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Review Article

Reactive oxygen and nitrogen species regulate porcine embryo development during pre-implantation period: A mini-review

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ABSTRACT

Significant porcine embryonic loss occurs during conceptus morphological elongation and attachment from d 10 to 20 of pregnancy, which directly decreases the reproductive efficiency of sows. A successful establishment of pregnancy mainly depends on the endometrium receptivity, embryo quality, and utero-placental microenvironment, which requires complex cross-talk between the conceptus and uterus. The understanding of the molecular mechanism regulating the uterine-conceptus communication during porcine conceptus elongation and attachment has developed in the past decades. Reactive oxygen and nitrogen species, which are intracellular reactive metabolites that regulate cell fate decisions and alter their biological functions, have recently reportedly been involved in porcine conceptus elongation and attachment. This mini-review will mainly focus on the recent researches about the role of reactive oxygen and nitrogen species in regulating porcine embryo development during the pre-implantation period.

1. Introduction

Pigs suffer 20% to 40% embryonic loss during the pre-implantation period from d 10 to 20 of pregnancy, which is a critical period for conceptus elongation and attachment, and placentation (Bazer and First, 1983). Conceptus trophectoderm and inner cell mass (ICM), and uterine luminal epithelium (LE) and glandular epithelium (GE), constitute the maternal–fetal interface during this period. The conceptus trophectoderm forms the placenta and the ICM gives rise to the embryo proper. The trophectoderm directly contacts the uterine histotrophic and provides nutrient substrates for the ICM (Houghton, 2006). A better understanding of the molecular mechanism regulating porcine conceptus elongation and attachment would provide new targets for decreasing early embryo loss and improving the efficiency of swine production. Factors including endocrine hormones, adhesions, inflammatory mediators, nutrients, environmental insults, and epigenetic modifications have been previously reported to affect cell proliferation, migration and endometrium receptivity at the porcine maternal–fetal interface (Bazer and Johnson, 2014; Bazer et al., 2014; Kong et al., 2019). However, the molecular mechanism of these effects remains yet to be investigated. Reactive oxygen and nitrogen species (ROS/RNS), which are involved in a series of signal transduction pathways and biological activities through redox-dependent regulation, have been reported to regulate embryo development and placentation. Furthermore, the excessive production or deficiency of ROS/RNS causes apoptosis and inhibits embryonic development in mice (Tranguch et al., 2003). Given the growing evidence regarding ROS/RNS biology in porcine conceptus elongation and attachment, this mini-review summarizes the recent researches about the role of ROS/RNS in regulating porcine embryo development during the pre-implantation period.

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2. Activation of redox pathways in porcine conceptus trophectoderm

