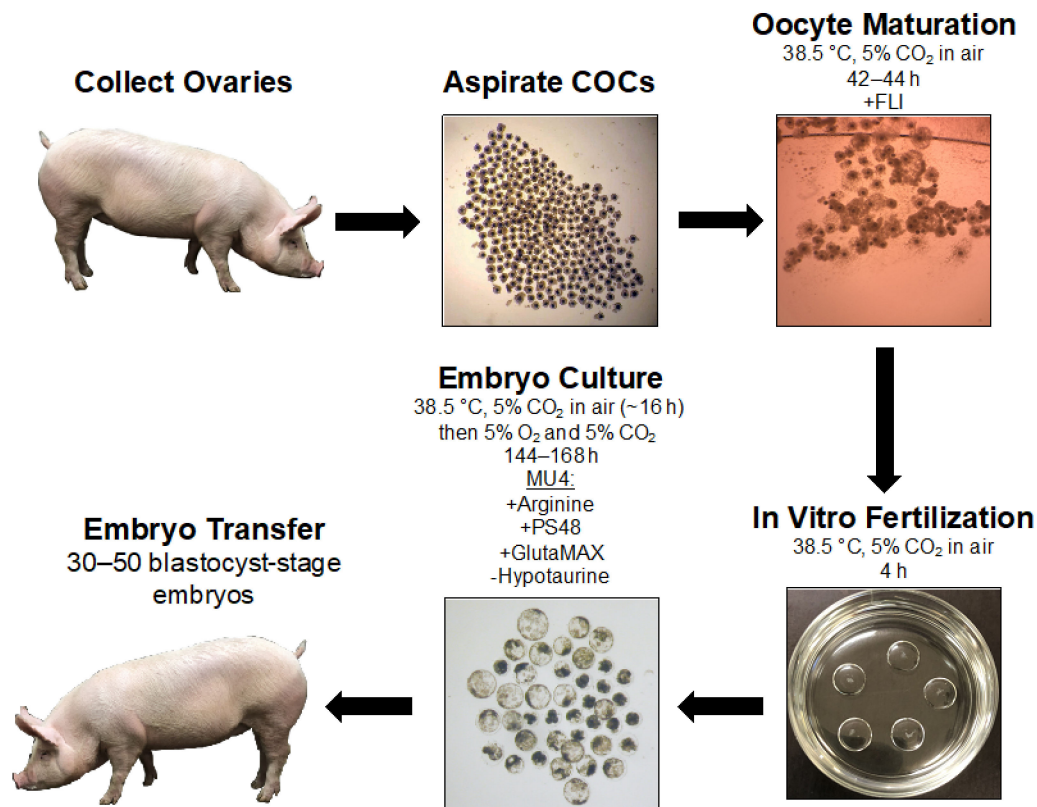


Figure 1. An overview of our current porcine embryo production pipeline. FLI: cocktail of fibroblast growth factor 2, leukemia inhibitory factor, and insulin-like growth factor 1. MU4 porcine embryo culture medium is a modified porcine zygote medium (PZM) that contains supplemental arginine, PS48, and GlutaMAX, and hypotaurine is removed from the formulation. Approximately 30–50 day 5 or 6 morula- and blastocyst-stage embryos are surgically transferred into a surrogate.

Herein, we provide an overview of the current porcine embryo culture system with a focus on the metabolic characteristics of porcine embryos. Moreover, measurements of embryo quality, stressors arising from the culture system, and parental factors that influence development are discussed.

2. Oocyte Quality and Maturation

2.1. Selection Criteria

In vitro maturation (IVM) of oocytes is the first critical step in the process of producing competent embryos that are able to produce viable offspring. While progress has been made to improve IVM of pig oocytes, the rate and the extent of maturation is considerably lower than in vivo-matured oocytes, suggesting a suboptimal maturation environment [13]. In vivo, the maternal ovarian follicle environment supplies the immature oocyte with the necessary components to develop and reach its developmental competence by maintaining an intimate relationship with its neighboring somatic cells. The bidirectional communication between the oocyte and cumulus cells allows for the oocyte to

gradually acquire the necessary molecular and cytoplasmic machinery needed to support embryo development [14,15].

Evidence suggests that the key factor in determining the proportion of oocytes that develop to the blastocyst stage appears to be the intrinsic quality of the oocyte that begins the in vitro production process [16]. Given this, the ability to select the most competent oocytes that are capable of being fertilized in vitro and produce healthy young is imperative. General selection parameters to determine oocyte competence in the pig are the age of the oocyte donor [17,18], follicle size [19], and the layers of cumulus cells and subsequent cumulus cell expansion during culture [20]. Oocytes that originate from small (<3 mm) follicles are less likely to mature to metaphase II (MII) or develop to the blastocyst stage compared to oocytes derived from medium (3–5 mm) and large (>5 mm) follicles [21]. Moreover, larger follicles (>5 mm) have been shown to have higher β -estradiol concentrations, and high quality COCs from these follicles have increased abundance of transcripts involved in ovarian steroidogenesis [22]. Historically, cumulus cell expansion has been considered to be required for successful maturation in vitro and often the degree of cumulus cell expansion is correlated with improved oocyte maturation [23,24]. As another factor to consider, oocyte quality is also impacted by the energy status of the female as feed restriction (50% per day) during the last two weeks of lactation decreased follicle size, concentration of steroids in the follicular fluid, and maturation to MII [22].

2.2. Supplementation of Growth Factors

A cocktail of growth factors, fibroblast growth factor 2 (FGF2), leukemia inhibitory factor (LIF), and insulin-like growth factor 1 (IGF1) (termed FLI) supplemented only during oocyte maturation of prepubertal gilt COCs, was found to provide a four-fold increase in the number of piglets born per oocyte collected [11]. These FLI-cultured COCs had a distinct mitogen-activated protein kinase (MAPK) activation pattern in the cumulus cells, displayed a higher degree of cumulus cell expansion, and accelerated the disruption of gap junctions compared to control COCs. However, a complementary study found that in the absence of gonadotropins but with supplementation of FLI, cumulus cells showed little expansion but maturation to MII was not impeded in oocytes from prepubertal gilts, and those oocytes were competent to produce healthy piglets [25]. While cumulus cell expansion was not needed to produce competent oocytes, cumulus cells are required as their removal at the beginning of maturation and even up to 24 h post-start of maturation impedes oocyte development to MII and the blastocyst stage after fertilization. Our knowledge and understanding about the relationship of the oocyte, cumulus cells, growth factors, and hormones are constantly evolving as a highly complex interaction is revealed. More research is needed to understand the molecular mechanisms and signaling events by which FLI can promote oocyte competence.

3. In Vitro Fertilization

Historically, the lack of fertilization of porcine oocytes in vitro [26] was followed by tremendous polyspermy [27]. The inability to achieve consistent monospermy has been a major problem for in vitro fertilization (IVF) of the pig. In the mid-1990s, Billy Day from the University of Missouri was quoted as saying, “For years, we tried to get the sperm in, and now, we can’t keep them out.” Biologically, the block to polyspermy in mammals is via two distinct mechanisms. The first is a membrane block that acts relatively quickly [28], and the second is a block at the level of the zona pellucida that takes minutes. The relative importance of these two mechanisms is different between mammalian species. In pigs, humans, and mice, both mechanisms are used. The first relatively quick membrane block appears to act by changing the binding characteristic of the plasma membrane of the oocyte, whereas the second zona pellucida block is a result of the release of cortical granule contents into the space between the oolemma and the zona pellucida. These contents (e.g., ovastacin [29], among others [30]) modify the proteins of the zona pellucida such that

sperm penetration is inhibited. Thus, these two mechanisms serve to complement each other in preventing multiple sperm from entering the oocyte cytoplasm.

In the pig, the block at the zona pellucida is relatively strong and is correlated with cortical granule release [31], while the membrane block is regulated by calreticulin [32]. Many efforts have been made to alter the fertilization conditions to improve monospermy rates. These efforts have included altering the concentration of sperm while varying the duration of exposure between the sperm and oocytes, adding follicular fluid [33] or substances found in oviductal fluid, such as deleted in malignant brain tumors 1 (DMBT1) [34], oviductal glycoprotein 1 (OVGP1) [35], secreted phosphoprotein 1 (SPP1) [36], or plasminogen [37,38], as well as trying to recreate the oviductal environment by adding oviductal fluid [39,40] or oviductal extracellular vesicles [41], among other things [42].

The problem of polyspermy encountered during IVF is generally confounded with in vitro-matured oocytes. Improvements in the quality of the oocytes will likely improve the success of monospermic fertilization. As in vitro-matured oocytes have a propensity to retain their transzonal projections, it was thought that these projections may interfere with sperm-oocyte binding and cortical granule exocytosis [43]. Retraction of those projections at the end of oocyte maturation may permit more timely and complete cortical granule exocytosis (the slower block to polyspermy), as well as altering the composition of the plasma membrane to provide a better fast block to polyspermy at the level of the plasma membrane. The aforementioned maturation system containing FLI has dramatically altered the timing of the retraction of the transzonal projections and improved the overall developmental competence of the oocytes [11]. As compared to other reports in the literature, polyspermy is less of a problem in our hands. A fertilization rate of 50–60% with monospermy of 70–80% is routinely achieved. Thus, on a per oocyte basis, the overall success is 35–50% monospermy. This range of monospermy corresponds well with our rates of development to the blastocyst stage (30–60%).

Another alternative to counteract polyspermy is to perform intracytoplasmic sperm injection (ICSI), which has led to the production of live piglets [44,45]. However, delays in cleavage divisions and decreased blastocyst formation of approximately 10–20% have been observed in ICSI-derived porcine embryos compared to those produced by IVF [46]. These decreases in development may be due to selection of damaged sperm and/or incomplete oocyte activation. Importantly, boar sperm selected by a Percoll gradient were shown to have higher abundance of phospholipase C- ζ (PLC ζ), a well-studied sperm-borne oocyte activation factor, and the use of Percoll-selected sperm for ICSI increased monospermic fertilization and oocyte activation as well as development to the blastocyst stage [47]. Several protocols use polyvinylpyrrolidone (PVP) for sperm immobilization before ICSI; however, this compound is toxic when injected into oocytes. When boar sperm were selected by hyaluronic acid (HA), known as physiological intracytoplasmic sperm injection (PICSI), versus PVP (conventional ICSI) or IVF, increases in development to the blastocyst stage and total cell numbers were observed [48].

4. Comparing Metabolic Characteristics of Mammalian Embryos

4.1. Carbohydrates

The metabolism of preimplantation embryos differs drastically from that of adult somatic cells. In normal conditions, somatic cells metabolize glucose through glycolysis, yielding two ATP, two NADH, and two pyruvate molecules. Pyruvate is further metabolized through the TCA cycle to produce electron donors, NADH and FADH₂, for generation of 30–36 ATP molecules by oxidative phosphorylation. In the precompaction embryo, ATP is generated by oxidative phosphorylation at a reduced but optimized level, which is sustained through oxidation of pyruvate, amino acids, and fatty acids [49]. A low level of pyruvate oxidation is deemed ideal to avoid excess production of reactive oxygen species (ROS) by the electron transport chain. After compaction, the primary metabolic pathway is glycolysis that is characterized by the Warburg effect (WE), which is a phenomenon first observed in cancer cells by Otto Warburg [50,51]. Under the WE, glycolytic intermediates

are shuttled towards the pentose phosphate pathway (PPP) and lactate production instead of the TCA cycle even in the presence of oxygen. The PPP supports rapid proliferation of cells by producing ribose-5-phosphate, a precursor for nucleotides, and NADPH, which is involved in redox regulation and lipid synthesis [52,53]. Recent studies have shown that glucose does not drive ATP generation during most stages of preimplantation development, but that it is metabolized through the PPP and the hexosamine biosynthesis pathway to control localization of transcription factors, yes-associated protein 1 (YAP1) and transcription factor AP-2 gamma (TFAP2C), for cell fate specification [54]. Thus, generation of biomass, such as DNA and membrane lipids, and orchestration of key events during development are potentially the main roles of glucose to support rapid proliferation during these early developmental stages.

Classically, pyruvate and lactate are known to be important carbohydrates during early stages of preimplantation development, and glucose becomes a key energy source after compaction [55–57]. Pig embryos have decreased metabolism of pyruvate compared to mouse and sheep embryos, pointing to a reliance on other energy sources, such as glucose or lipids [58–61]. Studies in several species, including mouse, hamster, sheep, cow, and human, have reported that glucose inhibits the development of embryos *in vitro* [62–66]. Glucose does not appear to be inhibitory for porcine preimplantation embryo development *in vitro*, but glucose in the presence of inorganic phosphate has been shown to be deleterious [67,68]. In porcine embryos, glucose metabolism minimally increases from the 1-cell to 8-cell stage (0.4 ± 0.1 to 3.1 ± 0.4 pmol/embryo/4 h) but notably increases in the compacted morula (49.5 ± 2.7 pmol/embryo/4 h) and further by the blastocyst stage (147 ± 12.0 pmol/embryo/4 h) [69]. Subsequent studies have also shown that glucose metabolism increases after compaction with glycolysis becoming a key metabolic pathway, but metabolic activity is significantly decreased in *in vitro*-produced embryos compared to *in vivo* counterparts [56,61]. However, porcine embryos can be cultured to the blastocyst stage in medium with no glucose but containing only pyruvate and lactate [70].

4.2. Amino Acids

Amino acids serve numerous functions in promoting the development of preimplantation embryos. Oviductal and uterine fluids contain varying concentrations of amino acids, which has influenced media formulations for several species [71–73]. Aside from protein synthesis, amino acids are involved in other activities, such as ATP production, nucleotide synthesis, lipid synthesis, antioxidant production, cell signaling, osmolarity, and pH regulation. Uptake of amino acids from the microenvironment by preimplantation embryos relies on the activity of different transport systems, which are expressed during different stages [74]. For example, in porcine oocytes, Na⁺-dependent transport of L-alanine and L-leucine was shown to be competitive and occurred by using the B^{0,+} system [75]. At the blastocyst stage, L-leucine was transported in a Na⁺-dependent manner through the L system. Transport of L-leucine into the embryo has been shown to be involved in activation of mechanistic target of rapamycin complex 1 (MTORC1) to promote trophoblast motility [76]. In support of this, treatment of mouse blastocyst-stage embryos with 200 nM rapamycin, an inhibitor of MTORC1, blocked outgrowth formation *in vitro* [74]. Sodium-dependent transport of L-aspartate and L-glutamate was not detected in porcine oocytes but was observed at the blastocyst stage, demonstrating that the X_{AG}⁻ system becomes active at later stages in development [77].

