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FGF9 の添加がブタ体外成熟・体外発生能に及ぼす影響

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Effects of FGF9 on porcine in vitro maturation and development after parthenogenetic activation

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Synopsis

Fibroblast growth factor 9 (FGF9) plays a key role in the regulation of cell differentiation, cell proliferation and embryonic development. It is also a survival factor for various cell types and interacts with two FGF receptors, FGFR2 and FGFR3. Furthermore, the absence of specific thecal-and oocyte-derived products during in vitro maturation (IVM) is suggested to be one reason for the reductions in the competency of oocytes used in assisted reproductive techniques. FGF9, as with some FGFs shown to promote oocyte competence and embryo development in several species is localize largely in theca cells, somewhat in the oocyte and its receptors in oocytes and cumulus cells. Therefore, to investigate the possible reproductive-related implications of FGF9 supplementation, its effects on IVM oocytes after parthenogenetic activation (PA) using the pig model were examined. Five approaches were taken to explore the function of FGF9 during in vitro embryo production (IVP). First, the maturation medium was supplemented with exogenous FGF9 to investigate its effects on maturation of GV oocytes measured by the extrusion of the first polar body hence MII oocytes. The results showed that FGF9 significantly accelerated MII rates in 50 ng/ml treated group ($p<0.05$) but had no visible change on embryo development after PA. However, the addition of FGF9 in both maturation and embryo culture media improved MII rates at 50 ng/ml ($p<0.05$), cleavage at 100 ng/ml ($p<0.05$) and blastocysts rate significantly ($p<0.05$) across treated groups after PA. Third, the addition of FGF9 in embryo culture alone revealed no visible effect. Fourth, RNA sequence analysis showed that FGF9 significantly up-regulated Thyroid hormone (TRH) providing insight into its possible mechanisms of action during IVM. With regards to oocyte quality, mitochondria membrane potential, mitochondria number and ROS levels were significantly increased at 100 ng/ml ($p<0.05$), reduced at 50 ng/ml ($p<0.05$) and increased across all FGF9-treated groups ($p<0.01$ and <0.05) respectively. Taken together, the present study determined that at least another thecal- and oocyte-derived product, FGF9, improves oocyte maturation and subsequent embryo development when provided during IVM and IVC. These findings, therefore, may be useful for porcine IVP system.

Keywords: FGF9, oocyte, embryo, parthenogenesis, IVM

1. INTRODUCTION

Pig (*Sus scrofa*) developmental model is studied extensively due to its commercial applications for meat production[1] and for biomedical research owing to similarities with human in various aspects[2] . Although advances have been made in devising porcine in vitro maturation (IVM) systems, lots of reports suggest that cytoplasmic maturation is incomplete[3] which means that IVM systems do not faithfully mimic the in vivo environment. To alleviate this phenomenon, the optimization of maturation media conditions has continued to receive great attention over the years. Growth factors (GFs) are generally considered to be important, and this can be attributed to the fact that numerous GFs, including fibroblast growth factors (FGFs), are present in the female reproductive tract fluids[4]. Furthermore, the absence of specific thecal- and oocyte-derived products during IVM may account for the depletions in the competence of oocytes used in ARTs in human[5] .

The FGFs, in addition to the specific members of the transforming growth factor-b(TGFB) superfamily such as BMP15 and GDF9, are another group of paracrine growth factors known for their ability to perform the same or similar biological function. At least 22 genes encode various FGFs that function as important paracrine regulators of proliferation, morphogenesis, and angiogenesis in various tissues. Within the oocytes and follicular somatic cells, several FGFs are expressed. For example, FGF8 is produced by the mouse oocyte and cooperates with BMP15 to promote glycolysis in cumulus cells in antral follicles. [1] FGF10 is produced by thecal cells and detected within the oocytes in cattle, and its primary receptor partner, FGF receptor 2b (FGFR2b), presides on granulosa cells[6] . Exploring the roles of these thecal- and oocyte-derived molecules have been of particular interest since IVM systems lack them.

