A comprehensive transcriptomic view on the role of *SMAD4* gene by RNAi-mediated knockdown in porcine follicular granulosa cells

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Abstract

As a key mediator of the transforming growth factor-beta (TGF- β) signaling pathway, which plays a pivotal role in regulating mammalian reproductive performance, Sma- and Mad-related protein 4 (*SMAD4*) is closely associated with the development of ovarian follicular. However, current knowledge of the genome-wide view on the role of *SMAD4* gene in mammalian follicular granulosa cells (GCs) is still largely unknown. In the present study, RNA-Seq was performed to investigate the effects of *SMAD4* knockdown by RNA interference (*SMAD4*-siRNA) in porcine follicular GCs. A total of 1025 differentially expressed genes (DEGs), including 530 upregulated genes and 495 downregulated genes, were identified in *SMAD4*-siRNA treated GCs compared with that treated with NC-siRNA. Furthermore, functional enrichment analysis indicated that upregulated DEGs in *SMAD4*-siRNA treated cells were mainly enriched in cell-cycle related processes, interferon signaling pathway, and immune system process, while downregulated DEGs in *SMAD4*-siRNA treated cells were mainly involved in extracellular matrix organization/disassembly, pathogenesis, and cell adhesion. In particular, cell cycle and TGF- β signaling pathway were discovered as the canonical pathways changed under *SMAD4*-silencing. Taken together, our data reveals *SMAD4* knockdown alters the expression of numerous genes involved in key biological processes of the development of follicular GCs and provides a novel global clue of the role of *SMAD4* gene in porcine follicular GCs, thus improving our understanding of regulatory mechanisms of *SMAD4* gene in follicular development.

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Introduction

The transforming growth factor-beta (TGF- β) signaling pathway has a virtual role of controlling multiple reproductive processes in the mammalian ovary, including follicular development, atresia, and ovulation (Rosairo *et al.* 2008, Yu *et al.* 2013, Hatzirodos *et al.* 2014). As a central component of the TGF- β signaling pathway, *SMAD4* is the only common-mediator SMAD (co-SMAD) of SMAD families and is shown to be expressed primarily in oocytes, thecal cells, and granulosa cells (GCs) at different stages of follicular development in mammalian (Lee *et al.* 2014, Xing *et al.* 2014), suggesting that *SMAD4* may play a crucial role in the reproductive process.

Previously, several groups mainly focused on *SMAD4* functions in follicular and embryo development. For example, conditional knockout of *Smad4* using *Amhr2*-Cre resulted in premature luteinization of GCs, decreased antral follicles, reduced ovulation rates, and impaired fertility in mice (Pangas *et al.* 2006); oocyte-conditional deletion of *Smad4* with *Gdf9*-iCre led to reduced litter size and a minor fertility reduction (Li *et al.* 2012); In mice *Smad4* GC-specific knock-out with *Cyp19*-Cre blocked luteinizing hormone (LH)

induced cumulus expansion and follicle rupture, which resulted in increased follicular atresia and impaired fertility (Yu *et al.* 2013). Moreover, ablation of *Smad4* caused sterility in female mice lacking Forkhead box L2 (FOXL2) in gonadotrope cells (Fortin *et al.* 2014) and reduced proportions of embryos in early bovine embryogenesis (Lee *et al.* 2014). Undoubtedly, these findings indicate that *SMAD4* affects the differentiation of GCs, follicular development, fertility, and embryogenesis.