The addition of essential amino acids to embryo culture media has been shown to be inhibitory for development to the blastocyst stage in mouse and cattle embryos [78,79]. However, Steeves and Gardner [80] demonstrated that presence of essential amino acids in medium with nonessential amino acids and glutamine was not inhibitory during cattle embryo cleavage and increased development to the blastocyst stage. In porcine parthenogenetic embryos cultured in modified Whitten's medium with polyvinyl alcohol (PVA), essential amino acids were inhibitory if present at the 1-cell stage but supported development to the blastocyst stage when added after 48 h of culture. Specifically, presence of

nonpolar essential amino acids, valine, leucine, isoleucine, and methionine, during the first 48 h of culture was shown to inhibit development past the 4-cell stage [81]. However, the porcine zygote medium (PZM) variants, containing both essential and nonessential amino acids, have been shown to support development of porcine embryos to the blastocyst stage [70].

Furthermore, transcriptional profiling of porcine blastocyst-stage embryos revealed that transcripts related to amino acid transport and metabolism were dysregulated in those cultured in vitro versus those derived in vivo [12]. In vitro-produced blastocyst-stage embryos had increased abundance of solute carrier 7A1 (*SLC7A1*), which encodes an arginine transporter, and supplementation of 1.69 mM arginine to PZM-3 decreased abundance of *SLC7A1* [82]. Arginine is metabolized for production of nitric oxide, and addition of nitric oxide synthase inhibitors to culture media has been shown to inhibit development of mouse and pig embryos [82,83]. Similarly, in vitro-produced porcine embryos had increased abundance of solute carrier 6A9 (*SLC6A9*), which encodes a glycine transporter, and supplementation of 10 mM glycine decreased *SLC6A9* abundance and improved development of blastocyst-stage embryos [84]. However, transfer of blastocyst-stage embryos cultured with 10 mM glycine did not result in pregnancies, unlike controls cultured with 0.1 mM glycine, showing that more cells at the blastocyst stage is not necessarily an indicator of developmental competence. Furthermore, early mouse and hamster embryo culture media formulations demonstrated the importance glutamine during cleavage stages to overcome the 2-cell block and for the development of blastocyst-stage embryos [62,85]. Glutamine has been shown to support development of porcine embryos from the 1- to 2-cell stage to the blastocyst stage in vitro without the presence of glucose [68]. Recently, glutamine supplementation to PZM-3 has been shown to correct abundance of transcripts related to glutamine transport and metabolism and increase activation of mTORC1 in porcine blastocyst-stage embryos [86,87]. Thus, RNA-sequencing is a powerful tool that can identify amino acid requirements of the embryo in culture, but viability in vivo must be assessed before supplementation of specific amino acids becomes common practice.

4.3. Lipids

In addition to carbohydrates, beta-oxidation of fatty acids is thought to contribute to sustaining oxidative phosphorylation in preimplantation embryos. Sturmey and Leese [60] demonstrated that oxygen consumption and ATP production increase at the early blastocyst stage, and ATP is mainly produced via aerobic metabolism. Treatment of mouse embryos with inhibitors of beta-oxidation, methyl palmoixirate and etomoxir, decreased the number of embryos reaching the blastocyst stage and total cell numbers within the embryos [88,89]. Interestingly, the inhibitory effects of etomoxir were decreased when porcine oocytes were matured in the presence of high glucose (4.0 mM) compared to low glucose (1.5 mM), thus other energy sources can compensate when beta-oxidation is blocked [90]. On the contrary, addition of L-carnitine, which is essential for uptake of fatty acids into the mitochondria, to embryo culture medium improved mitochondrial activity and development in embryos of several species [88,91–93].

Porcine oocytes and embryos have a dark appearance compared to other species due to presence of abundant lipid. Mouse oocytes were determined to have approximately 4 ng of fatty acids; sheep and cattle oocytes have a slightly darker appearance and about 89 and 63 ng of fatty acids, respectively; pig oocytes, almost black in appearance, have about 156 ng of fatty acids [49,94,95]. The most common fatty acids in pig oocytes are palmitic (16:0), stearic (18:0), and oleic (18:1n-9) acids [95]. Compared to other species, the large amount of lipid in pig oocytes is potentially the result of increased message for diacylglycerol acyltransferase 1 (*DGAT1*), which encodes an enzyme involved in the transfer of a fatty acyl CoA to a diacylglycerol to form triglycerides [96]. Moreover, inhibition of stearoyl-coenzyme A desaturase 1 (*SCD1*), which introduces a cis double bond in saturated fatty acids, decreased development to the blastocyst stage and lipid droplet

formation in porcine parthenogenetic embryos; however, cotreatment with 100 μ M oleic acid rescued development [97]. Sturmey and Leese [60] observed that triglyceride levels did not change from cleavage stages to the blastocyst stage, indicating that beta-oxidation might not be a key metabolic pathway in the presence of other energy sources. However, Romek et al. [98] noted that the volume of lipid droplets per unit of cytoplasm decreased at the blastocyst stage in both in vitro-produced and in vivo-derived porcine embryos, demonstrating controversy in this area.

The large amount of lipid in porcine oocytes and embryos has been identified as a barrier to vitrification and survival after thawing. In addition, porcine oocytes have a greater percentage of polyunsaturated fatty acids compared to ruminants, which further impairs survival after freezing [95]. L-carnitine supplementation, centrifugation in a high-osmolarity solution to separate lipids from the cytoplasm, and removal of lipids by micromanipulation have been shown to be effective methods of increasing the cryotolerance of porcine oocytes and embryos [99–102].

5. Strategies for Improving Current Culture Systems

5.1. Media Formulations and Supplements

Several media formulations exist that support development of porcine zygotes to the blastocyst stage, including modified Whitten's medium [103], North Carolina State University (NCSU)-23 medium [104], modified CZB medium [105], Beltsville embryo culture medium (BECM)-3 [106], and porcine zygote medium (PZM) variants [70]. Currently, NCSU-23 and PZM variants are most commonly used for porcine embryo culture. NCSU-23 contains glucose and glutamine as primary energy sources as well as taurine and hypotaurine to mediate osmolarity but lacks pyruvate, lactate, and all other amino acids [104]. PZM variants were formulated based on the composition of porcine oviductal fluid, containing pyruvate and lactate instead of glucose, and they lack taurine but include all other amino acids. Furthermore, PZM-3 contains bovine serum albumin (BSA), similar to NCSU-23, but culturing IVF or somatic cell nuclear transfer (SCNT)-derived embryos in PZM-3 was shown to improve development to the blastocyst stage and increase the number of cells in blastocyst-stage embryos compared to NCSU-23 [70,107]. PZM-4 substitutes PVA for BSA in an attempt to create a chemically defined medium [70]. Bovine serum albumin can contain undefined components, such as citrate and lipids, and different lots need to be tested before being routinely added to culture media. When embryos were cultured in PZM-4 compared to PZM-3, no difference in development was detected, and live piglets were born after embryo transfer [70]. However, subsequent studies demonstrated decreases in development to the blastocyst stage by using PZM-4 compared to PZM-3 [108].

Additives to PZM-3, such as amino acids and small molecules, have been shown to further improve porcine embryo development. For example, supplemental concentrations of arginine increased development to the blastocyst stage, improved embryo quality, and modulated transcript abundance related to arginine transport, resulting in a new medium named MU1 [82]. MU2 was developed after addition of 5-(4-Chloro-phenyl)-3-phenyl-pent-2-enoic acid (PS48) was shown to be able to replace BSA and improved development through stimulation of phosphoinositide 3 kinase (PI3K) to increase phosphorylation of v-akt murine thymoma viral oncogene homolog (AKT) [109]. Afterwards, MU3 was developed by replacing 1 mM glutamine with 3.75 mM GlutaMAX, an L-alanyl-L-glutamine dipeptide, which improved development to the blastocyst stage and increased mitochondrial activity [86]. Currently, MU4 has been implemented, which does not contain hypotaurine as removal of this component did not have a negative effect when embryo culture is conducted at 5% O₂ [110], and hypotaurine is a relatively expensive ingredient.

5.2. Morphological and Chromosomal Quality

Embryos developing in vivo are exposed to the oviductal and uterine epithelia, which are involved in provision of nutrients, removal of toxins, and production of antioxidant systems [111]. Embryos of several species fertilized and cultured in vitro demonstrate

a delay of about one cell division to the blastocyst stage compared to in vivo-derived embryos [12,112–114]. Total cell numbers in porcine blastocyst-stage embryos produced in vitro are decreased and have higher ratios of trophoctoderm to inner cell mass [12,112]. Conventionally, morphology of an embryo has been the main criterion for determining quality [115]. However, morphology should not necessarily be the primary selection standard as porcine embryos cultured with supplemental glycine exhibited improved development and cell numbers but failed to establish pregnancies after 11 embryo transfers [84]. When the synchronization protocol was altered to transfer embryos cultured with supplemental glycine on an earlier day of standing estrus (typically performed on day 3, 4, or 5 of standing estrus), one pregnancy was obtained (unpublished data), indicating that these embryos may have increased developmental competence. Similarly, addition of N-methyl-D-aspartic acid (NMDA) and homocysteine (HC) to PZM-4 increased the percentage of porcine embryos that developed to the blastocyst stage and sizes of the embryos over PZM-4 alone, but 16 transfers of embryos cultured with only NMDA or NMDA and HC did not result in any live piglets [108]. The use of time lapse monitoring of development in vitro has been shown to increase pregnancy rates over conventional morphological assessment [116]. This technology allows for the consideration of other factors, such as timing of first cleavage and number of blastomeres after the first cleavage, which have both been shown to be more reliable indicators of quality [117,118]. The ability of porcine embryos to reach the morula stage before 102 h was correlated with increases in reaching the blastocyst stage, and the percentage of fragmentation negatively correlated with developmental progression [119].

Furthermore, chromosomal abnormalities can occur during development that significantly increase the chances of pregnancy loss or defects after birth. By using comparative genomic hybridization, approximately 14% of in vivo-derived porcine embryos (72 h after insemination) were observed to be aneuploid [120]. However, about 39% of in vitro-produced porcine blastocyst-stage embryos demonstrated chromosomal abnormalities [121]. Assessment of chromosomes 6 and 7 in in vitro-produced bovine blastocyst-stage embryos revealed that 72% of the embryos contained cells that were polyploid; however, the proportion of abnormal cells within each embryo was generally low (<10% of the total cells) [122]. More recent studies have observed high occurrence chromosomal aberrations in at least one blastomere of IVM-IVF bovine embryos as compared to in vivo-derived embryos (85% vs. 19%) [123] and that entire parental genomes can segregate into different cell lineages during cleavage divisions [124].

5.3. Mitochondrial Function

Mitochondria are dynamic organelles with important roles during fertilization and preimplantation development. From the zygote to expanded blastocyst stage, mitochondrial morphology changes from spherical with minimal cristae to elongated with numerous transverse cristae, demonstrating a major shift in metabolic activity during preimplantation development [125]. Mitochondrial membrane potential ($\Delta\Psi_m$) is formed by the pumping of protons across the inner membrane during oxidative phosphorylation and is commonly used as an indirect measure of mitochondrial function and developmental progression in preimplantation embryos. For example, JC-1 is a cationic, lipophilic dye that remains in its monomeric form at low (<100 mV) $\Delta\Psi_m$ and fluoresces green but forms aggregates inside mitochondria at high (>140 mV) $\Delta\Psi_m$ and fluoresces red [126]. Thus, the ratio of red to green fluorescence is an indicator of mitochondrial membrane potential and hence activity within an embryo. JC-10, which has better water solubility than JC-1, can effectively visualize mitochondrial activity in porcine blastocyst-stage embryos derived by IVF and SCNT [86,127], and glutamine supplementation into the culture medium increased the mitochondrial activity of the IVF-derived embryos [78]. Additionally, MitoTracker™ Green is non-fluorescent in aqueous solutions but accumulates in the mitochondrial matrix regardless of $\Delta\Psi_m$ and fluoresces green to measure total mitochondrial mass. MitoTracker™ Red or Orange are oxidized by molecular oxygen in mitochondria to emit a red-orange

fluorescence that can be used to measure oxidative activity [128]. MitoTracker™ Red has been used to determine mitochondrial activity of porcine IVF-derived blastocyst-stage embryos after culture with supplemental glycine; however, no difference in mitochondrial activity was observed [84].

5.4. Transcriptional Profiling

Transcriptional profiling has been used to elucidate “the needs” of the embryo. Studies using serial analysis of gene expression (SAGE) and microarrays revealed differences in abundance for transcripts related to cellular metabolism and transcriptional regulation in in vitro-produced porcine blastocyst-stage embryos compared to in vivo-derived counterparts [129,130]. By using next-generation sequencing (NGS), Bauer et al. [12] identified 1170 differentially abundant transcripts between in vivo-derived and in vitro-produced porcine blastocyst-stage embryos. Analysis of these data revealed that amino acid transport and metabolism were perturbed in in vitro-produced embryos. Specifically, message for an arginine transporter, *SLC7A1*, and a glycine transporter, *SLC6A9*, were upregulated in in vitro-produced embryos by 63- and 25-fold, respectively. As mentioned previously, supplementation of arginine to 1.69 mM increased development to the blastocyst stage and decreased abundance of *SLC7A1* to levels observed in in vivo-derived embryos [82]. Likewise, glycine supplementation to 10 mM improved developmental parameters of the embryos and decreased abundance of *SLC6A9*; however, pregnancies were not established after 11 transfers with embryos cultured in supplemental glycine [84]. Additionally, supplementation of glutamine improved development of porcine embryos and corrected abundance of transcripts related to glutamine transport and metabolism [78]. The use of RNA-sequencing datasets for defining and improving embryo culture is still in its infancy stages as there are numerous pathways yet to explore for improving media formulations.

