Stearoyl-coenzyme A desaturase 1 is required for lipid droplet formation in pig embryo

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Abstract

Lipid droplets (LD) provide a source of energy, and their importance during embryogenesis has been increasingly recognized. In particular, pig embryos have larger amounts of intercellular lipid bilayers than other mammalian species, suggesting that porcine embryos are more dependent on lipid metabolic pathways. The objective of the present study was to detect the effect of stearoyl-coenzyme A desaturase 1 (SCD1) on LD formation and to associate these effects with the mRNA abundance of LD formation-related genes (*SREBP*, *ARF1*, *COPG2*, *PLD1* and *ERK2*) in *in vitro*-produced porcine embryos. To determine the effect of SCD1 on LD formation and related genes, we examined the effects of SCD1 inhibition using CAY10566 (an SCD1 inhibitor, 50 μ M) on parthenogenetic embryos. SCD1 inhibition downregulated the mRNA levels of LD formation-related genes and embryo development. Our results revealed that SCD1 functions in the regulation of LD formation via phospholipid formation and embryo development. In addition, we treated parthenogenetic embryos with oleic acid (100 μ M), which led to a significant increase in the blastocyst formation rate, LD size and number compared to controls. Remarkably, the adverse effects of the SCD1 inhibitor could be counteracted by oleic acid. These data suggest that porcine embryos can use exogenous oleic acid as a metabolic energy source.

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Introduction

Mammalian oocyte-, egg- and cleavage-stage embryos rely almost exclusively on the metabolism of pyruvate and fatty acids (FAs) by mitochondria, which then produce energy in the form of ATP (Acton *et al.* 2004, Dumollard *et al.* 2008). FA metabolism is a major energy source for mitochondria and is particularly important in oocytes and preimplantation embryos. Lipid droplets (LDs) are unique organelles that store lipids and are essential for cellular energy and membrane production (Walther & Farese 2012, Thiam *et al.* 2013). LDs consist of a neutral lipid core primarily composed of triglycerides coated with a phospholipid monolayer. The importance of LDs as an energy source during embryogenesis has been increasingly recognized. To grow, proliferate and survive, the embryo must evolve mechanisms to quickly respond to lipid requirements. Pig embryos in particular contain numerous large LDs as well as large amounts of intercellular lipid bilayers compared to other species, suggesting that porcine embryos are more dependent on lipid metabolic pathways (Leese 2012). Studies have reported that LD degradation during embryo maturation can affect embryo development (Dunning *et al.* 2010,

Aardema *et al.* 2017). However, no studies have examined the development status of porcine embryos in relation to LD formation and degradation.

Previous studies have identified different populations of LDs in cells based on differences in their size, proteins and lipid composition (Wolins *et al.* 2005, Hsieh *et al.* 2012, Wilfling *et al.* 2013). LDs can be classified into two types according to size and stage of the LD life cycle. Initial LDs are formed in the endoplasmic reticulum (ER), presumably through a budding process, and appear to range from 300 to 600 nm in diameter (Wilfling *et al.* 2013). Initial LDs are thought to bud and detach from the ER in mammalian cells. A subset of initial LDs can be converted into expanding LDs with distinct protein compositions, including triglyceride synthesis enzymes that mediate their expansion (Wilfling *et al.* 2013). The ADP-ribosylation factor 1 (Arf1) and coatamer (a coat protein complex; COPI) machinery is required for this transition. Depletion of Arf1/COPI proteins from cells leads to the formation of relatively uniform LDs of a characteristic size that exhibit impaired lipolysis (Beller *et al.* 2008, Guo *et al.* 2008). We identified changes in coatamer subunit