Meanwhile, our recent studies have found high expression levels of *SMAD4* occurred in GCs and oocytes from primary, preantral and antral follicles, and miRNAs could play a proapoptotic effect by targeting *SMAD4* in porcine follicular GCs (Liu *et al.* 2014), further confirming that *SMAD4* has an important role in ovary function, particularly in regulating follicular GC growth, apoptosis, and steroidogenesis in porcine ovary (Liu *et al.* 2014, Xing *et al.* 2014). However, as co-SMAD involved in multiple reproductive processes, functional evidences of the regulatory mechanisms of *SMAD4* gene from the above studies are still limited. Most recently, transcriptome profiling has been proved as an effective

and widely used approach to identify critical genes and their related pathways. As such, the objective of this study was to estimate the changes of gene expression and analyze their related biological processes under *SMAD4*-silencing using RNA sequencing technology (RNA-Seq), thus providing genome-wide insights of the role of *SMAD4* gene in porcine follicular GCs.

Materials and methods

Animal

Animal samples were obtained as described previously (Liu *et al.* 2014). The ovaries of mature sows were collected at a local slaughterhouse. All animal experiments were approved by the Animal Ethics Committee at Nanjing Agricultural University, China.

Cell culture

GCs collected from ovarian follicles (3–5 mm diameter) were seeded into T25 flasks and cultured at 37°C and 5% CO₂ in DMEM/F-12 medium (Gibco) containing 15% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin.

RNA interference

The small interfering RNA (siRNA) sequences of both NC-siRNA and *SMAD4*-siRNA are designed and given in [Supplementary Table S1](#), see section on [supplementary data](#) given at the end of this article. Cultures of GCs were transfected with *SMAD4*-specific or NC nonsense siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions.

cDNA library preparation and Ion Torrent Proton sequencing

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and its integrities and qualities were assessed by Bioanalyzer 2100 (Agilent Technologies) and kept at –80°C. RNA integrity number (RIN) of NC-siRNA and *SMAD4*-siRNA groups was more than 8.0. RNA samples from NC-siRNA and *SMAD4*-siRNA treatment groups ($n=3$ per group) were pooled with equal quantities, respectively, and used for cDNA library preparation. The cDNA libraries for single-end sequencing were prepared using Ion Total RNA-Seq Kit v2.0 (Life Technologies) according to the manufacturer's instructions and then processed for the Ion Proton sequencing. First, samples were diluted and mixed. Then the mixture was processed on OneTouch 2 instrument and enriched on OneTouch 2 ES station (Life Technologies). After that, the enriched mixture samples were loaded on to 1 P1v2 Proton Chip and sequenced on Ion Torrent Proton Sequencers (Life Technologies).

Gene mapping

The raw sequencing data were evaluated by FASTQC and then filtered by excluding adaptor sequences, reads with >5%

ambiguous bases, and low-quality reads containing more than 20 percent of bases with qualities of lesser than 13. Next, MapSplice program (v.2.1.8) (Wang *et al.* 2010a) was used for aligning the clean reads to the current pig reference genome (Sscrofa 10.2).

Gene expression analysis

UQRPKM value (Miecznikowski *et al.* 2012, Dillies *et al.* 2013) and DEGseq algorithm (Wang *et al.* 2010b) were used to measure the gene expression levels and filter the differentially expressed genes (DEGs), respectively. The criteria filtering DEGs was as follows: (1) fold Change >1.5 or <0.667; (2) FDR<0.05; (3) UQRPKM value >1 in both NC-siRNA and *SMAD4*-siRNA samples. Hierarchical clustering analysis of DEGs was performed using Cluster 3.0 with average linkage and Euclidean distance metric and then was visualized using Java TreeView (version 1.1.6r2, Stanford University, Stanford, CA, USA).

Functional enrichment analysis

Gene ontology (GO) and pathway analysis of DEGs were conducted using the Gene Ontology (<http://www.geneontology.org/>) and KEGG (<http://www.genome.jp/kegg/>), respectively. Fisher's exact test was applied to identify the significant GO categories or pathways, and FDR was used to correct the *P*-values. Terms with corrected *P*-values less than 0.05 were considered as significant or enriched terms.