Drummond et al [7] and Zhong *et al* [8] determined the localization of FGF9 in rat theca cell and mouse oocyte, respectively and that FGFR2 and FGFR3 utilized by FGF9 are expressed in granulosa cells, cumulus cells and oocytes, indicating a paracrine or autocrine involvement of FGF9 in oocyte development. FGF9, as with most FGFs shown to promote oocyte competence and embryo development in several species,[5 9,10,11,12,13] is localized largely in theca cells, somewhat in oocytes, its receptors in the oocytes and cumulus cells. However, similar biological function has not been reported for FGF9. Therefore, to investigate the possible reproductive-related implications of FGF9 supplementation, its effects on IVM oocytes after parthenogenetic activation (PA) using the pig model were examined.

2. Materials and methods

Oocyte collection and IVM

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in saline within 2 hours at 32°C–35°C. Cumulus–oocyte complexes (COCs) were aspirated from follicles with a diameter of 2–8 mm using an 18 gauge needle and a disposable 10 ml syringe. COCs were washed three times in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered tissue culture medium (TCM)-199. Groups of 30-50 COCs were matured in 500 uL of HP-POM (IFP1011P; Japan) containing 10IU eCG and 10IU hCG under mineral oil for 20-22h and without hormones for another 20-22h at 39°C in a humidified atmosphere of 5% (v/v) CO_2 and 95% (v/v) air. The maturation medium was supplemented with 0, 10, 50, 100 ng /ml FGF9 (PEPROTECH, USA); reconstituted according to the manufacturer's description, stored and diluted just before use. Cumulus cells were removed by pipetting and the extrusion of the first polar body was recorded as an indication of nuclear maturation.

Parthenogenetic activation and embryo culture

Activation of metaphase II (MII) stage oocytes was performed by the method described by Machebe et al (2019) [14] with slight modification. The MII oocytes were oriented in an electro-chamber (Iwaki, Japan) filled with 0.28M mannitol solution supplemented with 0.01% PVA, 0.1mM $MGSO_4$ and 0.25mM $CaCl_2$. Oocytes were then subjected to two direct current pulses of 100V/mm for 20μsec in a 0.1s interval using a LF101 Electro Fusion generator (Nepagene, Japan). Thereafter, activated oocytes were washed and cultured in

mPZM5 medium containing 5μg/ml CB for 4h. Oocytes were then washed three times in mPZM5 and cultured in sets of 10 in 30 μ l droplets (10 gametes per drop) of mPZM5 covered with pre-warmed mineral oil for up to 7 days in a humidified atmosphere of 5% CO_2. Cleavage and blastocyst rates were assessed at 48 h and 96 h, respectively.

Determination of mitochondrial number and reactive oxygen species (ROS) levels

MII oocytes after treatment were treated with MitoTracker Deep Red FM (Invitrogen Molecular Probes, M22426) or ROS Detection Assay Kit (BioVision) according to a manufacture's instruction. Briefly, oocytes were incubated in medium supplemented with 0.5 μmol/L MitoTracker Deep Red or 1μmol/L ROS Label in a dark environment under humidified air with 5% CO2, for 30 min at 39°C, followed by three washes with DPBS or PZM5. The oocytes were observed under a fluorescent microscope (abs/em ～ 644/665 μ m or 480-520 nm / 570-600 nm). The fluorescence intensity of the images was analyzed using Image J software.

mRNA sequencing

After in vitro maturation, the total RNAs of the COC pellets were extracted using RNAiso Plus (Cat# 9108, Takara Bio) and the RNA pellets were dissolved in 20 μL of Milli-Q Water, and the integrity of the RNA were checked using Agilent RNA 6000 Nano Kit (Cat# 5067-1511; Agilent) on the Bioanalyzer (Agilent). The RNA Integrity Number (RIN) of all samples was 10, thus, the RNAs of all samples could be subjected to library preparations for mRNA-seq. Using 625 ng of the total RNA each sample, the libraries were created using NEBNext Ultra II RNA Library Prep Kit for Illumina and NEBNext Poly(A) mRNA Magnetic Isolation Module (Cat# E7770S and E7490L, New England Biolabs), according to the manufacturer's instructions, and the final PCR cycle was 12. Concentrations and size distributions of the libraries were measured using an Agilent DNA 7500 kit (Cat#5067-1506, Agilent) on Bioanalyzer. All samples were passed for analyses on NGS equipment. The libraries were pooled and the concentrations were adjusted to 1 nM. The pooled libraries were subjected to denaturation and neutralization. Subsequently, the libraries were diluted to 1.8 pM and then applied for an NGS run on the NextSeq 500 System (Illumina) using NextSeq500/550 v2.5 (75 Cycles) Kits Cat#20024906, Illumina). The sequencing was performed with paired-end reads of 36 bases. After the sequencing run, FASTQ files were exported and basic information of the NGS run data was checked on CLC Genomics Workbench 21.0.3 software (CLC, QIAGEN). In the results, the PHRED-score as a quality score of the reads over 20 was confirmed for 99.7% of all reads, indicating the success of the run. The read number was approximately 52 to 57 million per sample as paired-end reads.Bioinformatics analysis was performed using the software CLC Genomics Workbench, version 20.0.3.