5.5. Metabolomics

Consumption or production of metabolites from the culture medium has been used as a noninvasive marker of embryo viability. Metabolomics assays are largely applied in IVF clinics to select embryos for transfer; however, these techniques are also useful for selection of embryos from agricultural species for cryopreservation or transfer. Metabolism of glucose, pyruvate, and lactate has been a canonical measure of preimplantation embryo competence, which was studied by using ultramicrofluorescence assays that measure NADH oxidation to represent pyruvate conversion to lactate or NADPH formation to represent glucose uptake [131]. Hardy et al. [132] observed that increased pyruvate consumption by early cleavage-stage human embryos correlated with increased development to the blastocyst stage. Regarding in vitro-produced porcine embryos, metabolism of glucose for glycolysis and pyruvate for the TCA cycle increased after compaction, indicating greater metabolic activity [61]. Glutamine and arginine have been shown to be consumed from culture media by human and pig embryos [133,134]. However, increased arginine consumption was associated with increased development to the blastocyst stage in pig embryos [133] but was associated with decreased development to the blastocyst stage in human embryos [134].

Currently, different platforms are being used to study metabolomic profiles of preimplantation embryos. Gas or liquid chromatography (GC or LC) coupled with mass spectrometry (MS) are ideal for analyzing metabolites in small volumes of culture media (5–10 µL). After derivatization, the mass of compounds allows for the identification of metabolites based on database information [52]. This technique has been successfully applied to porcine embryos to determine the production and consumption of metabolites from the medium after glutamine supplementation [86]. Culturing embryos with supplemental glutamine increased leucine consumption from the medium as well as activation of mTORC1 [78,79]. Additionally, nuclear magnetic resonance (NMR) spectroscopy has been used to analyze metabolites in spent culture media; however, this technique requires larger sample volumes (25 µL) [135,136].

5.6. Microfluidics

Changing the type of culture system may provide methods to better customize the environment surrounding the developing embryos. Most current systems are static in a single atmosphere. The ability to continuously modulate the composition of the culture medium as the needs of the embryo change may provide dramatic improvements in developmental competence. Microfluidic devices are in development, and there are reports in the literature specific to pigs. Such devices have been reported to reduce osmotic stress [137], remove the zona pellucida [138], facilitate fertilization and reduce polyspermy [139,140], and customize the culture environment to improve development [141,142]. Moreover, microfluidic devices may improve tolerance of embryos to cryopreservation as addition and removal of cryoprotectants inflicts osmotic shock that may be detrimental to development. Use of a microfluidic device to gradually change the osmotic pressure during cryoprotectant addition or removal may increase the cryosurvival of pig oocytes or embryos [137].

5.7. Extended Culture

By day 12 of gestation, porcine embryos have undergone several changes in morphology, including spherical, ovoid, tubular, and filamentous forms, transitioning from tubular to approximately 100 μm in length in 1 to 2 h. Methods of culturing porcine embryos past the blastocyst stage as a measure of competence have been met with challenges that have hindered progress in this area. Spherical embryos encapsulated in double-layered alginate beads were able to attain a tubular form with increased estradiol-17 β production in the culture medium [143]. However, the embryos did not elongate into filamentous forms; thus, maternally secreted factors and extracellular matrix components may be required to initiate this process. Chemical modifications to the alginate hydrogels, such as covalent attachment of RGD peptides or incorporation of 0.1 $\mu\text{g}/\text{mL}$ SPP1, increased survival and promoted morphological changes [144]. Nonetheless, full elongation of porcine embryos *in vitro* has not been achieved and will require further investigation.

5.8. Cryopreservation

In many species, cryopreservation of sperm, embryos, and somatic cells is straightforward. Cryopreservation of pig somatic cells, such as fibroblast cells, can be achieved with conventional freezing systems used for other species. In contrast, pig sperm and embryos present some unique challenges. In all likelihood, the cell type with most variable survivability is boar sperm. Variation in viability is season-, breed-, boar- and ejaculate-specific. Commercial application of artificial insemination has historically been by using fresh semen. Since pigs are litter bearing, considerable genetic selection pressure can be placed on the offspring resulting from artificial insemination. As genetic progress can be made by using fresh semen, the lowered success rates from cryopreserved semen does not justify the lower farrowing rate (~20–30%) and decrease in litter size as compared to fresh semen [145].

While cryopreservation of semen presents difficulties, embryos are even more challenging. It is thought that the high lipid content of pig embryos presents the main obstacle to successful cryopreservation. To circumvent this problem, Nagashima [102,146] developed a method to centrifuge the early embryo, thus stratifying the lipids within the zona pellucida. Then, the lipids could be removed via micromanipulation, and the resulting embryos were cryopreserved by using conventional methods. One would reason that the lipids were necessary for development of the embryo; however, in at least one report, development was enhanced after removal [99]. Lipid removal by micromanipulation is labor-intensive. High speed centrifugation to completely separate the lipids within the zona pellucida has been used as a high throughput lipid removal technique for cryopreservation of *in vitro*-produced embryos [147]. Solid surface vitrification of oocytes or zygotes may be another alternative as 15 piglets were derived from 8 embryo transfers [148].

Although few reports on conventional cryopreservation of porcine embryos exist, those reports of successful cryopreservation of early pig embryos have not been widely

repeatable, and the industry has not adopted the transfer of frozen embryos as a method of improving genetics or moving genetics around the world. As expected, in vitro-derived embryos are less viable than in vivo-derived embryos, and thus do not survive the rigors of cryopreservation to the same extent as in vivo-derived embryos. Continual efforts are being made to improve the quality of oocytes matured and embryos cultured in vitro. Improvements, such as adding FLI to the oocyte maturation system, may result in increases in cryosurvival in the pig as has been observed in cattle [149]. Two recent reports describe the production of piglets after cryopreservation and embryo transfer. After transferring 553 embryos to 35 surrogates, 59 piglets from 14 litters were produced ($59/553 = 10\%$) [150]. In another study, 180 embryos were transferred to 12 surrogates, producing 37 piglets from 8 sows ($37/180 = 21\%$) [151]. Unfortunately, the number of embryos cryopreserved is not well described in either case. Thus, the percentages listed above may not fully describe the success of cryopreservation as it may be concluded that more embryos were cryopreserved than were transferred. The question of viability is then raised, i.e., were only the embryos of good quality cryopreserved and transferred? If so, then the percentages above may represent an overestimation of the true success of these technologies.

6. Environmental Stressors Arising from the Culture System

6.1. Oxygen Tension and Reactive Oxygen Species

During oxidative phosphorylation, ROS, such as superoxides, form as a result of premature leaking of electrons from the electron transport chain to molecular oxygen [152]. Therefore, mitochondria are the main source of ROS within cells, which can act as signaling molecules for differentiation or cause membrane, protein, and DNA damage at higher concentrations, potentially leading to apoptosis. Interestingly, Dalvit et al. [153] observed a significant increase in ROS concentrations from the oocyte at MII to the two-cell stage in bovine. ROS levels continued to increase until the late morula stage but decreased significantly during blastocyst formation. In vitro-produced embryos are more susceptible to ROS formation and damage compared to in vivo-derived embryos because of external stressors, such as fluctuating oxygen tension, exposure to visible light, media contaminants, and absence of maternal antioxidant systems [111]. Supplementation of antioxidants to porcine embryo culture media has been shown to improve development and reduce ROS [154,155], but these studies were conducted by using atmospheric (21%) O₂ during the culture period which promotes ROS formation.

Oxygen tension during the culture period has an impact on subsequent embryonic development (Figure 2). One- and two-cell stage porcine embryos cultured in 5% O₂ and 5% CO₂ developed to the blastocyst stage at a rate of 50%, whereas culture in 2% or 21% O₂ and 5% CO₂ resulted in less than 10% development to the blastocyst stage [156]. Culture in 5% O₂ instead of atmospheric (21%) O₂ shifted the global patterns of gene expression in mouse embryos to be more similar to those derived in vivo [157]. Additionally, amino acid turnover, as an indicator of viability, by human embryos was lower when cultured at 5% O₂ compared to 21% O₂ [158]. Culture in low (5%) O₂ increased cell numbers in in vitro-produced porcine blastocyst-stage embryos and the abundance of transaldolase 1 (TALDO1) and pyruvate dehydrogenase kinase 1 (PDK1), which are signatures of a Warburg Effect-like metabolism [51]. As mentioned previously, lowering the oxygen tension to 5% O₂ eliminated the need to add hypotaurine, which functions as an antioxidant, to porcine embryo culture medium as removal of this component did not impair development nor increase apoptosis [110].

6.2. Temperature

Most porcine in vitro culture systems use a physiological temperature of 38.5 °C for the incubation (Figure 2). However, several studies have investigated the effects of increased temperature, or heat stress, on oocyte maturation and development in vitro. Heat stress during in vitro maturation may model the effects of summer months or fever in sows on oocyte or embryo quality. Porcine COCs exposed to heat stress (41.5 °C) for 24 h

had decreased cumulus expansion, progression to MII, and development after fertilization [159]. Supplementation of antioxidants during maturation, including astaxanthin, melatonin, and resveratrol, has been shown to mitigate effects of heat stress and decrease apoptosis [160–162].

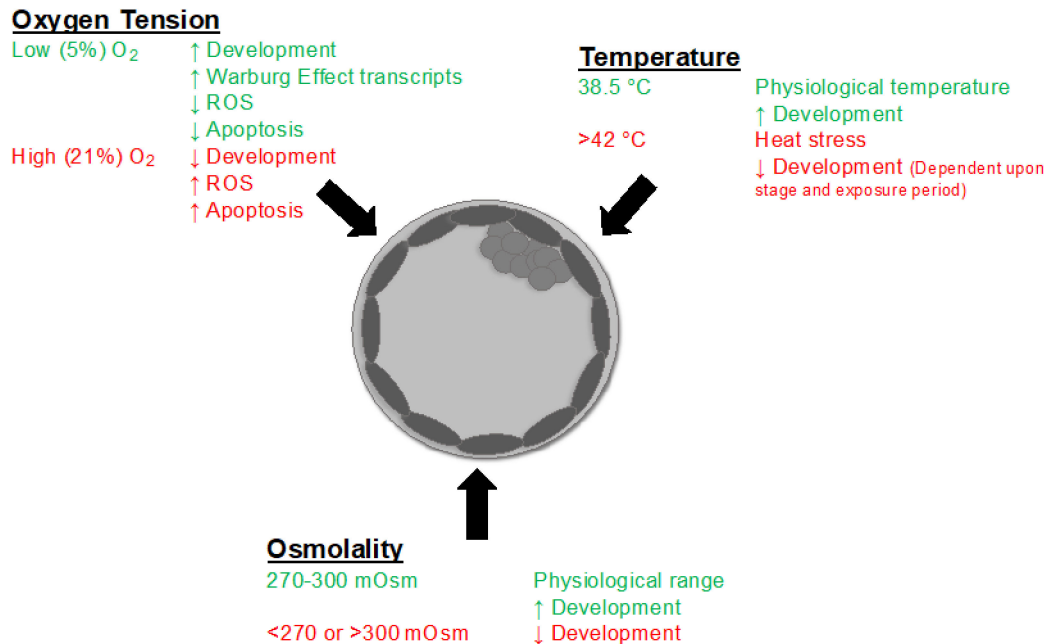


Figure 2. A summary of environmental stressors on porcine embryo development in vitro. Positive conditions are in green, and negative conditions are in red.

Increasing the incubator temperature above 43 °C during the embryo culture period has been shown to decrease development of porcine embryos in a temporal manner, but abundance of heat shock protein 70 was not increased after heat stress exposure [163]. When IVF-derived porcine embryos were exposed to 42 °C for 9 h after the late 1-cell stage, decreases in development to the blastocyst stage and increases in apoptotic nuclei were observed [164]. However, heat shock at the same conditions directly after fertilization increased the rate of cleavage and tended to increase development to the blastocyst stage compared to the non-heat shock controls [165]. Similarly, increases in development of parthenogenetic embryos was observed when heat shock occurred directly after oocyte activation [165], and subsequent analyses revealed that dephosphorylation of MAPK was increased in the heat-shocked embryos compared to controls [164].

6.3. Osmolality

The osmolalities of porcine oviductal and uterine fluids range from 290 to 320 mOsm during the first five days of gestation [73]. The osmolalities of the most commonly used culture media, PZM-3 and NCSU-23, are 288 and 291 mOsm, respectively [70]. Li et al. [73] demonstrated that culture of porcine embryos below 270 mOsm or above 300 mOsm, by altering the NaCl concentration, decreased development to the blastocyst stage (Figure 2) [73]. Contrarily, Hwang et al. [166] observed that increasing osmolality up to 320 mOsm with NaCl or sucrose did not impair development of IVF-derived embryos but surprisingly decreased transcript abundance of BCL2 associated X protein (*BAX*), a proapoptotic gene, compared to the PZM-3 control. Particular amino acids can also act as osmolytes, including glycine and proline. When 10 mM glycine was added to MU1 porcine embryo culture medium at 260, 275, or 300 mOsm, no difference in development to the blastocyst stage was detected [84]. However, culture with supplemental glycine increased the total cell numbers of blastocyst-stage embryos at 275 mOsm, demonstrating a beneficial effect on embryo quality at this osmolality [84].

7. Maternal and Paternal Factors Influencing Development

7.1. Gilt-Versus Sow-Derived Oocytes

The production of pig embryos *in vitro* starts with oocytes from either prepubertal gilts or sexually mature sows. Collection of ovaries at the abattoir is dependent upon the type of facility. Some plants slaughter only gilts while others focus on sows and sausage production. It is generally accepted that *in vitro*-matured oocytes derived from sows are more developmentally competent than oocytes derived from prepubertal gilts [10,167–169]. The diameter of the oocyte, thickness of the zona pellucida, and the perivitelline space were found to be larger in sow oocytes compared in gilt oocytes [170]. However, culture conditions can be modified to improve the quality of *in vitro*-matured oocytes derived from prepubertal gilts to a level similar to oocytes derived from sexually mature sows. For example, addition of fibroblast growth factor 2, leukemia inhibitory factor, and insulin like growth factor 1 (termed FLI) to the maturation medium quadrupled the production of offspring on a per oocyte basis [11]. Indeed, in the presence of FLI, gonadotropins are unnecessary during oocyte maturation [25].