gamma-2 (COPG2), a COPI component, in response to CAY10566 and oleic acid (OA). Stearoyl-coenzyme A desaturase (SCD) is responsible for converting saturated FAs into mono-unsaturated fatty acids (MUFAs) and also plays an important role in regulating FA composition. Although the number of SCD isoforms differs among species, SCD1 is a major isoform expressed in the lipogenic cells of all species studied to date (Hsieh *et al.* 2012). In addition, SCD1 plays an important role in regulating FA synthesis and metabolism by participating in multiple biological processes. FA metabolism appears to be essential for preimplantation development in all mammalian embryos (Sturmey *et al.* 2009), including those with relatively low lipid contents. Furthermore, a link between SCD activity and LDs has been observed in various cell lines cultured from goat mammary epithelial cells (Ren *et al.* 2018), mouse hepatocytes (Lounis *et al.* 2017) and human hepatoma cells (Lyn *et al.* 2014). Shi and colleague showed that SCD1-inhibited or -removal samples exhibited difficulties in LD formation (Shi *et al.* 2013). However, recent studies have revealed that the cytotoxic effects caused by the inhibition of FA synthesis can be reversed by exogenous FA supplementation. In particular, exogenous OA restored globally decreased levels of cell lipids in cells undergoing blockage of SCD activity, indicating that active lipid synthesis is required for the FA-mediated restoration of proliferation in SCD1-inhibited cells (Hess *et al.* 2010). Regulators of LDs include the family of transcriptional regulators of lipid synthetic genes, known as SREBPs. In particular, the presence of SREBPs leads to an increase in LD proteins in fatty liver dystrophic mice (Hall *et al.* 2010). Several studies have also demonstrated a positive correlation between LD levels and SREBP1 expression (Pai *et al.* 2013, Fukunishi *et al.* 2014, Min *et al.* 2014).

The objective of the present study was to assess stage-specific LD profiles during early development of *in vitro*-produced porcine embryos and to associate these profiles with the mRNA levels of LD formation-related genes and with the effect of SCD1.

Materials and methods

Ethics statement

The experimental use of pigs was approved by the Institutional animal care and use committee, Seoul National University (SNU-140328-2).

In vitro embryo production

Mature oocytes were obtained using the method described in our previous study (Lee *et al.* 2016). Briefly, prepubertal gilt ovaries collected in Anseong, Kyunggi Province, Korea, were used in this experiment. The follicular fluid and cumulus-oocyte complexes (COCs) were aspirated using an 18-gauge needle and then pooled to obtain sediments. Sediments were washed with TL-HEPES-PVA medium, and oocytes with compact

cumulus cells and granulated cytoplasm were selected for *in vitro* maturation. The washed COCs were cultured in a tissue culture medium (TCM-199; Life Technologies, Carlsbad, CA, USA) containing 10 ng/mL epidermal growth factor, 1 mg/mL insulin, and 10% porcine follicular fluid for 44 h at 39.8°C at 5% CO₂ and 100% humidity. The COCs were treated with 4 IU/mL of the hormones Q6 equine chorionic gonadotropin and human chorionic gonadotropin (Intervet, Cambridge, UK) for the first 22 h. The COCs were then matured under hormone-free conditions. To generate parthenotes, cumulus-free oocytes were activated with an electric pulse (1.0 kV/cm for 60 ms) in activation medium (280 mM mannitol, 0.01 mM CaCl₂, 0.05 mM MgCl₂) using a BTX Electro-cell Manipulator (BTX, CA, USA), followed by 4 h of incubation in PZM3 medium containing 2 mmol/L 6-dimethylaminopurine. Subsequently, the zygotes were transferred in groups of 35–70 to wells with 500 µL PZM3 medium for 7 days with or without DMSO, CAY10566 (50 µM) or OA (O1008, 112-80-1, 100 µM) from Sigma (purity, 99%).

Immunocytochemistry

Each stage of embryos without zona pellucida was fixed in 4% paraformaldehyde for 30 min at 4°C. Fixed samples were permeabilized using 1% Triton X-100 for 5 min at room temperature and washed three times with phosphate-buffered saline (PBS). The embryos were blocked using 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature, and primary antibodies targeting SCD1 (ab39969, rabbit-igG; Abcam) were added overnight at 4°C. The primary antibody was diluted 1:200 in PBS containing 1% BSA. Embryos were washed three times with PBS containing 0.1% Tween-20 before incubation with the fluorescent-conjugated secondary antibodies anti-rabbit-IgG (green, 1:500, A11008; Invitrogen) and anti-rabbit-IgG (red, 1:500, A11012; Invitrogen), which were diluted in a blocking solution, for 2 h at room temperature. The samples were washed three times with PBS containing 0.1% Tween-20, and nuclei were stained using 0.1% Hoechst 33342 (Molecular Probes) for 10 min. After washing three times with PBS, samples were mounted on a slide glass. Stained samples were visualized under a microscope (Eclipse TE2000-U; Nikon), and captured images were processed using a Nikon digital sight DS-L1.