3. Results

Table 1 showed the in vitro development of porcine parthenotes from oocytes matured in IVM and in vitro culture (IVC) medium with or without FGF9. When FGF9 was added in IVM medium only, there was a significant difference in MII rates ($p<0.05$) at 50 ng/ml FGF9 but no visible effect in the cleavage or blastocyst rates regardless of the concentration of FGF9 used ($P>0.05$). The percentage of parthenotes reaching the blastocyst stage was slightly higher in the 50 ng/ml treated group (16%) compared with the control (14.5%), but no significant difference. When FGF9 was added in IVC medium only, no significant difference in cleavage and blastocyst rates suggesting that embryos need to be pretreated in FGF9-supplemented maturation medium. When FGF9 was added in both IVM and IVC medium, FGF9 did not only improve maturation rate at 50 ng/ml (63% vs 56.9% in control), but also cleavage rate at 100 ng/ml (60% vs 50.9% in control) and blastocyst rate in FGF9 treated groups (27.3%, 27.3% and 20.8% vs 16.4% in control). $P<0.05$

Table 1 The *in vitro* development of porcine parthenotes from oocytes matured in IVM and IVC medium with or without FGF9

Timing of addition of FGF9		Concentration of FGF9 added (ng/ml)	Number of oocytes				
IVM	IVC		(COCs*) cultured	matured (%)	activated (%)	cleaved (%)	developed to Blastocyst stage (%)
○	X	0 (control)	199	97 (48.7)	83 (85.6)	51 (61.5)	12 (14.5)
		10	199	107 (53.8)	95 (88.8)	49 (51.6)	10 (10.5)
		50	192	142 (74)**	112 (78.9)	53 (49.3)	18 (16)
		100	192	108 (56.3)	93 (86)	48 (51.6)	8(9)
X	○	0 (control)			85	56 (70)	23 (29)
		10		-	85	64 (75.3)	24 (28.2)
		50			85	57 (67.1)	20 (23.5)
		100			85	53 (66.3)	20 (25)
○	○	0 (control)	116	66 (56.9)	55 (83)	28 (50.9)	9 (16.4)
		10	98	55 (56)	55 (100)	38 (69.1)	15 (27.3)*
		50	106	67 (63)*	55 (82.1)	33 (60)	15 (27.3)*
		100	101	55 (54)	53 (96.4)	32 (60)*	11 (20.8)*

* cumulus–oocyte complexes
**significantly different from control in the same column ($p<0.05$)

Mitochondria number and ROS (Reactive Oxygen Species) levels

Fig 1 showed the mitochondria number and Reactive Oxygen Species (ROS) levels of oocytes in untreated (0 ng/ml) and treated (10-100 ng/ml)groups. Total of 72 oocytes (18 per group) was analyzed for mitochondria number. The proportion of oocytes with significantly increased was found in the 100ng ($p<0.01$) treatment group, in relation to control. Total of 80 oocytes (20 per group) was analyzed for ROS level. All FGF9 treated groups, in comparison to the control (0 ng/ml), had a significantly increased levels of ROS within the oocytes. The 10 ng/ml ($p<0.05$) and 50 and 100ng groups ($p<0.01$) was significantly different from the control (0 ng/ml).