ROS/RNS are reactive molecules mainly including superoxide ($O_2\cdot$), hydrogen peroxide (H_2O_2), hydroxyl ($OH\cdot$), nitric oxide (NO) and peroxynitrite ($ONOO\cdot$) (Fig. 1), which play a central role in signaling transduction pathways, metabolic control and functional maintenance. Oxygen consumption is low during the porcine embryo cleavage period, but oxygen consumption, adenosine triphosphate (ATP) and lactate production increase at the porcine blastocyst stage, indicating a high energy metabolism during the blastocyst period (Sturmey and Leese, 2003). The trophectoderm cells (containing elongated and tubular mitochondria with well-developed cristae structures and deeply stained membrane) were reported to have higher energy metabolism and metabolic requirements than ICM (containing spherical mitochondria with few cristae structures) in pre-implantation embryos of humans and mice (Hashimoto et al., 2017; Houghton, 2006). This suggests that high mitochondrial metabolic activity in the trophectoderm is primarily responsible for blastocyst development during the pre-implantation period. Therefore, it is possible that this high metabolic activity determines ROS production, as the inhibition of mitochondrial oxidative phosphorylation reduces ROS generation and promotes porcine embryo development in vitro (Machaty et al., 2000). A previous study of pigs indicated that gene expression of glutathione peroxidase 1 (*GPX1*), microsomal glutathione S-transferase 1 (*MGST1*) and cytoplasmic copper-zinc superoxide dismutase (*SOD1*) increased during conceptus elongation (Blomberg et al., 2005). Similarly, a serum proteome-based study of pigs also demonstrated that GPX3 and copper-containing acute-phase protein increased during this period (De et al., 2019). Mun et al. (2017) indicated that decreased ROS impaired porcine embryo development during the early in vitro culture phase, but improved developmental competence during the late in vitro culture phase. They also found that embryonic ROS was closely associated with porcine trophectoderm (pTr) cell proliferation and differentiation, instead of ICM (Mun et al., 2017). In ovine conceptus trophectoderm, the knockdown of NO synthase-3 (*NOS3*, synthesis of NO) resulted in small and underdeveloped conceptus (Wang et al., 2014). Furthermore, dietary supplementation with 0.8% L-arginine (precursor of NO) reduced the embryonic survival rate between d 0 and 25 of gestation, but increased embryonic development and survival between d 14 and 25 (Li et al., 2010, 2014), which suggests that RNS is also essential for porcine conceptus elongation and development. These results indicate that the ROS/RNS levels play a key role in porcine conceptus elongation and development during the pre-implantation period.

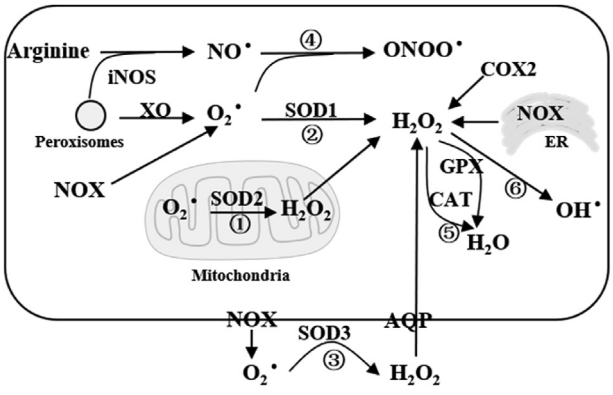

Fig. 1. ROS production and their modulators. ① The $O_2\cdot$ from mitochondrial respiratory chain is catalyzed by SOD2 to form H_2O_2. ②③ The $O_2\cdot$ from NOX and XO are catalyzed by SOD1 and SOD3 to produce H_2O_2. ④ $O_2\cdot$ can also react with NO from arginine decomposition to produce $OONO\cdot$. ⑤ H_2O_2 could be converted into water by CAT and GPX. ⑥ H_2O_2 can also react wth iron (2+) to form OH^- through Fenton reaction. Due to the high reactivity of H_2O_2, $O_2\cdot$, $ONOO^-$ and OH^-, they can result in oxidative injury of protein, DNA and lipids and cell death. $O_2\cdot$ = superoxide; H_2O_2 = hydrogen peroxide; $OH\cdot$ = hydroxyl; $NO\cdot$ = nitric oxide; $ONOO\cdot$ = peroxynitrite; iNOS = inducible NO synthase; SOD1 = cytoplasmic copper-zinc superoxide dismutase; SOD2 = mitochondrial Mn superoxide dismutase; SOD3 = extracellular copper-zinc superoxide dismutase; GPX = glutathione peroxidase; CAT = catalase; NOX = NADPH oxidases; XO = xanthine oxidase; COX2 = cyclooxygenase-2.