Nile Red staining

A number of lipid dyes have been used to stain LDs in mammalian oocytes, including Nile Red and BODIPY 493/503 (Genicot *et al.* 2005, del Collado *et al.* 2016). Nile Red is commonly used for porcine and bovine oocytes that have large LDs (Leroy *et al.* 2005a, Sturmey *et al.* 2006). The Nile Red staining method of embryos is described elsewhere (Sudano *et al.* 2016). Briefly, a sample of each early denuded embryo development stage (2–3, 4, 6–8, morula and blastocyst (BL) stages) was randomly selected during the experimental replications and stained with Nile Red (Molecular Probes). Denuded embryos were washed in a solution of 0.1% polyvinylpyrrolidone in PBS (PVP-PBS) and fixed in 4% formaldehyde in PVP-PBS solution for 1 h. A stock solution

was prepared by dissolving Nile Red in dimethyl sulfoxide at a concentration of 1 mg/mL and stored at 20°C. Embryos were stained with a working solution of 15 mg/mL of Nile Red in PVP-PBS solution for 1 h in the dark at room temperature. Final concentrations were obtained by diluting the stock with PVP-PBS. After washing three times with PBS, samples were mounted on a glass slide. Stained samples were visualized under a microscope for intensity (Eclipse TE2000-U; Nikon) and for size and number (confocal laser scanning microscope; Leica). Captured images were processed using the LAS X Software and Nikon digital sight DS-L1. Fluorescence intensity and size were quantified using ImageJ 1.51j8 software (Wayne Rasband; National Institutes of Health, Washington DC, USA).

Quantitative RT-PCR

Pooled embryos, each stage of *in vitro*-produced embryos (2–3-cell, $n=20$; 4-cell, $n=20$; 6–8-cell, $n=20$; morula, $n=10$; and BL, $n=5$), and single blastocysts were processed with Dynabeads mRNA DIRECT Kit (Invitrogen) following the manufacturer's instructions. The zona pellucida was removed using Tyrod's acid before mRNA extraction. cDNA was synthesized using a High-Capacity RNA-to-cDNA Kit (Applied Biosystems). Extracted cDNA samples were amplified using a DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific) containing 1–2 pmol of each primer set listed in Table 1 in a 10 μ L reaction volume. Amplification and detection were conducted using the ABI 7300 Real-Time PCR system (Applied Biosystems) under the following conditions: one cycle of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension for 1 min (annealing/extension temperatures dependent on each primer set). The dissociation curves were analyzed, and the amplified products were loaded onto gels to confirm the specificity of the PCR products. The relative expression level was calculated by normalizing the threshold cycle (Ct) values of each gene to that of the reference gene GAPDH via the delta-delta Ct method.

Table 1 Primer sequences used for real-time PCR.

Genes	Sequences	AT (°C)	Size (bp)
<i>SCD1</i>	5'-GCCACCTTCTCTCGTTACG-3' 5'-CCTCACCCACAGCTCCCAAT-3'	60	142
<i>SREBP-1c</i>	5'-GCGAGTCAAGACCAGTCTCC-3' 5'-TCCCATCCACGAAGAAACG-3'	60	156
<i>ARF1</i>	5'-GGACCTTCCCAATGCCATGA-3' 5'-GAGCTGATTGGACAGCCAGT-3'	60	148
<i>COPG2</i>	5'-GACGAGGAGTCTGCTAGTGG-3' 5'-TTCTGTGGCTTCCGTAGTTC-3'	56	180
<i>PLD1</i>	5'-CAGACACCACTTGCACAACG-3' 5'-AACTTCGGATGGAGCCTGTG-3'	56	188
<i>ERK2</i>	5'-CAAACCTTCAAACCTGCTGC-3' 5'-TACTCCGTCAGGAACCTGT-3'	60	111
<i>ACTB</i>	5'-GTGGACATCAGGAAGGACCTCTA-3' 5'-ATGATCTTGATCTTCATGGTGCT-3'	63	131

AT, annealing temperature.