Quantitative real-time RT-PCR

The same RNA samples from NC-siRNA and *SMAD4*-siRNA groups applied for RNA-Seq were used for quantitative real-time RT-PCR (qRT-PCR). The primer sequences of 11 DEGs including 5 upregulated genes and 6 downregulated genes were shown in [Supplementary Table S2](#). First, total RNA was reverse transcribed using the M-MLV reverse transcriptase (Takara Bio). Then, qRT-PCR reactions were performed with a standard SYBR Green PCR kit (Takara Bio) and StepOne Software v2.0 (ABI, Carlsbad, CA, USA) by using the following parameters: 95°C for 30 s followed by 40 cycles of 95°C for 5 s, annealing for 5 s, and 72°C for 30 s. Porcine GAPDH was used as the internal control and assays were run in triplicate. The comparative $2^{-\Delta\Delta CT}$ method was used to calculate the expression level differences.

Western blot

Western blot was carried out to measure protein expression levels of *SMAD4* as previously described by Wang *et al.* (2011). In brief, GCs were collected after 48-h transfection and were extracted via cell lysis buffer. Bradford method was used to quantify total protein concentration. Then each sample was separated in a 12% SDS-PAGE gel and was transferred onto a nitrocellulose membrane. Next, the membrane was blocked 2 h at room temperature with 2% bovine serum albumin in PBS and was then incubated at 4°C overnight with a diluted (1:2000) monoclonal anti-*SMAD4* antibody or anti-GAPDH

antibody (internal control), followed by a secondary goat anti-rabbit antibody (1:2000) for 2 h at room temperature. Protein signals were visualized using SuperSignal West Pico chemiluminescent substrate, and the signal intensity was quantified by Densitometric analysis.

Statistical analysis

Statistical analyses were performed using SPSS v17.0 software (SPSS). The results were showed as mean $\pm$ S.E.M. Statistical differences between NC-siRNA and SMAD4-siRNA groups were examined using the Student's *t*-test. *P*-values less than 0.05 and 0.01 were considered as significant and extremely significant difference, respectively.

Data accessibility

The datasets have been submitted to the Gene Expression Omnibus (GEO) database of NCBI (accession number GSE76833).

Results

The effect of RNA interference of SMAD4 in porcine follicular GCs

The expression levels of SMAD4 detected by qRT-PCR in SMAD4-siRNA treated cells were extremely significant lower than that of cells with NC-siRNA treatment ($P < 0.01$) (Fig. 1A), which was consistent with the data from protein expression of SMAD4 in SMAD4-siRNA treated cells (Fig. 1B), indicating that SMAD4-siRNA could be used for the further experiment.

Characteristics of RNA-Seq

To obtain a global view on the role of SMAD4 gene in porcine follicular GCs, comparative transcriptome analyses between NC-siRNA and SMAD4-siRNA treatment groups were performed in our study. In total, 28.48 and

Table 1 Statistics of the sequencing reads mapping to the reference Sscrofa 10.2 genome.

Terms	NC-siRNA	SMAD4-siRNA
All reads	28,485,053	28,498,799
Unmapped reads	4,120,919	4,481,121
Mapped reads	24,364,134	24,017,678
Mapping rate	0.855	0.843
Unique mapping	23,296,428	22,894,748
Unique mapping rate	0.818	0.803
Repeat mapping	1,068,315	1,123,535

28.49 million high quality clean reads from NC-siRNA and SMAD4-siRNA samples were acquired, respectively (Table 1). For each sample, ~84% reads and ~80% unique reads could be mapped in the current pig genome (Sscrofa 10.2), respectively (Table 1). After gene mapping, 15,344 mRNAs were discovered to be expressed in at least one group (Supplementary Table S3).