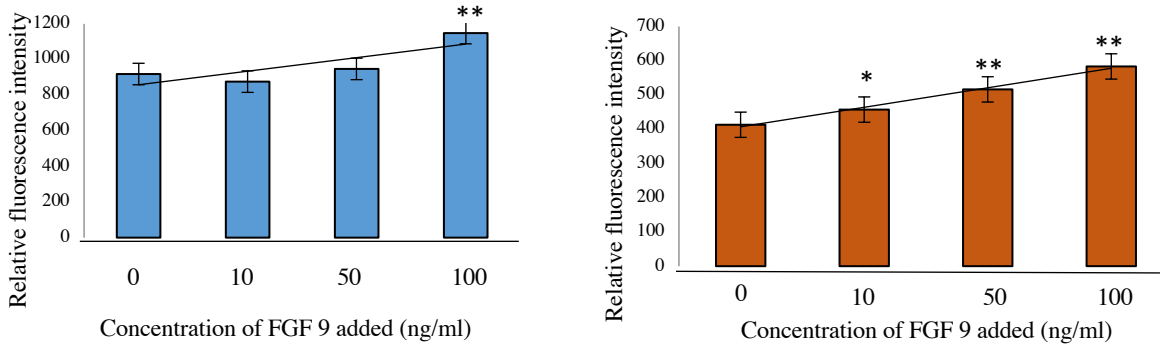

Fig.1 Mitochondrial activity (left) and Reactive Oxygen Species (ROS) levels (right) of oocytes in untreated (0 ng/ml) and treated (10-100 ng/ml) groups
Significantly different from control (*$p<0.05$, **$p<0.01$)

Comprehensive gene analysis in COC

1860 COC was obtained and multiplied by the 91 and 115 eggs from each group. RNA libraries were prepared from COC of untreated and 50 ng/ml FGF9-treated IVM group and sequenced using NextSeq500.

After adapter trimming and quality filtering, an average of 47% of the quality controlled (QC) reads was mapped to the *Sus scrofa* reference genome and 53% were uniquely mapped. As shown in Fig.2, heat map of expression values of selected genes in each sample, a total of 102 genes were found in which 48 genes and 54 genes were upregulated and downregulated respectively while 30 genes were uncharacterized indicated as 'novel genes'. Fig. 3 showed pathway of function enrichment analysis, showing difference in especially metabolic functions. Four genes form those genes like TRH, SLC16A3, CCDC80 and PDK4, include genes related to glucose metabolism regulator (PDK4), embryonic skeletal growth and secretory-promoting factors (TRH), general protein synthesis (RPL36A), and spindle check point and apoptosis (LCMT1) are focused. In 50 ng/ml FGF9 treated group, these genes except for PDK4 were upregulated at -2 to 2.55 times in fold change. PDK4 is an enzyme involved in the metabolic pathway from pyruvate to acetylcholine A. It is suggested that glycolysis might be significantly converted by the addition of FGF9. TRH is a thyrotropin-releasing hormone, but it is also known to have a function of releasing other hormones such as prolactin. The expression level of TRH is high even in the control group, and the fold change is 2.55 times higher by adding FGF9, suggesting that FGF9 may directly enhance the expression of TRH and affect other unknown substances. CCDC80 has a role as an antioxidant, and the ROS level was increased in this experiment, suggesting that it is linked to this. MCT4 (SLC16A3) is the third member of the monocarboxylic acid transporter (MCT) family and is involved in the transport of metabolically important monocarboxylic acids such as lactic acid, pyruvate, acetic acid and ketone bodies. ELGN2 is an α-ketoglutaric acid-dependent hydroxylase, a superfamily of heme iron-free proteins. It has been shown that EGLN2 depletion reduces mitochondrial respiration in breast cancer under normal and hypoxia. Although there is a lack of information on the network of FGF9 within the oocytes, studies from other cell types portray that FGF9 appears to act in concert with other FGFs to influence biological activities[15]. In the mammalian oocyte, however, several FGFs are found within the oocytes and the surrounding somatic cells of which their exogenous effects of some on IVM and embryo development have been positively demonstrated. The basic FGF2 is the most widely studied of the FGF family and has preference for has preference for the same receptors as FGF9; FGFR2 and FGFR3. Given the data from the above studies, it is possible that FGF9 network in oocyte is not only similar to that of FGF2 but also actively involve several pathways hence regulating oocyte metabolism.