7.2. Sperm Quality

Sperm quality is defined in three major categories: sperm motility, sperm morphology, and sperm biomarker-defined health. Sperm motility can be objectively measured using computer-assisted sperm analysis (CASA) systems [171]. These are typically phase-contrast microscopes fitted with cameras and attached to a computer to perform motility calculations. Software on these computers can likewise perform sperm morphology assessment. Common sperm morphology abnormalities include retained cytoplasmic droplets (proximal and distal in relationship to the sperm midpiece), distal midpiece refluxes, and coiled tails.

There has been increasing attention to evaluating sperm health and function from a biomarker perspective. The advantage to evaluating sperm with biomarkers is that it can reveal sperm quality characteristics that cannot be evaluated under optical microscopes (conventional light microscopy) alone. Biomarkers can be assessed by using microscopes equipped with epifluorescence or through flow cytometry. While there are several characteristics that can be analyzed, we will mention those that are relevant along with manufacturer in parentheses. A common sperm health attribute to assess is acrosomal integrity. Fluorescent conjugated lectins can be used for this. In pig, a common lectin to assess acrosome integrity is *Arachis hypogaea*/peanut agglutinin (Invitrogen Lectin PNA, Alexa Fluor™ 488) [172]. Plasma membrane integrity can give insight to sperm viability [173] and/or if there has been capacitation-associated plasma membrane remodeling [174]. This is commonly analyzed using propidium iodide (Invitrogen). Mitochondrial membrane potential can be assessed using JC-1 (Invitrogen) [175], while capacitation indicating calcium influx status can be evaluated using Fluo 4 NW (Invitrogen) [176]. An additional capacitation status indicator is the efflux of zinc, which can be analyzed using FluoZin™-3 AM (Invitrogen) [174]. Misfolded sperm protein content can be evaluated for with PROTEOSTAT aggresome kit (Enzo Life Sciences) [177] along with sperm proteins marked for recycling via the ubiquitin proteasome pathway with antibodies against ubiquitin [178]. Reactive oxygen species can be indicated by 2',7'-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen) [179] or membrane lipid peroxidation from ROS by 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid, C11-BODIPY (BODIPY, Invitrogen) [180]. Lastly, DNA fragmentation can be evaluated by acridine orange (Acros Organics) [181] or TUNEL (Promega DeadEnd™ Colorimetric TUNEL System) [182].

While it is assumed that lower quality sperm can be used for successful fertilization in IVF compared to artificial insemination, sperm quality is still important. To emphasize this, recent studies have shown that flow cytometer (FC)-sorted sperm result in reduced IVF rates [183]. It is well understood that FC-sorted sperm have reduced sperm quality [184,185]. Even though sperm may undergo successful penetration of the zona pellucida, a series of events are necessary to activate the oocyte. Two sperm-oocyte activat-

ing factors implicated in activating the oocyte are phospholipase C zeta (PLC ζ) [186] and postacrosomal WW domain-binding protein (PAWP) [187]. After sperm-oocyte activation, billions of zinc ions are released, termed the zinc spark (Xenopus [188], cattle [189], and mice [190]). While the zinc spark has not been reported in pigs, we previously reported that the zinc chelator, TPEN, activates pig oocytes [191]. Recent data have shown that zinc inhibits sperm zona pellucida proteinases, such as matrix metalloproteinase-2 (MMP2), and the 26S proteasome [192]. Altogether, sperm-oocyte zinc signaling might be a new polyspermy defense mechanism [174,193].

7.3. Epigenetics and In Vitro Production of Porcine Embryos

Creating or culturing pig embryos in vitro changes the developmental potential [112] as well as the transcriptional [12] and epigenetic profile [194–196] of the resulting embryos. Some would argue that the change in transcription is due to changes in DNA methylation. While there are reports of epigenetic control over transcription, it is not clear if the changes in DNA methylation, for example, are the result of or the cause of changes in transcription [197–200]. In pigs, epigenetic regulation of development is not limited to DNA methylation and includes histone modifications, such as acetylation [201], methylation [202,203], and RNA methylation [204]. Much work has been reported on epigenetic regulation in pig development and after SCNT [205,206]. Increasing histone acetylation by inhibiting deacetylase inhibitors improves the development of SCNT embryos and corrects the expression of some genes [201,207,208]. Presumably changes in the culture environment can bring the transcriptional profile and epigenetic marks, such as DNA methylation and histone acetylation, to a more ‘in vivo’ level.

8. Conclusions and Perspectives

Progress in the in vitro production of porcine embryos has been steady; however, with advances in technologies, such as deep sequencing and metabolomics, the conditions required for healthy oocytes and embryos will be revealed more rapidly. In turn, improvements in media formulations and implementation of dynamic culture systems have great potential for dramatically increasing developmental outcomes. Nevertheless, environmental stressors and parental factors that cannot be controlled will continuously pose challenges for the culture of porcine embryos. These challenges will be faced directly to improve embryo viability as porcine models are becoming more important and applicable in agriculture and human medicine.

Funding: Funding was provided by the United States Department of Agriculture, National Institute of Food and Agriculture (Award numbers 2019-67011-29543, 2019-67012-29714, and 2020-67015-31016), and a grant from the Rural Development Administration, Republic of Korea. Funding for the National Swine Resource and Research Center is from the National Institute of Allergy and Infectious Disease, the National Institute of Heart, Lung and Blood, and the Office of the Director (U42OD011140).

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Whyte, J.J.; Meyer, A.E.; Spate, L.D.; Benne, J.A.; Cecil, R.; Samuel, M.S.; Murphy, C.N.; Prather, R.S.; Geisert, R.D. Inactivation of porcine interleukin-1beta results in failure of rapid conceptus elongation. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 307–312. [[CrossRef](#)] [[PubMed](#)]
2. Meyer, A.E.; Pfeiffer, C.A.; Brooks, K.E.; Spate, L.D.; Benne, J.A.; Cecil, R.; Samuel, M.S.; Murphy, C.N.; Behura, S.; McLean, M.K.; et al. New perspective on conceptus estrogens in maternal recognition and pregnancy establishment in the pigdagger. *Biol. Reprod.* **2019**, *101*, 148–161. [[CrossRef](#)] [[PubMed](#)]
3. Pfeiffer, C.A.; Meyer, A.E.; Brooks, K.E.; Chen, P.R.; Milano-Foster, J.; Spate, L.D.; Benne, J.A.; Cecil, R.F.; Samuel, M.S.; Ciernia, L.A.; et al. Ablation of conceptus PTGS2 expression does not alter early conceptus development and establishment of pregnancy in the pigdagger. *Biol. Reprod.* **2020**, *102*, 475–488. [[CrossRef](#)]

4. Chen, P.R.; Lucas, C.G.; Cecil, R.F.; Pfeiffer, C.A.; Fudge, M.A.; Samuel, M.S.; Zigo, M.; Seo, H.; Spate, L.D.; Whitworth, K.M.; et al. Disrupting porcine glutaminase does not block preimplantation development and elongation nor decrease mTORC1 activation in conceptuses. *Biol. Reprod.* **2021**, *165*. [[CrossRef](#)]
5. Lai, L.; Kang, J.X.; Li, R.; Wang, J.; Witt, W.T.; Yong, H.Y.; Hao, Y.; Wax, D.M.; Murphy, C.N.; Rieke, A.; et al. Generation of cloned transgenic pigs rich in omega-3 fatty acids. *Nat. Biotechnol.* **2006**, *24*, 435–436. [[CrossRef](#)] [[PubMed](#)]
6. Whitworth, K.M.; Rowland, R.R.; Ewen, C.L.; Tribble, B.R.; Kerrigan, M.A.; Cino-Ozuna, A.G.; Samuel, M.S.; Lightner, J.E.; McLaren, D.G.; Mileham, A.J.; et al. Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. *Nat. Biotechnol.* **2016**, *34*, 20–22. [[CrossRef](#)] [[PubMed](#)]
7. Whitworth, K.M.; Rowland, R.R.R.; Petrovan, V.; Sheahan, M.; Cino-Ozuna, A.G.; Fang, Y.; Hesse, R.; Mileham, A.; Samuel, M.S.; Wells, K.D.; et al. Resistance to coronavirus infection in amino peptidase N-deficient pigs. *Transgenic Res.* **2019**, *28*, 21–32. [[CrossRef](#)]
8. Rogers, C.S.; Hao, Y.; Rokhlina, T.; Samuel, M.; Stoltz, D.A.; Li, Y.; Petroff, E.; Vermeer, D.W.; Kabel, A.C.; Yan, Z.; et al. Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer. *J. Clin. Investig.* **2008**, *118*, 1571–1577. [[CrossRef](#)]
9. Koppes, E.A.; Redel, B.K.; Johnson, M.A.; Skvorak, K.J.; Ghaloul-Gonzalez, L.; Yates, M.E.; Lewis, D.W.; Gollin, S.M.; Wu, Y.L.; Christ, S.E.; et al. A porcine model of phenylketonuria generated by CRISPR/Cas9 genome editing. *JCI Insight* **2020**, *5*. [[CrossRef](#)]
10. Lai, L.; Kolber-Simonds, D.; Park, K.W.; Cheong, H.T.; Greenstein, J.L.; Im, G.S.; Samuel, M.; Bonk, A.; Rieke, A.; Day, B.N.; et al. Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* **2002**, *295*, 1089–1092. [[CrossRef](#)]
11. Yuan, Y.; Spate, L.D.; Redel, B.K.; Tian, Y.; Zhou, J.; Prather, R.S.; Roberts, R.M. Quadrupling efficiency in production of genetically modified pigs through improved oocyte maturation. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E5796–E5804. [[CrossRef](#)] [[PubMed](#)]
12. Bauer, B.K.; Isom, S.C.; Spate, L.D.; Whitworth, K.M.; Spollen, W.G.; Blake, S.M.; Springer, G.K.; Murphy, C.N.; Prather, R.S. Transcriptional profiling by deep sequencing identifies differences in mRNA transcript abundance in in vivo-derived versus in vitro-cultured porcine blastocyst stage embryos. *Biol. Reprod.* **2010**, *83*, 791–798. [[CrossRef](#)] [[PubMed](#)]
13. Spate, L.D.; Brown, A.N.; Redel, B.K.; Whitworth, K.M.; Murphy, C.N.; Prather, R.S. Dickkopf-related protein 1 inhibits the WNT signaling pathway and improves pig oocyte maturation. *PLoS ONE* **2014**, *9*, e95114. [[CrossRef](#)]
14. Russell, D.L.; Gilchrist, R.B.; Brown, H.M.; Thompson, J.G. Bidirectional communication between cumulus cells and the oocyte: Old hands and new players? *Theriogenology* **2016**, *86*, 62–68. [[CrossRef](#)]
15. Norris, R.P.; Ratzan, W.J.; Freudzon, M.; Mehlmann, L.M.; Krall, J.; Movsesian, M.A.; Wang, H.; Ke, H.; Nikolaev, V.O.; Jaffe, L.A. Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development* **2009**, *136*, 1869–1878. [[CrossRef](#)]
16. Lonergan, P.; Rizos, D.; Gutierrez-Adan, A.; Fair, T.; Boland, M.P. Oocyte and embryo quality: Effect of origin, culture conditions and gene expression patterns. *Reprod. Domest. Anim.* **2003**, *38*, 259–267. [[CrossRef](#)]
17. Armstrong, D.T. Effects of maternal age on oocyte developmental competence. *Theriogenology* **2001**, *55*, 1303–1322. [[CrossRef](#)]
18. Lechniak, D.; Warzych, E.; Pers-Kamczyc, E.; Sosnowski, J.; Antosik, P.; Rubes, J. Gilts and sows produce similar rate of diploid oocytes in vitro whereas the incidence of aneuploidy differs significantly. *Theriogenology* **2007**, *68*, 755–762. [[CrossRef](#)]
19. Lee, J.B.; Lee, M.G.; Lin, T.; Shin, H.Y.; Lee, J.E.; Kang, J.W.; Jin, D.-I. Effect of oocyte chromatin status in porcine follicles on the embryo development in vitro. *Asian-Australas J. Anim. Sci.* **2019**, *32*, 956–965. [[CrossRef](#)]
20. Chen, L.; Russell, P.T.; Larsen, W.J. Functional significance of cumulus expansion in the mouse: Roles for the preovulatory synthesis of hyaluronic acid within the cumulus mass. *Mol. Reprod. Dev.* **1993**, *34*, 87–93. [[CrossRef](#)]
21. Marchal, R.; Vigneron, C.; Perreau, C.; Bali-Papp, A.; Mermillod, P. Effect of follicular size on meiotic and developmental competence of porcine oocytes. *Theriogenology* **2002**, *57*, 1523–1532. [[CrossRef](#)]
22. Costermans, N.G.J.; Soede, N.M.; van Tricht, F.; Blokland, M.; Kemp, B.; Keijer, J.; Teerds, K.J. Follicular fluid steroid profile in sows: Relationship to follicle size and oocyte quality. *Biol. Reprod.* **2020**, *102*, 740–749. [[CrossRef](#)] [[PubMed](#)]
23. Yokoo, M.; Kimura, N.; Sato, E. Induction of oocyte maturation by hyaluronan-CD44 interaction in pigs. *J. Reprod. Dev.* **2010**, *56*, 15–19. [[CrossRef](#)] [[PubMed](#)]
24. Nevoral, J.; Orsák, M.; Klein, P.; Petr, J.; Dvořáková, M.; Weingartová, I.; Vyskočilová, A.; Zámstná, K.; Krejčová, T.; Jílek, F. Cumulus Cell Expansion, Its Role in Oocyte Biology and Perspectives of Measurement: A Review. *Sci. Agric. Bohem.* **2015**, *45*, 212–225. [[CrossRef](#)]
25. Redel, B.K.; Spate, L.D.; Yuan, Y.; Murphy, C.N.; Roberts, R.M.; Prather, R.S. Neither gonadotropin nor cumulus cell expansion is needed for the maturation of competent porcine oocytes in vitro. *Biol. Reprod.* **2021**. [[CrossRef](#)]
26. Nagai, T.; Niwa, K.; Iritani, A. Effect of sperm concentration during preincubation in a defined medium on fertilization in vitro of pig follicular oocytes. *J. Reprod. Fertil.* **1984**, *70*, 271–275. [[CrossRef](#)]
27. Abeydeera, L.R.; Day, B.N. In vitro penetration of pig oocytes in a modified Tris-buffered medium: Effect of BSA, caffeine and calcium. *Theriogenology* **1997**, *48*, 537–544. [[CrossRef](#)]
28. Evans, J.P. Preventing polyspermy in mammalian eggs—Contributions of the membrane block and other mechanisms. *Mol. Reprod. Dev.* **2020**, *87*, 341–349. [[CrossRef](#)]
29. Burkart, A.D.; Xiong, B.; Baibakov, B.; Jimenez-Movilla, M.; Dean, J. Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. *J. Cell Biol.* **2012**, *197*, 37–44. [[CrossRef](#)]