Statistical analysis

The data obtained in this study were analyzed using the GraphPad Prism statistical program (GraphPad Software). Data on developmental rates were arcsine-transformed and then analyzed using analysis of variance and the Newman–Keuls multiple comparison test. Relative transcription levels in embryos were analyzed using the unpaired Student's *t*-test. All data are expressed as the means $\pm$ standard error of the mean. A probability of $P < 0.05$ was considered statistically significant.

Results

Identification of SCD1 mRNA and protein levels at different porcine embryo stages

To avoid saturated FA stress, stearic acid is converted into mono-unsaturated OA by SCD. To determine the expression levels of SCD1 in different embryonic stages, SCD1 protein and mRNA levels were observed for all stages of embryos using immunocytochemistry staining and qRT-PCR, respectively. SCD1 protein expression was detected during embryogenesis (Fig. 1A). In addition, SCD1 protein expression had decreased

Figure 1 Changes in the SCD1 gene during porcine embryonic stages. Immunocytochemistry for SCD1 expression in different stages of *in vitro*-produced porcine embryos. The nuclei of embryos were counterstained with Hoechst 33342 (orange) (A). Real-time quantification of SCD1 expression in different embryonic stages (B). Different superscript letters indicate significant differences ($P < 0.05$). Scale bar: 100 μ m.

at the BL stage. Interestingly, the intensity of SCD1 protein level at the two-cell stage did not differ from the intensity at approximately the morulae embryonic stage. In addition, the SCD1 mRNA expression level was significantly higher at the four-cell stage and decreased gradually as the embryo developed (Fig. 1B).

OA rescues the effect of SCD1 depletion

To determine the importance of SCD1 in porcine embryo development, embryos were cultured either with or without CAY10566 during the entire culture period. OA had a positive effect on embryo development without toxicity at low concentration (Fig. 2A, B and C), similar to previous results (Aardema *et al.* 2011). As expected, inhibition of SCD1 had a detrimental effect on embryonic development (Fig. 2D). To confirm the role of OA in an SCD1-inhibited environment,

SCD1-inhibited embryos were treated with OA. Remarkably, the negative effects of exposure to CAY10566 were completely counteracted by simultaneous exposure to OA during development (Fig. 2E). The amount of SCD1 protein in each group was also examined (Fig. 2F). The intensity of SCD1 protein levels in each group indicated that the expression of SCD1 was reduced in the CAY10566 treatment group and that the expression of SCD1 was restored in the group supplemented with OA (Fig. 2G).

Effect of SCD1 inhibition on lipid droplet formation-related genes

Research has demonstrated that phospholipase D1 (PLD1) and extracellular signal-regulated kinase 2 (ERK2) are essential for the increased LD formation triggered by insulin stimulation (Andersson *et al.* 2006).

Figure 2 Effect of CAY10566 (SCD1 inhibitor) and oleic acid on embryos. Percentage of parthenogenetic embryo blastocyst rate were counted at day 7 of culture from embryos exposed to CAY10566, oleic acid (OA) and CAY10566 with OA treated simultaneously (168 h). Dose-dependent effect of oleic acid on blastocyst formation rate (A, B and C). Added 100 μ M of OA group showed an increase of embryo development rate without toxicity (A and B), but toxicity was confirmed at higher (250–500 μ M) concentrations (C). Dose-dependent effect of CAY10566 on blastocyst formation rate (D). Effect of oleic acid on CAY10566-treated parthenogenetic embryo blastocyst rate. Low concentration of CAY10566 has no effect on blastocyst rate (1–25 μ M, data not shown) (E). Protein levels of the SCD1 gene in each embryo stage (2C; 2cell, 4C; 4cell, 6C; 6cell, Mo; Morulae) (F) and mean fluorescence intensity of SCD1 (G). Different superscript letters indicate significant differences between the control (con), DMSO (vehicle control, V.con), CAY10566-treated (CAY) and CAY10566+oleic acid (CAY+OA) groups ($P < 0.05$). DMSO was used only in CAY and OA was in liquid form. Scale bar: 100 μ m. Each group has 3–4 replicates.