The major sites responsible for intracellular ROS/RNS production are the mitochondrial electron transport chain, endoplasmic reticulum (ER) system, NADPH oxidases (NOX), NOS and peroxisomes (Fig. 1) (Sies and Jones, 2020). The contribution of ROS production in embryos is different depending on the species, the stage of development, and the culture conditions in the pre-implantation period (Guérin et al., 2001). Of those, mitochondria are multifunctional organelles that regulate ATP generation, citric acid cycle and signaling transduction. Mitochondrial DNA is also particularly susceptible to ROS because of lacking of self-repairing systems, which leads to structural changes and dysfunction (Yakes and Houten, 1997). The accumulation of dysfunctional mitochondria within cells results in the overproduction of ROS. Mitochondrial quality control such as biogenesis, dynamics (fission and fusion), and mitophagy serves as a mechanism to regulate the mitochondrial homeostasis and ROS production (Willems et al., 2015). Sirtuins (SIRT) and peroxisome proliferator-activated receptor-γ co-activator alpha (PGC1α) regulate mitochondrial biogenesis at a transcriptional level, and activate the downstream mitochondrial transcription factor A (TFAM) and nuclear respiratory factors (NRF) to maintain the cellular redox balance (Scarpulla et al., 2012). Our previous study showed that the increase of ROS activated the *SIRT1*/*PGC1α*/*NRF* pathways, suggesting that ROS act as important regulators to maintain mitochondrial biogenesis in pTr cells (Luo et al., 2019). SIRT3, a mitochondrial protein deacetylase that is responsible for detoxifying ROS by PGC1α, promoted AMPK-mTOR-dependent autophagy pathways and decreased GPX4-dependent ferroptosis in pTr cells, which indicates that mitochondrial biogenesis is a pro-survival mechanism that responds to excessive ROS (Han et al., 2020). A recent study demonstrated that melatonin promoted the expression of implantation-related genes, including adiponectin receptor 1 and 2 (*ADIPOR1* and *ADIPOR2*), cyclin D1, and an insulin-like growth factor receptor. It also caused the proliferation of cell nuclear antigens in pTr and LE cells through regulation of SIRT1, which suggests that ROS-SIRT1 pathway positively regulate porcine uterine-conceptus interactions (Bae et al., 2020b).

2.1. ROS and endocrine system during pre-implantation period

Porcine conceptus estrogen, and ovarian progesterone and prostaglandins are essential for maternal recognition and maintenance of pregnancy, conceptus mobility, elongation and implantation during the pre-implantation period (Bazer and Johnson, 2014). A previous study indicated that the expression of steroidogenic genes including cytochrome P450 family 11 (*CYP11A1*), steroidogenic acute regulatory protein (*STAR*) and aromatase (*CYP19A1*) increased during porcine conceptus elongation (Blomberg et al., 2005). Both CYP11A1 and STAR are located in mitochondria. CYP11A1 is the enzyme responsible for the cleavage of cholesterol to produce pregnenolone, and STAR is responsible for transporting cholesterol across the mitochondrial membrane, which indicates that mitochondria are the sites that link steroid hormone synthesis and ROS production (Chow et al., 2017). Thus, mitochondrial dysfunction may lead to a decreased intracellular or extracellular secretion of steroid hormones. Furthermore, estradiol is able to regulate mitochondrial oxidative

phosphorylation, dynamics and redox homeostasis through estrogen receptors α and β, which are also located in mitochondria (Klinge, 2020). Estrogen increased $O_2\cdot$ production by decreasing SOD activity and increasing inducible NOS (iNOS) expression. Progesterone decreased $O_2\cdot$ production by increasing SOD activity and endothelial NOS (eNOS), as well as iNOS expression during implantation in the uterus of mice and rats (Laloraya et al., 1996; Ogando et al., 2003). The gene expression of *SOD1* was increased but mitochondrial Mn superoxide dismutase (*SOD2*) was decreased in the porcine filamentous conceptuses (Blomberg et al., 2005). It is probable that the decrease of SOD2 is attributable to the increase of estrogen production during implantation period, however this needs to be verified. Recently, low-dose N-acetyl-L-cysteine (NAC, a ROS scavenger) was reported to decrease pTr cell progesterone production, and increase estradiol production, *CYP19A1* and *NOS3* gene expression (Ding et al., 2021). Taken together, these results indicate close interactions between the endocrine system and redox homeostasis during the pre-implantation period.