30. Fahrenkamp, E.; Algarra, B.; Jovine, L. Mammalian egg coat modifications and the block to polyspermy. *Mol. Reprod. Dev.* **2020**, *87*, 326–340. [[CrossRef](#)]
31. Wang, W.H.; Machaty, Z.; Abeydeera, L.R.; Prather, R.S.; Day, B.N. Parthenogenetic activation of pig oocytes with calcium ionophore and the block to sperm penetration after activation. *Biol. Reprod.* **1998**, *58*, 1357–1366. [[CrossRef](#)] [[PubMed](#)]
32. Saavedra, M.D.; Mondejar, I.; Coy, P.; Betancourt, M.; Gonzalez-Marquez, H.; Jimenez-Movilla, M.; Aviles, M.; Romar, R. Calreticulin from subolemmal vesicles affects membrane regulation of polyspermy. *Reproduction* **2014**, *147*, 369–378. [[CrossRef](#)] [[PubMed](#)]
33. Funahashi, H.; Day, B.N. Effects of follicular fluid at fertilization in vitro on sperm penetration in pig oocytes. *J. Reprod. Fertil.* **1993**, *99*, 97–103. [[CrossRef](#)] [[PubMed](#)]
34. Teijeiro, J.M.; Marini, P.E. The effect of oviductal deleted in malignant brain tumor 1 over porcine sperm is mediated by a signal transduction pathway that involves pro-AKAP4 phosphorylation. *Reproduction* **2012**, *143*, 773–785. [[CrossRef](#)] [[PubMed](#)]
35. Coy, P.; Canovas, S.; Mondejar, I.; Saavedra, M.D.; Romar, R.; Grullon, L.; Matas, C.; Aviles, M. Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 15809–15814. [[CrossRef](#)]
36. Hao, Y.; Mathialagan, N.; Walters, E.; Mao, J.; Lai, L.; Becker, D.; Li, W.; Critser, J.; Prather, R.S. Osteopontin reduces polyspermy during in vitro fertilization of porcine oocytes. *Biol. Reprod.* **2006**, *75*, 726–733. [[CrossRef](#)]
37. Coy, P.; Jimenez-Movilla, M.; Garcia-Vazquez, F.A.; Mondejar, I.; Grullon, L.; Romar, R. Oocytes use the plasminogen-plasmin system to remove supernumerary spermatozoa. *Hum. Reprod.* **2012**, *27*, 1985–1993. [[CrossRef](#)]
38. Mondejar, I.; Grullon, L.A.; Garcia-Vazquez, F.A.; Romar, R.; Coy, P. Fertilization outcome could be regulated by binding of oviductal plasminogen to oocytes and by releasing of plasminogen activators during interplay between gametes. *Fertil. Steril.* **2012**, *97*, 453–461. [[CrossRef](#)]
39. Umehara, T.; Tsujita, N.; Goto, M.; Tonai, S.; Nakanishi, T.; Yamashita, Y.; Shimada, M. Methyl-beta cyclodextrin and creatine work synergistically under hypoxic conditions to improve the fertilization ability of boar ejaculated sperm. *Anim. Sci. J.* **2020**, *91*, e13493. [[CrossRef](#)]
40. Kim, N.H.; Funahashi, H.; Abeydeera, L.R.; Moon, S.J.; Prather, R.S.; Day, B.N. Effects of oviductal fluid on sperm penetration and cortical granule exocytosis during fertilization of pig oocytes in vitro. *J. Reprod. Fertil.* **1996**, *107*, 79–86. [[CrossRef](#)]
41. Harris, E.A.; Stephens, K.K.; Winuthayanon, W. Extracellular Vesicles and the Oviduct Function. *Int. J. Mol. Sci.* **2020**, *21*, 8280. [[CrossRef](#)]
42. Zigo, M.; Manaskova-Postlerova, P.; Zuidema, D.; Kerns, K.; Jonakova, V.; Tumova, L.; Bubenickova, F.; Sutovsky, P. Porcine model for the study of sperm capacitation, fertilization and male fertility. *Cell Tissue Res.* **2020**, *380*, 237–262. [[CrossRef](#)]
43. Galeati, G.; Modina, S.; Lauria, A.; Mattioli, M. Follicle somatic cells influence pig oocyte penetrability and cortical granule distribution. *Mol. Reprod. Dev.* **1991**, *29*, 40–46. [[CrossRef](#)] [[PubMed](#)]
44. Probst, S.; Rath, D. Production of piglets using intracytoplasmic sperm injection (ICSI) with flowcytometrically sorted boar semen and artificially activated oocytes. *Theriogenology* **2003**, *59*, 961–973. [[CrossRef](#)]
45. Yong, H.Y.; Hao, Y.; Lai, L.; Li, R.; Murphy, C.N.; Rieke, A.; Wax, D.; Samuel, M.; Prather, R.S. Production of a transgenic piglet by a sperm injection technique in which no chemical or physical treatments were used for oocytes or sperm. *Mol. Reprod. Dev.* **2006**, *73*, 595–599. [[CrossRef](#)] [[PubMed](#)]
46. Nakai, M.; Ozawa, M.; Maedomari, N.; Noguchi, J.; Kaneko, H.; Ito, J.; Onishi, A.; Kashiwazaki, N.; Kikuchi, K. Delay in cleavage of porcine embryos after intracytoplasmic sperm injection (ICSI) shows poorer embryonic development. *J. Reprod. Dev.* **2014**, *60*, 256–259. [[CrossRef](#)]
47. Nakai, M.; Suzuki, S.I.; Ito, J.; Fuchimoto, D.I.; Sembon, S.; Noguchi, J.; Onishi, A.; Kashiwazaki, N.; Kikuchi, K. Efficient pig ICSI using Percoll-selected spermatozoa; evidence for the essential role of phospholipase C-zeta in ICSI success. *J. Reprod. Dev.* **2016**, *62*, 639–643. [[CrossRef](#)]
48. Casillas, F.; Betancourt, M.; Cuello, C.; Ducolomb, Y.; Lopez, A.; Juarez-Rojas, L.; Retana-Marquez, S. An efficiency comparison of different in vitro fertilization methods: IVF, ICSI, and PICSI for embryo development to the blastocyst stage from vitrified porcine immature oocytes. *Porc. Heal. Manag.* **2018**, *4*, 16. [[CrossRef](#)] [[PubMed](#)]
49. Bradley, J.; Swann, K. Mitochondria and lipid metabolism in mammalian oocytes and early embryos. *Int. J. Dev. Biol.* **2019**, *63*, 93–103. [[CrossRef](#)]
50. Warburg, O. On the origin of cancer cells. *Science* **1956**, *123*, 309–314. [[CrossRef](#)]
51. Redel, B.K.; Brown, A.N.; Spate, L.D.; Whitworth, K.M.; Green, J.A.; Prather, R.S. Glycolysis in preimplantation development is partially controlled by the Warburg Effect. *Mol. Reprod. Dev.* **2012**, *79*, 262–271. [[CrossRef](#)] [[PubMed](#)]
52. Krisher, R.L.; Heuberger, A.L.; Paczkowski, M.; Stevens, J.; Pospisil, C.; Prather, R.S.; Sturmey, R.G.; Herrick, J.R.; Schoolcraft, W.B. Applying metabolomic analyses to the practice of embryology: Physiology, development and assisted reproductive technology. *Reprod. Fertil. Dev.* **2015**, *27*, 602–620. [[CrossRef](#)] [[PubMed](#)]
53. Rieger, D. Relationships between energy metabolism and development of early mammalian embryos. *Theriogenology* **1992**, *37*, 75–93. [[CrossRef](#)]
54. Chi, F.; Sharpely, M.S.; Nagaraj, R.; Roy, S.S.; Banerjee, U. Glycolysis-Independent Glucose Metabolism Distinguishes TE from ICM Fate during Mammalian Embryogenesis. *Dev. Cell* **2020**, *53*, 9–26.e24. [[CrossRef](#)]

55. Biggers, J.D.; Whittingham, D.G.; Donahue, R.P. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc. Natl. Acad. Sci. USA* **1967**, *58*, 560–567. [[CrossRef](#)]
56. Gandhi, A.P.; Lane, M.; Gardner, D.K.; Krisher, R.L. Substrate utilization in porcine embryos cultured in NCSU23 and G1.2/G2.2 sequential culture media. *Mol. Reprod. Dev.* **2001**, *58*, 269–275. [[CrossRef](#)]
57. Martin, K.L.; Leese, H.J. Role of developmental factors in the switch from pyruvate to glucose as the major exogenous energy substrate in the preimplantation mouse embryo. *Reprod. Fertil. Dev.* **1999**, *11*, 425–433. [[CrossRef](#)]
58. Gardner, D.K.; Lane, M.; Batt, P. Uptake and metabolism of pyruvate and glucose by individual sheep preattachment embryos developed in vivo. *Mol. Reprod. Dev.* **1993**, *36*, 313–319. [[CrossRef](#)]
59. Leese, H.J.; Barton, A.M. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J. Reprod. Fertil.* **1984**, *72*, 9–13. [[CrossRef](#)]
60. Sturmey, R.G.; Leese, H.J. Energy metabolism in pig oocytes and early embryos. *Reproduction* **2003**, *126*, 197–204. [[CrossRef](#)]
61. Swain, J.E.; Bormann, C.L.; Clark, S.G.; Walters, E.M.; Wheeler, M.B.; Krisher, R.L. Use of energy substrates by various stage preimplantation pig embryos produced in vivo and in vitro. *Reproduction* **2002**, *123*, 253–260. [[CrossRef](#)]
62. Chatot, C.L.; Ziomek, C.A.; Bavister, B.D.; Lewis, J.L.; Torres, I. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J. Reprod. Fertil.* **1989**, *86*, 679–688. [[CrossRef](#)]
63. Schini, S.A.; Bavister, B.D. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. *Biol. Reprod.* **1988**, *39*, 1183–1192. [[CrossRef](#)]
64. Conaghan, J.; Hardy, K.; Handyside, A.H.; Winston, R.M.; Leese, H.J. Selection criteria for human embryo transfer: A comparison of pyruvate uptake and morphology. *J. Assist. Reprod. Genet.* **1993**, *10*, 21–30. [[CrossRef](#)] [[PubMed](#)]
65. Takahashi, Y.; First, N.L. In vitro development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **1992**, *37*, 963–978. [[CrossRef](#)]
66. Thompson, J.G.; Simpson, A.C.; Pugh, P.A.; Tervit, H.R. Requirement for glucose during in vitro culture of sheep preimplantation embryos. *Mol. Reprod. Dev.* **1992**, *31*, 253–257. [[CrossRef](#)] [[PubMed](#)]
67. Hagen, D.R.; Prather, R.S.; Sims, M.M.; First, N.L. Development of one-cell porcine embryos to the blastocyst stage in simple media. *J. Anim. Sci.* **1991**, *69*, 1147–1150. [[CrossRef](#)]
68. Petters, R.M.; Johnson, B.H.; Reed, M.L.; Archibong, A.E. Glucose, glutamine and inorganic phosphate in early development of the pig embryo in vitro. *J. Reprod. Fertil.* **1990**, *89*, 269–275. [[CrossRef](#)]
69. Flood, M.R.; Wiebold, J.L. Glucose metabolism by preimplantation pig embryos. *J. Reprod. Fertil.* **1988**, *84*, 7–12. [[CrossRef](#)]
70. Yoshioka, K.; Suzuki, C.; Tanaka, A.; Anas, I.M.; Iwamura, S. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biol. Reprod.* **2002**, *66*, 112–119. [[CrossRef](#)]
71. Gardner, D.K.; Leese, H.J. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J. Reprod. Fertil.* **1990**, *88*, 361–368. [[CrossRef](#)] [[PubMed](#)]
72. Hugentobler, S.A.; Diskin, M.G.; Leese, H.J.; Humpherson, P.G.; Watson, T.; Sreenan, J.M.; Morris, D.G. Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. *Mol. Reprod. Dev.* **2007**, *74*, 445–454. [[CrossRef](#)] [[PubMed](#)]
73. Li, R.; Whitworth, K.; Lai, L.; Wax, D.; Spate, L.; Murphy, C.N.; Rieke, A.; Isom, C.; Hao, Y.; Zhong, Z.; et al. Concentration and composition of free amino acids and osmolalities of porcine oviductal and uterine fluid and their effects on development of porcine IVF embryos. *Mol. Reprod. Dev.* **2007**, *74*, 1228–1235. [[CrossRef](#)]
74. Van Winkle, L.J. Amino acid transport regulation and early embryo development. *Biol. Reprod.* **2001**, *64*, 1–12. [[CrossRef](#)]
75. Prather, R.S.; Peters, M.S.; Van Winkle, L.J. Alanine and leucine transport in unfertilized pig oocytes and early blastocysts. *Mol. Reprod. Dev.* **1993**, *34*, 250–254. [[CrossRef](#)] [[PubMed](#)]
76. Van Winkle, L.J.; Tesch, J.K.; Shah, A.; Campione, A.L. System B₀+ amino acid transport regulates the penetration stage of blastocyst implantation with possible long-term developmental consequences through adulthood. *Hum. Reprod. Updat.* **2006**, *12*, 145–157. [[CrossRef](#)]
77. Prather, R.S.; Peters, M.S.; Van Winkle, L.J. Aspartate and glutamate transport in unfertilized pig oocytes and blastocysts. *Mol. Reprod. Dev.* **1993**, *36*, 49–52. [[CrossRef](#)]
78. Gardner, D.K.; Lane, M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol. Reprod.* **1993**, *48*, 377–385. [[CrossRef](#)]
79. Pinyopummintr, T.; Bavister, B.D. Effects of amino acids on development in vitro of cleavage-stage bovine embryos into blastocysts. *Reprod. Fertil. Dev.* **1996**, *8*, 835–841. [[CrossRef](#)]
80. Steeves, T.E.; Gardner, D.K. Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol. Reprod.* **1999**, *61*, 731–740. [[CrossRef](#)]
81. Van Thuan, N.; Harayama, H.; Miyake, M. Characteristics of preimplantation development of porcine parthenogenetic diploids relative to the existence of amino acids in vitro. *Biol. Reprod.* **2002**, *67*, 1688–1698. [[CrossRef](#)] [[PubMed](#)]
82. Redel, B.K.; Tessanne, K.J.; Spate, L.D.; Murphy, C.N.; Prather, R.S. Arginine increases development of in vitro-produced porcine embryos and affects the protein arginine methyltransferase-dimethylarginine dimethylaminohydrolase-nitric oxide axis. *Reprod. Fertil. Dev.* **2015**, *27*, 655–666. [[CrossRef](#)] [[PubMed](#)]
83. Tranguch, S.; Steuerwald, N.; Huet-Hudson, Y.M. Nitric oxide synthase production and nitric oxide regulation of preimplantation embryo development. *Biol. Reprod.* **2003**, *68*, 1538–1544. [[CrossRef](#)] [[PubMed](#)]