The activity of ERK2 appears to be essential for the PLD1-mediated increase in LD formation. The above-mentioned LD formation-related genes are lipogenic genes, and their expression is decreased by stress of the ER, which is directly connected to mitochondria in lipid synthesis (Marchi *et al.* 2014) caused by SCD1 deficiency (AlJohani *et al.* 2017). Based on these findings, we hypothesized that SCD1-inhibited embryos would have lower developmental potential as a result of their more deficient utilization of intracellular LDs compared to normal embryos. To investigate this possibility, we compared the above genes in SCD1-deficient, control and OA-addition treatments. As expected, SCD1-inhibited embryos exhibited lower mRNA expression compared to normal embryos (Fig. 3A). In contrast,

mRNA expression in OA-treated embryos was higher than that in control group (Fig. 3B). In the group supplemented with OA, deficient gene expression due to SCD1 inhibition was restored to the level of normal embryos (Fig. 3C).

Effect of SCD1 inhibition on lipid droplet formation

We hypothesized that SCD1-inhibited embryos would have lower developmental potential as a result of their more deficient utilization of intracellular LDs compared to normal embryos. To confirm this hypothesis, normal, SCD1-inhibited and OA-supplemented embryos were stained with Nile Red, and confocal images were used to determine the size and number of LDs. Embryos

Figure 3 Transcription levels of lipid droplet formation-related genes. Transcription levels of lipid droplet formation-related genes shown for control SCD1-inhibited blastocysts (CAY10566) (A), oleic acid-treated group (B) and CAY10566 with oleic acid-treated blastocysts (C). Each group of blastocysts was pulled at day 7 after parthenogenesis. Each group has three replicates. Different superscript letters indicate significant differences between the control (CON), CAY10566-treated (CAY), oleic acid-treated (OA) and CAY10566+oleic acid (CAY+O) groups ($P < 0.05$).

Figure 4 Lipid droplet formation of CAY10566- and oleic acid-treated embryos. Porcine embryos at different stages stained with Hoechst 33342, fixed and observed under z-stack of confocal microscopy. Nile Red fluorescence indicating lipid droplets (A). Stage-dependent effect of CAY10566 and oleic acid on lipid droplet number (B). Lipid droplet size was calculated from (C). Different superscript letters indicate significant differences between the control (CON), oleic acid- (OA), CAY10566- (CAY) and CAY10566 + oleic acid-treated groups (CAY + OA) ($P < 0.05$). Scale bar: 50 µm.

from the three treatments differed significantly in the size and number of LDs. When embryos were exposed to OA, the number of LDs increased, whereas in the simultaneous presence of a SCD1 inhibitor, the number of LD was drastically reduced (Fig. 4A and B). However, the number of LDs at the BL stage did not differ among treatments (Fig. 4B). We observed an increase in the size and number of LDs in OA-supplemented embryos following the same pattern as the previously described transcription levels (Fig. 4C).

Discussion

In embryo development, several potential problems are related to embryonic metabolism, and once failure occurs, damage to the embryo is difficult to reverse. For these reasons, a balance is necessary between the energy production required for embryonic development and the rate at which that energy will be used. Lipids serve as signaling molecules that contribute to key events during embryonic development, implantation and post-implantation growth (Burnum *et al.* 2009). However, elements of additions to media regarding lipid metabolism content or FA composition have been rarely reported.

The role of SCD1 in embryo development

To understand the importance of SCD1 in pig embryo development, we examined the effect of the inhibition of SCD1 in embryos on LD formation, which plays an important role in embryonic development. In bovine

specimens, SCD activity in cumulus cells appears to be a prerequisite for a functional and protective barrier of cumulus cells that prevents negative effects of saturated FAs on the developmental competence of the oocyte (Aardema *et al.* 2017). Indeed, mice that are genetically deficient for SCD1 are resistant to diet-induced weight gain and hepatic steatosis and demonstrate increased insulin sensitivity (Miyazaki *et al.* 2007, Ntambi *et al.* 2002). Mice lacking SCD1 are lean and resistant to diet-induced obesity (Flowers & Ntambi 2008, Ntambi *et al.* 2002). Moreover, they exhibit reduced FA and TAG synthesis in response to high dietary carbohydrates (Miyazaki *et al.* 2004) and also have increased fat oxidation rates in various tissues (Flowers & Ntambi 2008). The present report demonstrates that SCD1 activity in porcine embryos is important for embryo development. More specifically, during embryonic development, SCD1-depleted groups exhibited less morula compaction than during normal embryo development (Cui *et al.* 2007). In addition, our findings confirm that FA oxidation is correlated with oxygen consumption by the embryo, which remains relatively constant up to the eight-cell stage, but suddenly increases between the eight-cell and morula stages in humans (Haggarty *et al.* 2006). However, the concentration of CAY10566 used in our study was higher than the previously reported concentrations of CAY10566 required for SCD1 inhibition, likely because more lipid metabolism-related substances occur in the cytoplasm of pig embryos compared to other