Knockout of conceptus *CYP19A1* gene decreased estrogen content, but had no effect on testosterone, and prostaglandin F2α (PGF2α) contents in the uterine flushings on d 14 and 17. Additionally, *CYP19A1*$^{-/-}$ embryos aborted between d 27 and 31 and exogenous estrogen failed to maintain pregnancy, which suggests that estrogen is not essential for conceptus elongation, attachment and placentation but for the maintenance of pregnancy beyond d 24 (Meyer et al., 2019). An analysis of endometrial transcriptome revealed that extracellular copper-zinc superoxide dismutase (*SOD3*) and *NOX3* were increased, but *MGST2* was decreased in *CYP19A1*$^{-/-}$ gilts from d 14 to 17, indicating that the change of uterine ROS levels were in response to a deficiency of conceptus estrogen, as MGST2 can bind glutathione and form a thiolate to reduce peroxide (Meyer et al., 2019; Ahmad et al., 2015). Prostaglandin biosynthesis involves the direct oxygenation of arachidonic acid via the lipoxygenase and cyclooxygenase (COX) pathways, which also involves ROS production such as hydroperoxyl radical. Furthermore, H_2O_2, NO and peroxide-initiated free radicals are involved in regulating COX activity and prostaglandin biosynthesis through different mechanisms (Panganamala et al., 1974; Kim (2011); Hemler and Lands, 1980), which suggest a reciprocal interaction between free radicals and prostaglandin biosynthesis. Prostaglandin E2 (PGE2) and PGF2α are the major prostaglandins responsible for luteotropic and luteolytic processes in mammals. Previously, PGF2α was reported as playing a luteolytic role through ROS-mediation mechanisms in luteal cells and corpus luteum of rats (Tanaka et al., 2000; Riley and Behrman, 1991). The treatment of porcine trophoblast cells in vitro with luteolytic PGF2α promoted cell proliferation, adhesion and migration, and an increased expression of endometrial angiogenic factors (Piotr et al., 2018; Kaczynski et al., 2020). This indicated a new role for PGF2α secreted by the conceptuses and endometrium in supporting pregnancy establishment during the implantation period. However, the depletion of the rate-limiting enzyme prostaglandin-endoperoxide synthase 2 (*PTGS2* or *COX2*) in conceptus had no effect on either PGF2α and PGE contents in the uterine flushings or conceptus elongation in pigs (Pfeiffer et al., 2020). Whether ROS were involved in this process remains unknown. Thus, further studies are still needed to clarify the potential mechanism between the endocrine system and ROS production during pre-implantation period.