84. Redel, B.K.; Spate, L.D.; Lee, K.; Mao, J.; Whitworth, K.M.; Prather, R.S. Glycine supplementation in vitro enhances porcine preimplantation embryo cell number and decreases apoptosis but does not lead to live births. *Mol. Reprod. Dev.* **2016**, *83*, 246–258. [[CrossRef](#)]
85. Carney, E.W.; Bavister, B.D. Stimulatory and inhibitory effects of amino acids on the development of hamster eight-cell embryos in vitro. *J. Assist. Reprod. Genet.* **1987**, *4*, 162–167. [[CrossRef](#)]
86. Chen, P.R.; Redel, B.K.; Spate, L.D.; Ji, T.; Salazar, S.R.; Prather, R.S. Glutamine supplementation enhances development of in vitro-produced porcine embryos and increases leucine consumption from the medium. *Biol. Reprod.* **2018**, *99*, 938–948. [[CrossRef](#)]
87. Chen, P.R.; Lucas, C.G.; Spate, L.D.; Prather, R.S. Glutaminolysis is involved in the activation of mTORC1 in in vitro-produced porcine embryos. *Mol. Reprod. Dev.* **2021**, *88*, 490–499. [[CrossRef](#)]
88. Dunning, K.R.; Cashman, K.; Russell, D.L.; Thompson, J.G.; Norman, R.J.; Robker, R.L. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biol. Reprod.* **2010**, *83*, 909–918. [[CrossRef](#)]
89. Hewitson, L.C.; Martin, K.L.; Leese, H.J. Effects of metabolic inhibitors on mouse preimplantation embryo development and the energy metabolism of isolated inner cell masses. *Mol. Reprod. Dev.* **1996**, *43*, 323–330. [[CrossRef](#)]
90. Lowe, J.L.; Bathgate, R.; Grupen, C.G. Effect of carbohydrates on lipid metabolism during porcine oocyte IVM. *Reprod. Fertil. Dev.* **2019**, *31*, 557–569. [[CrossRef](#)]
91. Kim, M.K.; Park, J.K.; Paek, S.K.; Kim, J.W.; Kwak, I.P.; Lee, H.J.; Lyu, S.W.; Lee, W.S. Effects and pregnancy outcomes of L-carnitine supplementation in culture media for human embryo development from in vitro fertilization. *J. Obstet. Gynaecol. Res.* **2018**, *44*, 2059–2066. [[CrossRef](#)]
92. Somfai, T.; Kaneda, M.; Akagi, S.; Watanabe, S.; Haraguchi, S.; Mizutani, E.; Dang-Nguyen, T.Q.; Geshi, M.; Kikuchi, K.; Nagai, T. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod. Fertil. Dev.* **2011**, *23*, 912–920. [[CrossRef](#)]
93. Knitlova, D.; Hulinska, P.; Jeseta, M.; Hanzalova, K.; Kempisty, B.; Machatkova, M. Supplementation of l-carnitine during in vitro maturation improves embryo development from less competent bovine oocytes. *Theriogenology* **2017**, *102*, 16–22. [[CrossRef](#)]
94. Loewenstein, J.E.; Cohen, A.I. Dry Mass, Lipid Content and Protein Content of the Intact and Zona-Free Mouse Ovum. *J. Embryol. Exp. Morphol.* **1964**, *12*, 113–121.
95. McEvoy, T.G.; Coull, G.D.; Broadbent, P.J.; Hutchinson, J.S.; Speake, B.K. Fatty acid composition of lipids in immature cattle, pig and sheep oocytes with intact zona pellucida. *J. Reprod. Fertil.* **2000**, *118*, 163–170. [[CrossRef](#)]
96. Jiang, Z.; Dong, H.; Zheng, X.; Marjani, S.L.; Donovan, D.M.; Chen, J.; Tian, X.C. mRNA Levels of Imprinted Genes in Bovine In Vivo Oocytes, Embryos and Cross Species Comparisons with Humans, Mice and Pigs. *Sci. Rep.* **2015**, *5*, 17898. [[CrossRef](#)] [[PubMed](#)]
97. Lee, D.K.; Choi, K.H.; Hwang, J.Y.; Oh, J.N.; Kim, S.H.; Lee, C.K. Stearoyl-coenzyme A desaturase 1 is required for lipid droplet formation in pig embryo. *Reproduction* **2019**, *157*, 235–243. [[CrossRef](#)]
98. Romek, M.; Gajda, B.; Krzysztofowicz, E.; Smorag, Z. Lipid content of non-cultured and cultured pig embryo. *Reprod. Domest. Anim.* **2009**, *44*, 24–32. [[CrossRef](#)] [[PubMed](#)]
99. Li, R.; Lai, L.; Wax, D.; Hao, Y.; Murphy, C.N.; Rieke, A.; Samuel, M.; Linville, M.L.; Korte, S.W.; Evans, R.W.; et al. Cloned transgenic swine via in vitro production and cryopreservation. *Biol. Reprod.* **2006**, *75*, 226–230. [[CrossRef](#)] [[PubMed](#)]
100. Li, R.; Murphy, C.N.; Spate, L.; Wax, D.; Isom, C.; Rieke, A.; Walters, E.M.; Samuel, M.; Prather, R.S. Production of piglets after cryopreservation of embryos using a centrifugation-based method for delipation without micromanipulation. *Biol. Reprod.* **2009**, *80*, 563–571. [[CrossRef](#)]
101. Lowe, J.L.; Bartolac, L.K.; Bathgate, R.; Grupen, C.G. Supplementation of culture medium with L-carnitine improves the development and cryotolerance of in vitro-produced porcine embryos. *Reprod. Fertil. Dev.* **2017**, *29*, 2357–2366. [[CrossRef](#)] [[PubMed](#)]
102. Nagashima, H.; Kashiwazaki, N.; Ashman, R.J.; Grupen, C.G.; Seamark, R.F.; Nottle, M.B. Removal of cytoplasmic lipid enhances the tolerance of porcine embryos to chilling. *Biol. Reprod.* **1994**, *51*, 618–622. [[CrossRef](#)] [[PubMed](#)]
103. Beckman, L.S.; Day, B.N. Effects of media NaCl concentration and osmolarity on the culture of early-stage porcine embryos and the viability of embryos cultured in a selected superior medium. *Theriogenology* **1993**, *39*, 611–622. [[CrossRef](#)]
104. Petters, R.M.; Wells, K.D. Culture of pig embryos. *J. Reprod. Fertil. Suppl.* **1993**, *48*, 61–73. [[PubMed](#)]
105. Pollard, J.W.; Plante, C.; Leibo, S.P. Comparison of development of pig zygotes and embryos in simple and complex culture media. *J. Reprod. Fertil.* **1995**, *103*, 331–337. [[CrossRef](#)]
106. Dobrinsky, J.R.; Johnson, L.A.; Rath, D. Development of a culture medium (BECM-3) for porcine embryos: Effects of bovine serum albumin and fetal bovine serum on embryo development. *Biol. Reprod.* **1996**, *55*, 1069–1074. [[CrossRef](#)]
107. Im, G.S.; Lai, L.; Liu, Z.; Hao, Y.; Wax, D.; Bonk, A.; Prather, R.S. In vitro development of preimplantation porcine nuclear transfer embryos cultured in different media and gas atmospheres. *Theriogenology* **2004**, *61*, 1125–1135. [[CrossRef](#)]
108. Spate, L.D.; Redel, B.K.; Brown, A.N.; Murphy, C.N.; Prather, R.S. Replacement of bovine serum albumin with N-methyl-D-aspartic acid and homocysteine improves development, but not live birth. *Mol. Reprod. Dev.* **2012**, *79*, 310. [[CrossRef](#)]
109. Spate, L.D.; Brown, A.; Redel, B.K.; Whitworth, K.M.; Prather, R.S. PS48 can replace bovine serum albumin in pig embryo culture medium, and improve in vitro embryo development by phosphorylating AKT. *Mol. Reprod. Dev.* **2015**, *82*, 315–320. [[CrossRef](#)]

110. Chen, P.R.; Spate, L.D.; Leffeler, E.C.; Benne, J.A.; Cecil, R.F.; Hord, T.K.; Prather, R.S. Removal of hypotaurine from porcine embryo culture medium does not impair development of in vitro-fertilized or somatic cell nuclear transfer-derived embryos at low oxygen tension. *Mol. Reprod. Dev.* **2020**. [[CrossRef](#)]
111. Guérin, P.; Mouatassim, S.E.; Ménézo, Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum. Reprod.* **2001**, *7*, 175–189. [[CrossRef](#)]
112. Machaty, Z.; Day, B.N.; Prather, R.S. Development of early porcine embryos in vitro and in vivo. *Biol. Reprod.* **1998**, *59*, 451–455. [[CrossRef](#)]
113. Sanfins, A.; Plancha, C.E.; Albertini, D.F. Pre-implantation developmental potential from in vivo and in vitro matured mouse oocytes: A cytoskeletal perspective on oocyte quality. *J. Assist. Reprod. Genet.* **2015**, *32*, 127–136. [[CrossRef](#)]
114. Ushijima, H.; Akiyama, K.; Tajima, T. Transition of cell numbers in bovine preimplantation embryos: In vivo collected and in vitro produced embryos. *J. Reprod. Dev.* **2008**, *54*, 239–243. [[CrossRef](#)]
115. Lindner, G.M.; Wright, R.W.J. Morphological evaluation of bovine embryos. *Theriogenology* **1983**, *20*, 407–416. [[CrossRef](#)]
116. Sugimura, S.; Akai, T.; Imai, K. Selection of viable in vitro-fertilized bovine embryos using time-lapse monitoring in microwell culture dishes. *J. Reprod. Dev.* **2017**, *63*, 353–357. [[CrossRef](#)]
117. Isom, S.C.; Li, R.F.; Whitworth, K.M.; Prather, R.S. Timing of first embryonic cleavage is a positive indicator of the in vitro developmental potential of porcine embryos derived from in vitro fertilization, somatic cell nuclear transfer and parthenogenesis. *Mol. Reprod. Dev.* **2012**, *79*, 197–207. [[CrossRef](#)] [[PubMed](#)]
118. Lundin, K.; Bergh, C.; Hardarson, T. Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Hum. Reprod.* **2001**, *16*, 2652–2657. [[CrossRef](#)]
119. Mateusen, B.; Van Soom, A.; Maes, D.G.; Donnay, I.; Duchateau, L.; Lequarre, A.S. Porcine embryo development and fragmentation and their relation to apoptotic markers: A cinematographic and confocal laser scanning microscopic study. *Reproduction* **2005**, *129*, 443–452. [[CrossRef](#)] [[PubMed](#)]
120. Hornak, M.; Hulinska, P.; Musilova, P.; Kubickova, S.; Rubes, J. Investigation of chromosome aneuploidies in early porcine embryos using comparative genomic hybridization. *Cytogenet. Genome Res.* **2009**, *126*, 210–216. [[CrossRef](#)] [[PubMed](#)]
121. McCauley, T.C.; Mazza, M.R.; Didion, B.A.; Mao, J.; Wu, G.; Coppola, G.; Coppola, G.F.; Di Bernardino, D.; Day, B.N. Chromosomal abnormalities in Day-6, in vitro-produced pig embryos. *Theriogenology* **2003**, *60*, 1569–1580. [[CrossRef](#)]
122. Viuff, D.; Rickords, L.; Offenber, H.; Hyttel, P.; Avery, B.; Greve, T.; Olsaker, I.; Williams, J.L.; Callesen, H.; Thomsen, P.D. A high proportion of bovine blastocysts produced in vitro are mixoploid. *Biol. Reprod.* **1999**, *60*, 1273–1278. [[CrossRef](#)]
123. Tsuiko, O.; Catteeuw, M.; Zamani Esteki, M.; Destouni, A.; Bogado Pascottini, O.; Besenfelder, U.; Havlicek, V.; Smits, K.; Kurg, A.; Salumets, A.; et al. Genome stability of bovine in vivo-conceived cleavage-stage embryos is higher compared to in vitro-produced embryos. *Hum. Reprod.* **2017**, *32*, 2348–2357. [[CrossRef](#)]
124. Destouni, A.; Zamani Esteki, M.; Catteeuw, M.; Tsuiko, O.; Dimitriadou, E.; Smits, K.; Kurg, A.; Salumets, A.; Van Soom, A.; Voet, T.; et al. Zygotes segregate entire parental genomes in distinct blastomere lineages causing cleavage-stage chimerism and mixoploidy. *Genome Res.* **2016**, *26*, 567–578. [[CrossRef](#)]
125. Sathananthan, A.H.; Trounson, A.O. Mitochondrial morphology during preimplantational human embryogenesis. *Hum. Reprod.* **2000**, *15* (Suppl. 2), 148–159. [[CrossRef](#)]
126. Reers, M.; Smiley, S.T.; Mottola-Hartshorn, C.; Chen, A.; Lin, M.; Chen, L.B. Mitochondrial membrane potential monitored by JC-1 dye. *Methods Enzymol.* **1995**, *260*, 406–417. [[CrossRef](#)]
127. Mordhorst, B.R.; Murphy, S.L.; Ross, R.M.; Benne, J.A.; Samuel, M.S.; Cecil, R.F.; Redel, B.K.; Spate, L.D.; Murphy, C.N.; Wells, K.D.; et al. Pharmacologic treatment of donor cells induced to have a Warburg effect-like metabolism does not alter embryonic development in vitro or survival during early gestation when used in somatic cell nuclear transfer in pigs. *Mol. Reprod. Dev.* **2018**, *85*, 290–302. [[CrossRef](#)]
128. Agnello, M.; Morici, G.; Rinaldi, A.M. A method for measuring mitochondrial mass and activity. *Cytotechnology* **2008**, *56*, 145–149. [[CrossRef](#)]
129. Miles, J.R.; Blomberg, L.A.; Krisher, R.L.; Everts, R.E.; Sonstegard, T.S.; Van Tassell, C.P.; Zuelke, K.A. Comparative transcriptome analysis of in vivo- and in vitro-produced porcine blastocysts by small amplified RNA-serial analysis of gene expression (SAR-SAGE). *Mol. Reprod. Dev.* **2008**, *75*, 976–988. [[CrossRef](#)] [[PubMed](#)]
130. Whitworth, K.M.; Agca, C.; Kim, J.G.; Patel, R.V.; Springer, G.K.; Bivens, N.J.; Forrester, L.J.; Mathialagan, N.; Green, J.A.; Prather, R.S. Transcriptional profiling of pig embryogenesis by using a 15-K member unigene set specific for pig reproductive tissues and embryos. *Biol. Reprod.* **2005**, *72*, 1437–1451. [[CrossRef](#)] [[PubMed](#)]
131. Clarke, R.N.; Baltz, J.M.; Lechene, C.P.; Biggers, J.D. Use of ultramicrofluorometric methods for the study of single preimplantation embryos. *Poult. Sci.* **1989**, *68*, 972–978. [[CrossRef](#)] [[PubMed](#)]
132. Hardy, K.; Hooper, M.A.; Handyside, A.H.; Rutherford, A.J.; Winston, R.M.; Leese, H.J. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Hum. Reprod.* **1989**, *4*, 188–191. [[CrossRef](#)] [[PubMed](#)]
133. Booth, P.J.; Humpherson, P.G.; Watson, T.J.; Leese, H.J. Amino acid depletion and appearance during porcine preimplantation embryo development in vitro. *Reproduction* **2005**, *130*, 655–668. [[CrossRef](#)] [[PubMed](#)]