Table 2 *In vitro* development of SCD1-inhibited embryos.

Group	No. embryos ($n = 3$)	No. cleaved (%) [§]	No. Mo-compaction (%) [‡]	Blastocyst (%)
CON	125	99 (79.3 ± 1.3)	50 (39.9 ± 1.4) ^a	43 (34.9 ± 3.1) ^a
V.con	123	95 (76.9 ± 2.1)	45 (37.4 ± 4.0) ^a	42 (34.3 ± 1.1) ^a
50 µM	133	102 (77.7 ± 3.7)	16 (11.6 ± 1.7) ^b	13 (9.9 ± 1.7) ^b
100 µM	125	92 (73.6 ± 2.8)	12 (9.6 ± 1.2) ^b	8 (6.4 ± 1.5) ^b

[§]The cleavage rate was counted after 2 days in culture; [‡]the morula (Mo) compaction rate was counted after 4 days in culture. The blastocyst rate was counted after 7 days in culture. Values with different letters (a and b) are significantly different ($P < 0.05$).

species and are possibly more complementary to the metabolism-related substances present in the culture medium. Nevertheless, our results clearly demonstrate that the depletion of SCD1 adversely affects pig embryo development similar to results observed for other species.

Correlation between SCD1 and lipid droplet formation

Triacylglycerides and cholesteryl esters are stored in LDs, which are highly ordered intracellular structures that are formed from the ER through a budding process. An abnormal status of the *SCD1* gene negatively affects this process (Farese & Walther 2009). A link between SCD activity and lipid droplet size was previously observed in cell lines cultured from patients with Berardinelli–Seip congenital lipodystrophy (Boutet *et al.* 2009). This study showed that patients with mutations in the *Seipin* gene had increased the percentage of saturated fatty acid in their lipids, reducing SCD activity and decreasing the size and amount of LD.

Role of OA in porcine preimplantation embryo

Mammalian oocytes and embryos efficiently incorporate and metabolize external FAs. Previous studies have observed that embryos are capable of taking up FAs from the environment (Roche 2006, Fouladi-Nashta *et al.* 2007). However, our data indicate that lipid uptake occurs during an earlier stage (Fig. 2). Therefore, it is possible that FA exposure directly influences the embryo rather than indirectly affecting granulosa and cumulus cells (Mu *et al.* 2001, Leroy *et al.* 2005b). The amount and size of LDs in the embryos in our experiment changed after exposure to OA during embryo development. This finding suggests that exogenous FAs directly positively affect embryos. However, the effects on LDs in embryos largely depended on the type and concentration of FAs to which the embryos were exposed during development. In bovines, saturated FAs negatively affected embryo development and LD size (Aardema *et al.* 2011). In our experiments, MUFAs also had a negative effect on embryo development at high concentrations. The developmental rate of blastocysts increased with increasing MUFA concentration, but the blastocyst cell number decreased at high concentrations (Fig. 2). Accordingly, unsaturated FA content may be an important factor in preimplantation embryo development. OA, the product of SCD1 activity, was shown to compete with the saturated FAs, block the abnormal lipid distribution and attenuate ER stress (Hapala *et al.* 2011, Peng *et al.* 2011). Remarkably, exogenous supplementation of OA, the direct product of SCD1 activity and rescued the developmental ability (Fig. 2). These data indicate that SCD1 is required for early embryonic development in pig.

Summary

We provide a comprehensive outline of LD formation and its effects on early porcine embryo development. These results are consistent with the general LD formation process of mammals. Our findings reveal that porcine embryos require OA for their development and are thus sensitive to SCD1 inhibition, which activates a cascade of events that negative effect on porcine embryo development.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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