2.2. ROS and inflammation during pre-implantation period

Early embryo implantation relies on a proinflammatory environment. During the pre-implantation period, porcine conceptuses secrete interleukin (IL)-1B and interferons (delta and gamma) to promote conceptus development and implantation (Bazer and Johnson, 2014). Gene expression of porcine *IL-1B*, IL-1 receptor type 1 (*IL-1RT1*), and the IL-1 receptor accessory protein (*IL-1RAP*) is increased during rapid trophoblastic elongation (Ross et al., 2003). Pig conceptus expresses a novel isoform IL-1B2, which binds to IL-1R1 on the uterine luminal epithelia to stimulate nuclear translocation of nuclear factor-kappa B and initiate a cascade of signaling pathways such as inflammation and cell adhesion (Mathew et al., 2015). Knockout of pig conceptus *IL-1B2* leads to the failure of rapid conceptus elongation, decreased estrogen secretion and increased gene expression of conceptus interferons delta and PGTS2, suggesting that the proinflammatory environment of the uterus in response to conceptus IL1B2 is important for conceptus elongation and attachment (Whyte et al., 2018). Inflammatory mediators such as cytokines and chemokines act in an autocrine and paracrine manner to recruit macrophages, dendritic cells, natural killer cells and T cells to the porcine endometrium during implantation and placentation (Kridli et al., 2016). Recent studies reported that the expression of cysteine-X-cysteine motif chemokine ligands 9 (*CXCL9*), *CXCL10*, *CXCL11* and CXC chemokine receptor type 3 (*CXCR3*) (Han et al., 2017), C–C motif chemokine ligand 2 (*CCL2*)-atypical chemokine receptor (Lim et al., 2018b), *CCL4-CCR5* (Lim et al., 2018c), *CCL5-CCR3* (Bae et al., 2020a), *CCL20-CCR6* (Park et al., 2019a), *CCL21-CCR7* (Bae et al., 2019), *CCL23-CCR1* (Jeong et al., 2017) and *CXCL12-CXCR4* (Han et al., 2018) genes was increased in porcine endometrium from d 10 to 30 during pregnancy. Inflammasome can activate caspase-1 to induce the proinflammatory cytokines IL-1β and IL-18 production. Porcine endometrial expression of caspase-1 and IL-18 was increased during the pre-implantation period (Ashworth et al., 2010). Previously, we reported that NAC inhibits H_2O_2-induced gene expression of *IL-1β* in pTr cells (Luo et al., 2019). Importantly, how the immune cells are recruited to the maternal–fetal interface and how the uterine epithelial immune response is activated remain unknown. ROS such as H_2O_2 are reported to act as a chemoattractant in chemokine-dependent T cell migration and are responsible for activating nucleotide-binding oligomerization domain-like receptors containing pyrin domain 3 (NLRP3) inflammasome (Sena and Chandel, 2012). Cytokines and inflammatory cells also appear to signal through ROS, and the release of ROS is probably another form of cross-talk when inflammatory responses are sustained (Sena and Chandel, 2012). Further studies are needed to explore the complex interplay between ROS and inflammation within the conceptus and endometrium during the pre-implantation period.

3. ROS/RNS regulate porcine conceptus development

3.1. ROS and environmental insults

Environmental insults can disrupt the intrauterine environment and have an adverse effect on fetal development as the embryos are particularly susceptible to the environmental changes. Exposure to insecticides, such as trichlorfon, fenbendazole, ivermectin, oxibendazole and etoxazole to pTr and uterine LE cells significantly inhibits cell proliferation and migration and promotes cell apoptosis, as accompanied by the loss of mitochondrial membrane potential. Additionally, it increases mitochondrial Ca^{2+} overload and ROS generation in LE. These effects may result in abnormal embryo development during early pregnancy (Lee et al., 2019; Lim et al., 2018a; Park et al., 2019b, c; Park et al., 2020). These results suggest that uterine ROS may be involved in abnormal porcine embryo development induced by toxin exposure during early pregnancy.

3.2. ROS and aquaporins

Aquaporins (AQP) are responsible for transport of water and non-polar solutes such as H_2O_2 across biological membranes, which play an important role in human cells (Miller et al., 2010). AQP1, 3, 5, and 9 were expressed in the LE and GE of porcine endometrium and trophectoderm cells during early gestation. The injection of estrogen, progesterone, and relaxin can induce AQP3 expression in pTr cells in vitro although conceptus does not have an estrogen receptor at this time (Zhu et al., 2018). Furthermore, *AQP3* in the ICM and trophectoderm of later blastocyst was highly expressed in porcine pre-implantation embryos (Wei et al., 2018). AQP may modulate H_2O_2 membrane permeability as a general way to control its downstream effects such as cell migration and inflammation in mammals (Meli et al., 2018). Whether the aquaporins-mediated H_2O_2 transport is involved in porcine conceptus elongation and uterine receptivity during early pregnancy is unknown.