134. Stokes, P.J.; Hawkhead, J.A.; Fawthrop, R.K.; Picton, H.M.; Sharma, V.; Leese, H.J.; Houghton, F.D. Metabolism of human embryos following cryopreservation: Implications for the safety and selection of embryos for transfer in clinical IVF. *Hum. Reprod.* **2007**, *22*, 829–835. [[CrossRef](#)]
135. D'Souza, F.; Uppangala, S.; Asampille, G.; Salian, S.R.; Kalthur, G.; Talevi, R.; Atreya, H.S.; Adiga, S.K. Spent embryo culture medium metabolites are related to the in vitro attachment ability of blastocysts. *Sci. Rep.* **2018**, *8*, 17025. [[CrossRef](#)]
136. Seli, E.; Botros, L.; Sakkas, D.; Burns, D.H. Noninvasive metabolomic profiling of embryo culture media using proton nuclear magnetic resonance correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil. Steril.* **2008**, *90*, 2183–2189. [[CrossRef](#)]
137. Guo, Y.; Yang, Y.; Yi, X.; Zhou, X. Microfluidic method reduces osmotic stress injury to oocytes during cryoprotectant addition and removal processes in porcine oocytes. *Cryobiology* **2019**, *90*, 63–70. [[CrossRef](#)]
138. Zeringue, H.C.; Wheeler, M.B.; Beebe, D.J. A microfluidic method for removal of the zona pellucida from mammalian embryos. *Lab. Chip.* **2005**, *5*, 108–110. [[CrossRef](#)]
139. Sano, H.; Matsuura, K.; Naruse, K.; Funahashi, H. Application of a microfluidic sperm sorter to the in-vitro fertilization of porcine oocytes reduced the incidence of polyspermic penetration. *Theriogenology* **2010**, *74*, 863–870. [[CrossRef](#)]
140. Clark, S.G.; Haubert, K.; Beebe, D.J.; Ferguson, C.E.; Wheeler, M.B. Reduction of polyspermic penetration using biomimetic microfluidic technology during in vitro fertilization. *Lab Chip* **2005**, *5*, 1229–1232. [[CrossRef](#)]
141. Krisher, R.L.; Wheeler, M.B. Towards the use of microfluidics for individual embryo culture. *Reprod. Fertil. Dev.* **2010**, *22*, 32–39. [[CrossRef](#)] [[PubMed](#)]
142. Wheeler, M.B.; Walters, E.M.; Beebe, D.J. Toward culture of single gametes: The development of microfluidic platforms for assisted reproduction. *Theriogenology* **2007**, *68* (Suppl. 1), S178–S189. [[CrossRef](#)] [[PubMed](#)]
143. Sargus-Patino, C.N.; Wright, E.C.; Plautz, S.A.; Miles, J.R.; Vallet, J.L.; Pannier, A.K. In vitro development of preimplantation porcine embryos using alginate hydrogels as a three-dimensional extracellular matrix. *Reprod. Fertil. Dev.* **2014**, *26*, 943–953. [[CrossRef](#)]
144. Laughlin, T.D.; Miles, J.R.; Wright-Johnson, E.C.; Rempel, L.A.; Lents, C.A.; Pannier, A.K. Development of pre-implantation porcine blastocysts cultured within alginate hydrogel systems either supplemented with secreted phosphoprotein 1 or conjugated with Arg-Gly-Asp Peptide. *Reprod. Fertil. Dev.* **2017**, *29*, 2345–2356. [[CrossRef](#)] [[PubMed](#)]
145. Silva, C.G.; Cunha, E.R.; Blume, G.R.; Malaquias, J.V.; Bao, S.N.; Martins, C.F. Cryopreservation of boar sperm comparing different cryoprotectants associated in media based on powdered coconut water, lactose and trehalose. *Cryobiology* **2015**, *70*, 90–94. [[CrossRef](#)] [[PubMed](#)]
146. Nagashima, H.; Kashiwazaki, N.; Ashman, R.J.; Grupen, C.G.; Nottle, M.B. Cryopreservation of porcine embryos. *Nature* **1995**, *374*, 416. [[CrossRef](#)] [[PubMed](#)]
147. Spate, L.D.; Murphy, C.N.; Prather, R.S. High-throughput cryopreservation of in vivo-derived swine embryos. *PLoS ONE* **2013**, *8*, e65545. [[CrossRef](#)]
148. Somfai, T.; Kikuchi, K. Vitrification of Porcine Oocytes and Zygotes in Microdrops on a Solid Metal Surface or Liquid Nitrogen. *Methods Mol. Biol.* **2021**, *2180*, 455–468. [[CrossRef](#)] [[PubMed](#)]
149. Stoecklein, K.S.; Ortega, M.S.; Spate, L.D.; Murphy, C.N.; Prather, R.S. Improved cryopreservation of in vitro produced bovine embryos using FGF2, LIF, and IGF1. *PLoS ONE* **2021**, *16*, e0243727. [[CrossRef](#)]
150. Tajima, S.; Motoyama, S.; Wakiya, Y.; Uchikura, K.; Misawa, H.; Takishita, R.; Hirayama, Y.; Kikuchi, K. Piglet production by non-surgical transfer of vitrified embryos, transported to commercial swine farms and warmed on site. *Anim. Sci. J.* **2020**, *91*, e13476. [[CrossRef](#)]
151. Hirayama, Y.; Takishita, R.; Misawa, H.; Kikuchi, K.; Misumi, K.; Egawa, S.; Motoyama, S.; Hasuta, Y.; Nakamura, Y.; Hashiyada, Y. Non-surgical transfer of vitrified porcine embryos using a catheter designed for a proximal site of the uterus. *Anim. Sci. J.* **2020**, *91*, e13457. [[CrossRef](#)]
152. Bigarella, C.L.; Liang, R.; Ghaffari, S. Stem cells and the impact of ROS signaling. *Development* **2014**, *141*, 4206–4218. [[CrossRef](#)] [[PubMed](#)]
153. Dalvit, G.C.; Cetica, P.D.; Pintos, L.N.; Beconi, M.T. Reactive oxygen species in bovine embryo in vitro production. *Biocell* **2005**, *29*, 209–212. [[CrossRef](#)] [[PubMed](#)]
154. Kang, H.G.; Lee, S.; Jeong, P.S.; Kim, M.J.; Park, S.H.; Joo, Y.E.; Park, S.H.; Song, B.S.; Kim, S.U.; Kim, M.K.; et al. Lycopene Improves In Vitro Development of Porcine Embryos by Reducing Oxidative Stress and Apoptosis. *Antioxidants* **2021**, *10*, 230. [[CrossRef](#)]
155. Yang, S.G.; Park, H.J.; Kim, J.W.; Jung, J.M.; Kim, M.J.; Jegal, H.G.; Kim, I.S.; Kang, M.J.; Wee, G.; Yang, H.Y.; et al. Mito-TEMPO improves development competence by reducing superoxide in preimplantation porcine embryos. *Sci. Rep.* **2018**, *8*, 10130. [[CrossRef](#)]
156. Berthelot, F.; Terqui, M. Effects of oxygen, CO₂/pH and medium on the in vitro development of individually cultured porcine one- and two-cell embryos. *Reprod. Nutr. Dev.* **1996**, *36*, 241–251. [[CrossRef](#)]
157. Rinaudo, P.F.; Giritharan, G.; Talbi, S.; Dobson, A.T.; Schultz, R.M. Effects of oxygen tension on gene expression in preimplantation mouse embryos. *Fertil. Steril.* **2006**, *86*, 1265.e1–1265.e36. [[CrossRef](#)] [[PubMed](#)]
158. Jeseta, M.; Cela, A.; Zakova, J.; Madr, A.; Crha, I.; Glatz, Z.; Kempisty, B.; Ventruba, P. Metabolic Activity of Human Embryos after Thawing Differs in Atmosphere with Different Oxygen Concentrations. *J. Clin. Med.* **2020**, *9*, 2609. [[CrossRef](#)]