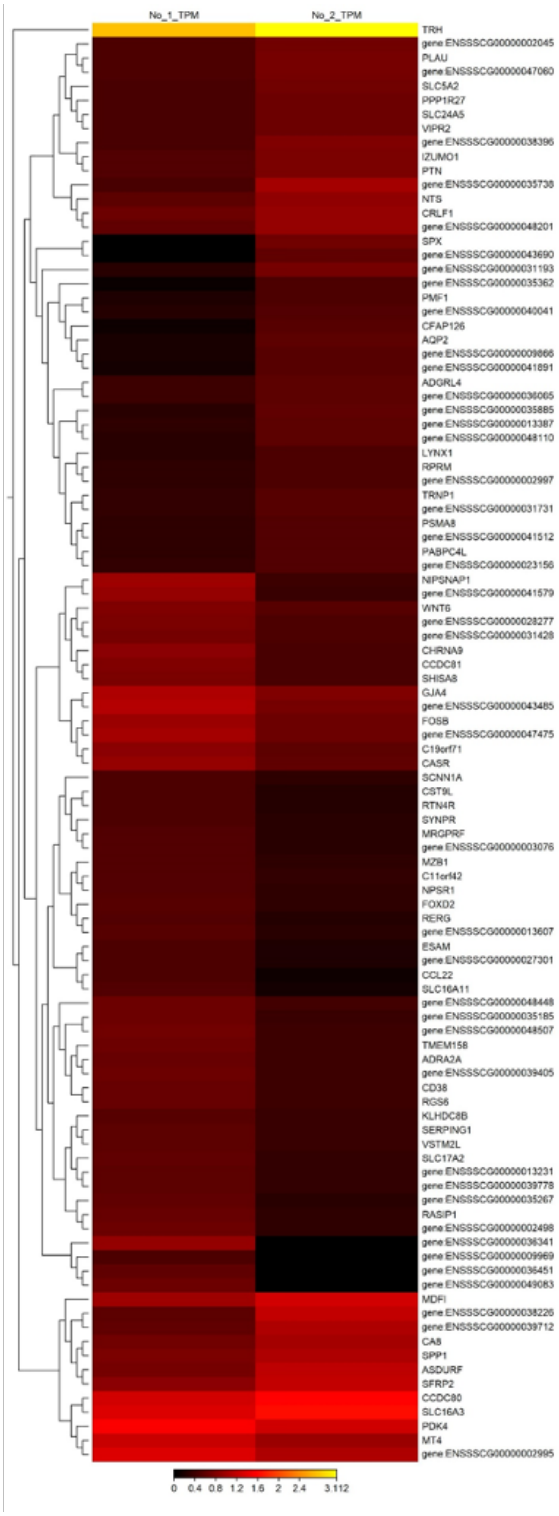

Fig.2 Heat map of expression values (normalized as TPMs)