3.3. ROS and hypoxia-inducible factors

Pre-implantation blastocysts were exposed to low oxygen tension during implantation in mammals such as monkeys, hamsters and rabbits (Fischer and Bavister, 1993). The decreased oxygen (hypoxia) can affect the embryo metabolism and development during pre-implantation in mice (Kelley and Gardner, 2019). Hypoxia-inducible factors (HIF), the redox sensitive transcription factors, are responsible for mediating the embryo adaptation in response to hypoxia. Hypoxia can increase ROS production, whereas the treatment of cells with H_2O_2 can result in HIF-1α accumulation (Thomas and Ashcroft, 2019). Low oxygen (5% O_2) increases pTr cell proliferation in a HIF-1α dependent manner but does not affect HIF-1β expression. In addition, depletion of *HIF-1α* reduces hypoxia-induced G1 arrest, p21 and p27 protein expression, and increases S phase cells (Jeong et al., 2016, 2018). Similarly, we reported that an extracellular H_2O_2 treatment increased pTr cell ROS production and the proportion of S and G2/M phase cells (Luo et al., 2018). Low oxygen tension-activated autophagy is a supplementary way to alleviate low oxygen stress, and promote porcine embryos development (Zhou et al., 2020b). This is in accordance with our previous study showing that ROS-induced autophagy regulates pTr cell proliferation and differentiation (Luo et al., 2019). However, the information about the precise relationship between ROS and HIF-1 protein stabilization in pre-implantation embryos is limited.

3.4. ROS and epigenetics

Epigenetics, including histone modification, DNA methylation and non-coding RNA, is involved in the fine tuning of the expression of genes responsible for the establishment of pregnancy without changes to the DNA sequence (Kong et al., 2019). MiRNA, such as miR-26a and miR-125b, have been detected in porcine uterine luminal flushing, and miR-92b-3p and miR-17-5p in serum increased during early pregnancy (Krawczynski et al., 2015; Zhou et al., 2020a). Furthermore, lncRNA-*ssc-miR*-132-mRNA