159. Yin, C.; Liu, J.; He, B.; Jia, L.; Gong, Y.; Guo, H.; Zhao, R. Heat stress induces distinct responses in porcine cumulus cells and oocytes associated with disrupted gap junction and trans-zonal projection colocalization. *J. Cell. Physiol.* **2019**, *234*, 4787–4798. [[CrossRef](#)]
160. Do, L.T.; Luu, V.V.; Morita, Y.; Taniguchi, M.; Nii, M.; Peter, A.T.; Otoi, T. Astaxanthin present in the maturation medium reduces negative effects of heat shock on the developmental competence of porcine oocytes. *Reprod. Biol.* **2015**, *15*, 86–93. [[CrossRef](#)]
161. Li, Y.; Wang, J.; Zhang, Z.; Yi, J.; He, C.; Wang, F.; Tian, X.; Yang, M.; Song, Y.; He, P.; et al. Resveratrol compares with melatonin in improving in vitro porcine oocyte maturation under heat stress. *J. Anim. Sci. Biotechnol.* **2016**, *7*, 33. [[CrossRef](#)]
162. Li, Y.; Zhang, Z.; He, C.; Zhu, K.; Xu, Z.; Ma, T.; Tao, J.; Liu, G. Melatonin protects porcine oocyte in vitro maturation from heat stress. *J. Pineal. Res.* **2015**, *59*, 365–375. [[CrossRef](#)]
163. Kojima, T.; Udagawa, K.; Onishi, A.; Iwahashi, H.; Komatsu, Y. Effect of heat stress on development in vitro and in vivo and on synthesis of heat shock proteins in porcine embryos. *Mol. Reprod. Dev.* **1996**, *43*, 452–457. [[CrossRef](#)]
164. Isom, S.C.; Lai, L.; Prather, R.S.; Rucker, E.B., 3rd. Heat shock of porcine zygotes immediately after oocyte activation increases viability. *Mol. Reprod. Dev.* **2009**, *76*, 548–554. [[CrossRef](#)] [[PubMed](#)]
165. Isom, S.C.; Prather, R.S.; Rucker Iii, E.B. Enhanced developmental potential of heat-shocked porcine parthenogenetic embryos is related to accelerated mitogen-activated protein kinase dephosphorylation. *Reprod. Fertil. Dev.* **2009**, *21*, 892–900. [[CrossRef](#)] [[PubMed](#)]
166. Hwang, I.S.; Park, M.R.; Moon, H.J.; Shim, J.H.; Kim, D.H.; Yang, B.C.; Ko, Y.G.; Yang, B.S.; Cheong, H.T.; Im, G.S. Osmolarity at early culture stage affects development and expression of apoptosis related genes (Bax-alpha and Bcl-xl) in pre-implantation porcine NT embryos. *Mol. Reprod. Dev.* **2008**, *75*, 464–471. [[CrossRef](#)] [[PubMed](#)]
167. Marchal, R.; Feugang, J.M.; Perreau, C.; Venturi, E.; Terqui, M.; Mermillod, P. Meiotic and developmental competence of prepubertal and adult swine oocytes. *Theriogenology* **2001**, *56*, 17–29. [[CrossRef](#)]
168. Bagg, M.A.; Nottle, M.B.; Grupen, C.G.; Armstrong, D.T. Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental potential of in vitro matured pre-pubertal and adult pig oocytes. *Mol. Reprod. Dev.* **2006**, *73*, 1326–1332. [[CrossRef](#)]
169. Bagg, M.A.; Nottle, M.B.; Armstrong, D.T.; Grupen, C.G. Relationship between follicle size and oocyte developmental competence in prepubertal and adult pigs. *Reprod. Fertil. Dev.* **2007**, *19*, 797–803. [[CrossRef](#)] [[PubMed](#)]
170. Hyun, S.H.; Lee, G.S.; Kim, D.Y.; Kim, H.S.; Lee, S.H.; Kim, S.; Lee, E.S.; Lim, J.M.; Kang, S.K.; Lee, B.C.; et al. Effect of maturation media and oocytes derived from sows or gilts on the development of cloned pig embryos. *Theriogenology* **2003**, *59*, 1641–1649. [[CrossRef](#)]
171. Amann, R.P.; Waberski, D. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology* **2014**, *81*, 5–17. [[CrossRef](#)]
172. Vazquez, J.M.; Martinez, E.; Pastor, L.M.; Roca, J.; Matas, C.; Calvo, A. Lectin histochemistry during in vitro capacitation and acrosome reaction in boar spermatozoa: New lectins for evaluating acrosomal status of boar spermatozoa. *Acta Histochem.* **1996**, *98*, 93–100. [[CrossRef](#)]
173. Garner, D.L.; Pinkel, D.; Johnson, L.A.; Pace, M.M. Assessment of spermatozoal function using dual fluorescent staining and flow cytometric analyses. *Biol. Reprod.* **1986**, *34*, 127–138. [[CrossRef](#)]
174. Kerns, K.; Zigo, M.; Drobnis, E.Z.; Sutovsky, M.; Sutovsky, P. Zinc ion flux during mammalian sperm capacitation. *Nature Communications* **2018**, *9*, 2061. [[CrossRef](#)]
175. Huo, L.-J.; Yue, K.-Z.; Yang, Z.-M. Characterization of viability, mitochondrial activity, acrosomal integrity and capacitation status in boar sperm during in vitro storage at different ambient temperatures. *Reprod. Fertil. Dev.* **2002**, *14*, 509. [[CrossRef](#)]
176. Hossain, M.S.; Johannisson, A.; Siqueira, A.P.; Wallgren, M.; Rodriguez-Martinez, H. Spermatozoa in the sperm-peak-fraction of the boar ejaculate show a lower flow of Ca²⁺ under capacitation conditions post-thaw which might account for their higher membrane stability after cryopreservation. *Anim. Reprod. Sci.* **2011**, *128*, 37–44. [[CrossRef](#)]
177. Kerns, K.; Jankovitz, J.; Robinson, J.; Minton, A.; Kuster, C.; Sutovsky, P. Relationship between the Length of Sperm Tail Mitochondrial Sheath and Fertility Traits in Boars Used for Artificial Insemination. *Antioxidants* **2020**, *9*, 1033. [[CrossRef](#)]
178. Lovercamp, K.W.; Safranski, T.J.; Fischer, K.A.; Manandhar, G.; Sutovsky, M.; Herring, W.; Sutovsky, P. Arachidonate 15-lipoxygenase and ubiquitin as fertility markers in boars. *Theriogenology* **2007**, *67*, 704–718. [[CrossRef](#)]
179. Guthrie, H.D.; Welch, G.R. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry1. *J. Anim. Sci.* **2006**, *84*, 2089–2100. [[CrossRef](#)]
180. Brouwers, J.F.; Silva, P.F.N.; Gadella, B.M. New assays for detection and localization of endogenous lipid peroxidation products in living boar sperm after BTS dilution or after freeze–thawing. *Theriogenology* **2005**, *63*, 458–469. [[CrossRef](#)]
181. Evenson, D.P.; Thompson, L.; Jost, L. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology* **1994**, *41*, 637–651. [[CrossRef](#)]
182. Parrilla, I.; Vazquez, J.; Oliver-Bonet, M.; Navarro, J.; Yelamos, J.; Roca, J.; Martinez, E. Fluorescence in situ hybridization in diluted and flow cytometrically sorted boar spermatozoa using specific DNA direct probes labelled by nick translation. *Reproduction* **2003**, *126*, 317–325. [[CrossRef](#)] [[PubMed](#)]
183. Steele, H.; Makri, D.; Maalouf, W.E.; Reese, S.; Kölle, S. Bovine Sperm Sexing Alters Sperm Morphokinetics and Subsequent Early Embryonic Development. *Sci. Rep.* **2020**, *10*, 1–13. [[CrossRef](#)] [[PubMed](#)]
184. Suh, T.K.; Schenk, J.L.; Seidel, G.E. High pressure flow cytometric sorting damages sperm. *Theriogenology* **2005**, *64*, 1035–1048. [[CrossRef](#)] [[PubMed](#)]

185. Umezū, K.; Hiradate, Y.; Numabe, T.; Hara, K.; Tanemura, K. Effects on glycoalyx structures of frozen-thawed bovine sperm induced by flow cytometry and artificial capacitation. *J. Reprod. Dev.* **2017**, *63*, 473–480. [[CrossRef](#)] [[PubMed](#)]
186. Saunders, C.M.; Larman, M.G.; Parrington, J.; Cox, L.J.; Royse, J.; Blayney, L.M.; Swann, K.; Lai, F.A. PLC ζ : A sperm-specific trigger of Ca²⁺ oscillations in eggs and embryo development. *Development* **2002**, *129*, 3533–3544. [[CrossRef](#)]
187. Wu, A.T.; Sutovsky, P.; Manandhar, G.; Xu, W.; Katayama, M.; Day, B.N.; Park, K.W.; Yi, Y.J.; Xi, Y.W.; Prather, R.S.; et al. PAWP, a Sperm-specific WW Domain-binding Protein, Promotes Meiotic Resumption and Pronuclear Development during Fertilization. *J. Biol. Chem.* **2007**, *282*. [[CrossRef](#)] [[PubMed](#)]
188. Seeler, J.F.; Sharma, A.; Zaluzec, N.J.; Bleher, R.; Lai, B.; Schultz, E.G.; Hoffman, B.M.; Labonne, C.; Woodruff, T.K.; O'Halloran, T.V. Metal ion fluxes controlling amphibian fertilization. *Nat. Chem.* **2021**, *13*, 683–691. [[CrossRef](#)]
189. Que, E.L.; Duncan, F.E.; Lee, H.C.; Hornick, J.E.; Vogt, S.; Fissore, R.A.; O'Halloran, T.V.; Woodruff, T.K. Bovine eggs release zinc in response to parthenogenetic and sperm-induced egg activation. *Theriogenology* **2019**, *127*, 41–48. [[CrossRef](#)]
190. Kim, A.M.; Bernhardt, M.L.; Kong, B.Y.; Ahn, R.W.; Vogt, S.; Woodruff, T.K.; O'Halloran, T.V. Zinc sparks are triggered by fertilization and facilitate cell cycle resumption in mammalian eggs. *ACS Chem. Bio* **2011**, *6*, 716–723. [[CrossRef](#)]
191. Lee, K.; Davis, A.; Zhang, L.; Ryu, J.; Spate, L.D.; Park, K.-W.; Samuel, M.S.; Walters, E.M.; Murphy, C.N.; Machaty, Z.; et al. Pig oocyte activation using a Zn²⁺ chelator, TPEN. *Theriogenology* **2015**, *84*, 1024–1032. [[CrossRef](#)] [[PubMed](#)]
192. Kerns, K.; Sharif, M.; Zigo, M.; Xu, W.; Hamilton, L.E.; Sutovsky, M.; Ellersieck, M.; Drobnis, E.Z.; Bovin, N.; Oko, R.; et al. Sperm Cohort-Specific Zinc Signature Acquisition and Capacitation-Induced Zinc Flux Regulate Sperm-Oviduct and Sperm-Zona Pellucida Interactions. *Int. J. Mol. Sci.* **2020**, *21*, 2121. [[CrossRef](#)] [[PubMed](#)]
193. Que, E.L.; Duncan, F.E.; Bayer, A.R.; Philips, S.J.; Roth, E.W.; Bleher, R.; Gleber, S.C.; Vogt, S.; Woodruff, T.K.; O'Halloran, T.V. Zinc sparks induce physiochemical changes in the egg zona pellucida that prevent polyspermy. *Integr. Biol.* **2017**, *9*, 135–144. [[CrossRef](#)] [[PubMed](#)]
194. Bonk, A.J.; Cheong, H.T.; Li, R.; Lai, L.; Hao, Y.; Liu, Z.; Samuel, M.; Fergason, E.A.; Whitworth, K.M.; Murphy, C.N.; et al. Correlation of developmental differences of nuclear transfer embryos cells to the methylation profiles of nuclear transfer donor cells in Swine. *Epigenetics* **2007**, *2*, 179–186. [[CrossRef](#)] [[PubMed](#)]
195. Bonk, A.J.; Li, R.; Lai, L.; Hao, Y.; Liu, Z.; Samuel, M.; Fergason, E.A.; Whitworth, K.M.; Murphy, C.N.; Antoniou, E.; et al. Aberrant DNA methylation in porcine in vitro-, parthenogenetic-, and somatic cell nuclear transfer-produced blastocysts. *Mol. Reprod. Dev.* **2008**, *75*, 250–264. [[CrossRef](#)] [[PubMed](#)]
196. Ivanova, E.; Canovas, S.; Garcia-Martinez, S.; Romar, R.; Lopes, J.S.; Rizos, D.; Sanchez-Calabuig, M.J.; Krueger, F.; Andrews, S.; Perez-Sanz, F.; et al. DNA methylation changes during preimplantation development reveal inter-species differences and reprogramming events at imprinted genes. *Clin. Epigenetics* **2020**, *12*, 64. [[CrossRef](#)] [[PubMed](#)]
197. Jost, J.P.; Saluz, H.P.; McEwan, I.; Feavers, I.M.; Hughes, M.; Reiber, S.; Liang, H.M.; Vaccaro, M. Tissue specific expression of avian vitellogenin gene is correlated with DNA hypomethylation and in vivo specific protein-DNA interactions. *Philos Trans. R Soc. Lond. B Biol. Sci.* **1990**, *326*, 231–240. [[CrossRef](#)]
198. Burbelo, P.D.; Horikoshi, S.; Yamada, Y. DNA methylation and collagen IV gene expression in F9 teratocarcinoma cells. *J. Biol. Chem.* **1990**, *265*, 4839–4843. [[CrossRef](#)]
199. Knust, B.; Bruggemann, U.; Doerfler, W. Reactivation of a methylation-silenced gene in adenovirus-transformed cells by 5-azacytidine or by E1A trans activation. *J. Virol.* **1989**, *63*, 3519–3524. [[CrossRef](#)]
200. Gerondakis, S.; Boyd, A.; Bernard, O.; Webb, E.; Adams, J.M. Activation of immunoglobulin mu gene expression involves stepwise demethylation. *EMBO J.* **1984**, *3*, 3013–3021. [[CrossRef](#)]
201. Zhao, J.; Ross, J.W.; Hao, Y.; Spate, L.D.; Walters, E.M.; Samuel, M.S.; Rieke, A.; Murphy, C.N.; Prather, R.S. Significant improvement in cloning efficiency of an inbred miniature pig by histone deacetylase inhibitor treatment after somatic cell nuclear transfer. *Biol. Reprod.* **2009**, *81*, 525–530. [[CrossRef](#)]
202. Jeong, P.S.; Sim, B.W.; Park, S.H.; Kim, M.J.; Kang, H.G.; Nanjidsuren, T.; Lee, S.; Song, B.S.; Koo, D.B.; Kim, S.U. Chaetocin Improves Pig Cloning Efficiency by Enhancing Epigenetic Reprogramming and Autophagic Activity. *Int. J. Mol. Sci.* **2020**, *21*, 4836. [[CrossRef](#)]
203. Zhao, C.; Shi, J.; Zhou, R.; He, X.; Yang, H.; Wu, Z. DZNep and UNC0642 enhance in vitro developmental competence of cloned pig embryos. *Reproduction* **2018**, *157*, 359–369. [[CrossRef](#)] [[PubMed](#)]
204. Yu, T.; Qi, X.; Zhang, L.; Ning, W.; Gao, D.; Xu, T.; Ma, Y.; Knott, J.G.; Sathanawongs, A.; Cao, Z.; et al. Dynamic reprogramming and function of RNA N(6)-methyladenosine modification during porcine early embryonic development. *Zygote* **2021**, 1–10. [[CrossRef](#)] [[PubMed](#)]
205. Zhao, J.; Whyte, J.; Prather, R.S. Effect of epigenetic regulation during swine embryogenesis and on cloning by nuclear transfer. *Cell Tissue Res.* **2010**, *341*, 13–21. [[CrossRef](#)]
206. Li, G.; Jia, Q.; Zhao, J.; Li, X.; Yu, M.; Samuel, M.S.; Zhao, S.; Prather, R.S.; Li, C. Dysregulation of genome-wide gene expression and DNA methylation in abnormal cloned piglets. *BMC Genom.* **2014**, *15*, 811. [[CrossRef](#)] [[PubMed](#)]

-
207. Whitworth, K.M.; Mao, J.; Lee, K.; Spollen, W.G.; Samuel, M.S.; Walters, E.M.; Spate, L.D.; Prather, R.S. Transcriptome Analysis of Pig In Vivo, In Vitro-Fertilized, and Nuclear Transfer Blastocyst-Stage Embryos Treated with Histone Deacetylase Inhibitors Postfusion and Activation Reveals Changes in the Lysosomal Pathway. *Cell. Reprogram* **2015**, *17*, 243–258. [[CrossRef](#)]
 208. Mao, J.; Zhao, M.T.; Whitworth, K.M.; Spate, L.D.; Walters, E.M.; O’Gorman, C.; Lee, K.; Samuel, M.S.; Murphy, C.N.; Wells, K.; et al. Oxamflatin treatment enhances cloned porcine embryo development and nuclear reprogramming. *Cell. Reprogram* **2015**, *17*, 28–40. [[CrossRef](#)]