of selected genes in each sample

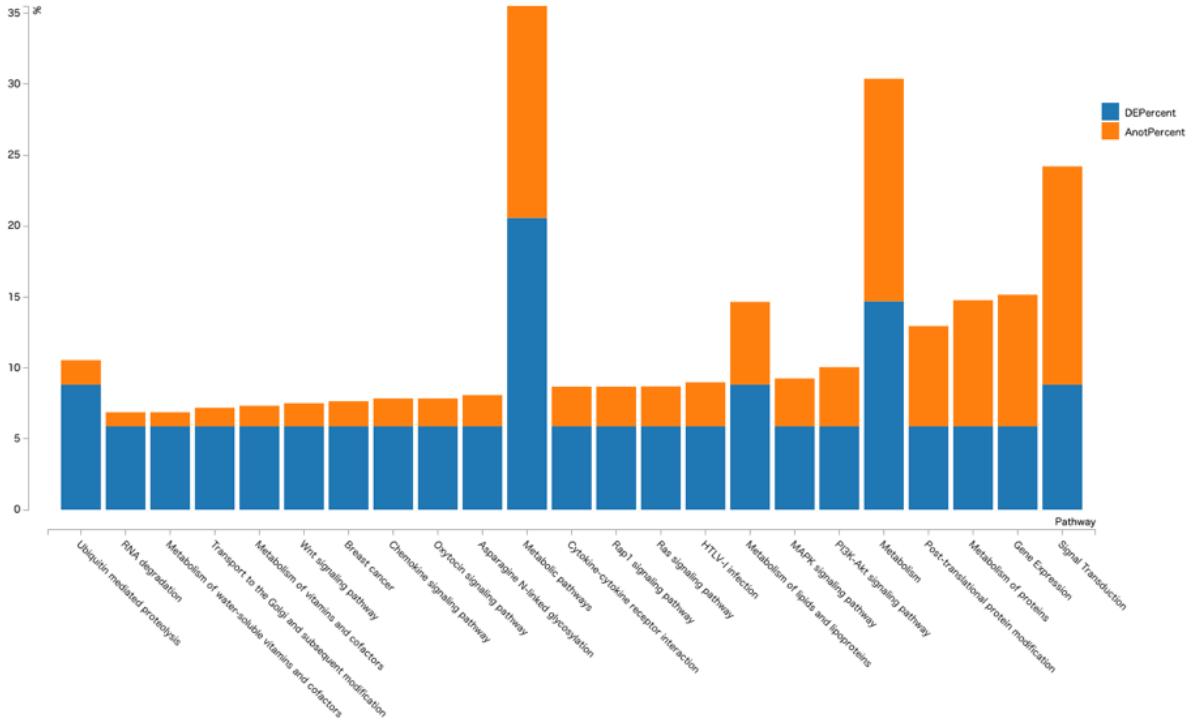

Figure 3 Barplot of percentages of significant DE genes (in blue) annotated to each functional category against whole genes (in orange).

4. Discussion

Unlike other growth factors, FGFs act in concert with heparin or heparan sulfate proteoglycan (HSPG) to activate FGFRs of cells and to induce the pleiotropic responses that lead to the variety of cellular responses induced by this large family of growth factors. Under in vivo conditions, a FGF9/FGFR paracrine and autocrine loop may promote follicle development[7]. Although FGF9 binds to both FGFR2 and FGFR3, its affinity is highest for FGFR3. However, both are expressed in the oocytes and cumulus cells. During IVM, it is possible that oocyte maturation may have been improved by FGF9/FGFR signaling. However, RNA seq data revealed other possible mechanisms by which FGF9 might have improved oocyte maturation during IVM such as the enhancement of the G protein-coupled estrogen receptor, GEPR, regulation of glucose metabolism, spindle checkpoint and apoptosis.

Oocyte maturation and embryo development are controlled by hormones as well as intra-ovarian factors such as cytokines and growth factors[16]. Thyroid hormones (TRH) may play a key role in the regulation of reproductive processes and are regarded as biological escalators of the gonadotropins stimulatory action [16]. Several evidence prove that TRH play a direct role in ovulation, early follicular development [17] and influenced human oocyte maturation [18]. Their receptors are localized in human oocyte, mural granulosa and cumulus cells while their regulatory enzyme are found in granulosa cells [17]. In combination with FSH, TRH increased the proliferation and inhibited the apoptosis of these cells by the PI3K/Akt pathway [18 19].

Oocyte maturation is also under the primary control by pituitary gonadotropin LH. Previous studies showed that LH or hCG has intrinsic effect on FSH and TSH activity, and that LH stimulated adenylate cyclase[20], an enzyme responsible for meiotic arrest through increased cAMP production necessary for the acquisition of oocyte competence. RNA-seq revealed that TRH was highly expressed in porcine oocytes treated in PMSG and hCG (FSH and LH respectively) supplemented medium. This high expression significantly

increased further with the addition of FGF9 to maturation medium, providing a mechanism of action for FGF9. TRH or TSH (Thyroid stimulating hormone) are primarily concerned with the general regulation of metabolism. Several metabolic pathways are activated by TRH including the G protein-coupled estrogen, GPER, pathway which plays a critical role in regulating re-entry into meiotic cell cycle [21]. Furthermore, TSH was shown to have acted on cumulus cell to convey a positive signal to the oocyte thus facilitated acquisition of competence, fertilization, and embryonic development in bovine IVM.