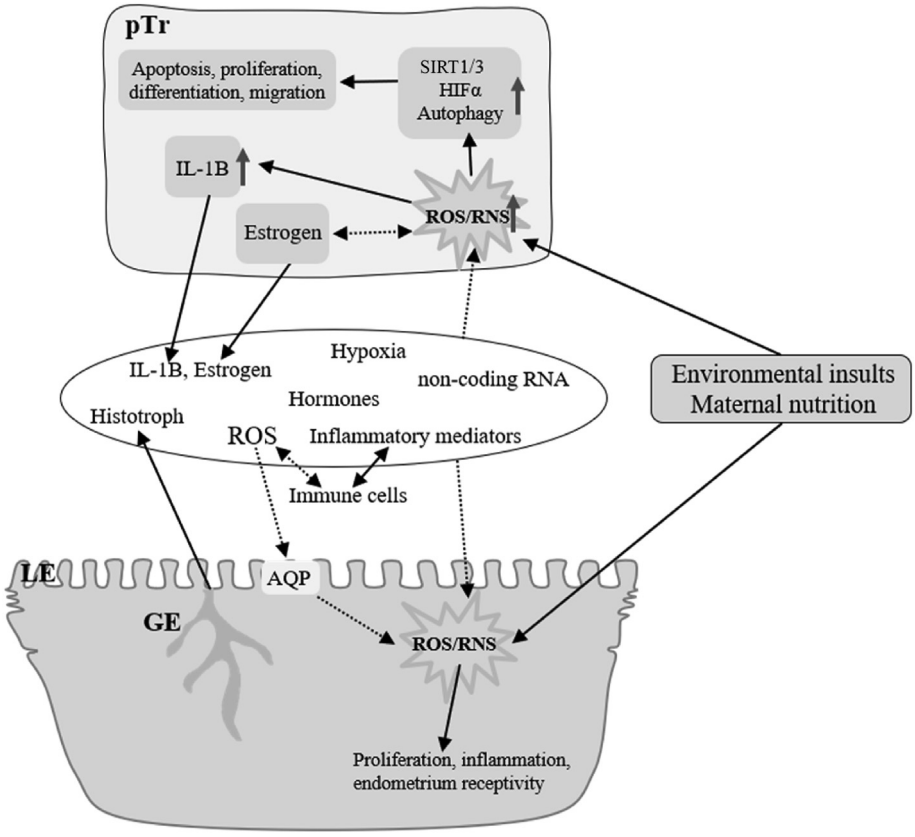

Fig. 2. Schematic pathway of the role of ROS in regulating porcine embryo development and uterine receptivity during pre-implantation period. The environmental insults, maternal nutrition and uterine microenvironment such as hypoxia and inflammation increase ROS production, which regulates cell proliferation, migration and attachment at the maternal-interface through activation of downstream pathways such as SIRT, autophagy, etc. The solid box indicates the reported regulation of porcine embryo development during pre-implantation period, and the dashed box indicates that the role still need to be determined. pTr = porcine trophectoderm; SIRT = sirtuin; HIFα = hypoxia-inducible factor α; IL = interleukin; ROS/RNS = reactive oxygen and nitrogen species; LE = luminal epithelium; GE = glandular epithelium; AQP = aquaporin.

interactions in porcine endometrium provide a regulatory network for the embryonic implantation process (Wang et al., 2017). Maternal estradiol exposure does not affect miRNA expression in porcine blastocysts, but causes DNA hypomethylation of the cyclin dependent kinase inhibitor 2D and phosphoserine aminotransferase 1 (*PSAT1*) genes in blastocysts (Bick et al., 2018; van der Weijden et al., 2018). Additionally, maternal malnutrition during embryonic development and implantation increased methylation levels of *ADIPOR2* and DNA (cytosine-5)-methyltransferase 1 (*DNMT1*), and decreased *DNMT1* expression in pre-implantation embryos of pigs (Zglejc-Waszak et al., 2019). These results suggest that maternal endocrine changes and nutrient availability mainly affect porcine embryo methylation. Indeed, an overall DNA demethylation after fertilization and remethylation around implantation takes place in the porcine embryos. Ten-eleven translocation (TET1-3) and thymine DNA glycosylase are the major enzymes responsible for DNA demethylation. Ascorbic acid treatment increases the development of porcine somatic cell nuclear transfer embryos through TET3-mediated demethylation (Zhao et al., 2017), which suggests that a decrease of ROS levels increase DNA demethylation of porcine embryos. SIRT1, a NAD-dependent lysine deacetylase, modulate histone H3 lysine 9 (H3K9) deacetylation and increases H3K9me3 in the zygotic pronuclei, which is beneficial for in vitro blastocyst formation (Adamkova et al., 2017). Low-dose oxidative stress also increased global levels of histone H3 trimethylation at lysine 4 (H3K4me3) and lysine 27 (H3K27me3), and the activity of class I/II histone deacetylase, suggesting that a low dose of ROS alters the activity of enzymes responsible for histone demethylation and deacetylation to maintain redox homeostasis (Niu et al., 2015). However, little information is available about the interactions between ROS and epigenetics in vivo during the porcine pre-implantation period.

4. Conclusion

Significant embryonic loss remains a serious problem in pig production. Although evidence has suggested that ROS plays a significant role in pTr cell fate decision and biological function (Fig. 2), our current understanding about the mechanisms of ROS in regulating porcine conceptus elongation during the pre-implantation period is still limited. For example, which kind of ROS plays a major role during porcine conceptus elongation and attachment? Where is the kind of ROS from? How do the specific targets (listed in Fig. 2) of ROS affect the porcine conceptus elongation and attachment? As the oxygen tension is low in the uterine environment during implantation, how can we simulate the uterine environment to investigate the role of physiological ROS levels in vitro? Because ROS are involved in different biological pathways and have a dual function, a more in-depth investigation of the mechanism of ROS in regulating porcine conceptus elongation and the application of specific ROS scavengers is essential for a better understanding of this physiological process.

Author contributions

Zhen Luo drafted the manuscript; **Jianbo Yao** and **Jianxiong Xu** revised and approved final version of manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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