Krisher et al (2007) [3]reported that porcine oocytes, unlike oocytes of other species, utilizes glucose as their primary energy substrate and that IVM porcine oocytes metabolized glucose via glycolytic pathway preferentially over glucose via PPP or glutamine via the Krebs cycle. In this study, treatment with or without FGF9 enhanced the expression of PDK4 gene which encodes PDK4 enzyme. PDK4 regulates glucose metabolism by reducing the conversion of pyruvate to acetyl-CoA. It follows that an increase in the enzyme levels leads to the production of more acetyl-coA and energy for basal metabolism while a reduction facilitates energy reservation through the production of more pyruvate. Therefore, FGF9 may have energy regulatory role during porcine IVM. Hypoxia-inducible factor is an evolutionarily conserved transcription factor that allows the cell to respond physiologically to low concentrations of oxygen [22]. Several pathways are induced by the action of this gene, EGNL2, including Oxygen-dependent proline hydroxylation of Hypoxia-inducible Factor Alpha (HIF1alpha), which positively influenced bovine COC maturation and subsequent embryo development. Although there is insufficient information on the role of EGNL2 in oocytes, FGF9 downregulated its expression suggesting a role of FGF9 in response to low oxygen during porcine IVM. It was clear that TBL1X encodes a protein similar to the WD40 group which mediates protein-protein interactions, signal transduction, RNA processing, gene regulation, vesicular trafficking, cytoskeletal assembly and may play a role in the control of cytotypic differentiation. The protein is found as a subunit in corepressor SMRT (silencing mediator for retinoid and thyroid receptors) complex along with histone deacetylase 3 protein. The RPL36A (ribosomal protein L36a) gene encodes a ribosomal protein that is a component of the 60S subunit, which in addition to the small 40S subunits make up the cytoplasmic ribosomes involved in the catalysis of protein synthesis. LCMT1 (leucine carboxyl methyltransferase 1-like;) is important for normal progression through mitosis via spindle checkpoint regulation, and cell survival by reducing apoptosis thus essential for embryonic development in mice [23]**.** Furthermore, RNA-seq provided even more insights into possible pathways stimulated by FGF9 to enhance oocyte developmental competence and embryo development. Since MAPK is one of the major activities controlling oocyte maturation [24] and the phosphorylation of ERK1/2 signaling pathway is proposed as the major trigger of meiosis resumption which is caused by the inhibitory action of ERK signaling on gap junction-mediated flow of cGMP [25], further research is recommended to provide more specific mode action of FGF9. Whether FGF9 may have also acted by enhancing the MAPK pathway as notable of FGFs, or as with FGF2 which has preference for the same receptors to enhance the activation of ERK1/2 signaling pathway in mouse COCs [25] remains an area to be explored.

In conclusion, the present study reveals that exogenous FGF9 facilitates the acquisition of oocyte developmental competence. In addition to the normal interaction between the FGF9 and its receptors within the COC, FGF9 may have influenced oocyte maturation and embryonic development through the expression and actions of relevant genes responsible for glucose metabolism in the mitochondria, spindle checkpoint regulation and apoptosis reduction.

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要約
繊維芽細胞増殖因子（FGF）は細胞や組織の増殖や分化に広く関わるが、FGF9 が哺乳類の未成熟卵に及ぼす影響については明らかになっていない。そこで、本研究では FGF9 がブタ未成熟卵の成熟能とその後の発生能に及ぼす影響を検討した。その結果、FGF9 を体外成熟培地に添加すると成熟率ならびに胚盤胞への発生率が有意に向上することがわかった。FGF9 処理卵と無処理卵との間で RNA シークエンス解析を行った結果、FGF 9 が多くの代謝経路を活性化することや抗酸化物質の活性を高めることが示された。また、ピルビン酸からアセチルコリン A に至る代謝経路に関連する酵素の発現量が低下しており解糖系優位に変化させた可能性が示唆された。今後は、FGF9 が卵子の生存性を向上させる機構や作用機序について検討する必要がある。

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