Identification of DEGs between NC-siRNA and SMAD4-siRNA in porcine follicular GCs

From 15,344 expressed mRNAs, 14,678 and 14,707 genes were found to be expressed in NC-siRNA and SMAD4-siRNA treated cells, respectively (Supplementary Table S3). Of these genes, 14,041 genes were expressed in both NC-siRNA and SMAD4-siRNA treatment groups, while 637 and 666 genes were expressed only in NC-siRNA and in SMAD4-siRNA treated cells, respectively (Fig. 2). After filtering, a total of 1025 differentially expressed genes (DEGs) including 530 upregulated genes and 495 downregulated genes were detected in porcine follicular GCs treated with SMAD4-siRNA as compared with cells treated with NC-siRNA (Fig. 2 and Supplementary Table S4).

Gene Ontology (GO) analysis of DEGs

Gene Ontology (GO) analysis showed that significant GO terms of all DEGs under SMAD4-silencing were mainly involved in type I interferon signaling pathway, mitotic cell cycle, mitotic nuclear division, immune system process, response to virus, cytokine-mediated signaling pathway, and extracellular matrix (ECM) organization (Supplementary Table S5). Furthermore, the significant GO terms of upregulated DEGs under SMAD4-silencing were mainly enriched in cell-cycle related processes, interferon signaling pathway, mitotic nuclear division, cytokine-mediated signaling, immune system process, and response to virus (Fig. 3 and Supplementary Table S6), while the main terms of downregulated DEGs under SMAD4-silencing were significantly involved in ECM organization/disassembly, pathogenesis, glycosaminoglycan metabolism process, cell adhesion, and axon guidance (Fig. 3 and Supplementary Table S7).

More importantly, cell-cycle and ECM related processes were found as the most enriched terms for upregulated

Figure 1 The effect of RNA interference of SMAD4 gene. (A) The expression of SMAD4 gene detected by qRT-PCR in porcine follicular GCs treated with NC-siRNA and SMAD4-siRNA. (B) The expression of SMAD4 protein detected by Western blot in porcine follicular GCs treated with NC-siRNA and SMAD4-siRNA.

Figure 2 RNA-Seq analyses of genes expressed in porcine follicular GCs treated with NC-siRNA and SMAD4-siRNA. (A) Venn diagram shows the overlap genes expressed in different groups. (B) The numbers of upregulated and downregulated differentially expressed genes (DEGs) under SMAD4-silencing.

and downregulated DEGs, respectively. Furthermore, the genes involved in cell-cycle and ECM related processes were shown in Figs 4 and 5, respectively. To verify the mRNA expression of genes related with these processes, qRT-PCR was performed using RNA samples from NC-siRNA and SMAD4-siRNA groups applied for RNA-Seq. Ten genes, including five cell-cycle-related genes (*CDK1*, *CCNA2*, *CCNB1*, *CCNB2*, and *TGFB1*), three ECM-related genes (*MMP9*, *FN1*, and *FBN1*), and two SMAD genes (*SMAD6* and *SMAD9*), were used for expression verification. Consistent with results of RNA-Seq, the expression of these five cell-cycle related genes were increased in SMAD4-siRNA groups compared with that of NC-siRNA groups (Fig. 4), while five ECM- and SMAD-related genes were significantly decreased in SMAD4-siRNA groups compared with that of NC-siRNA groups (Fig. 5), indicating the strong consistence between RNA-Seq and qRT-PCR data.

Pathway analysis of DEGs

Pathway enrichment results showed that significant pathways of all DEGs were mainly involved in cell cycle, HTLV-I infection, TGF- β signaling pathway, and viral carcinogenesis (Supplementary Table S8). Furthermore,

Figure 3 Gene ontology (GO) of upregulated and downregulated DEGs under SMAD4-silencing. (A) Top 15 significant GO terms (biological processes) associated with upregulated DEGs. (B) Top 15 significant GO terms (biological processes) associated with downregulated DEGs. The vertical axis represents the GO terms, while the horizontal axis shows the $-\log_2(P\text{-value})$ of the significant terms.

the significant enrichment pathways of upregulated DEGs under SMAD4-silencing were mainly related with cell cycle, antigen processing and presentation, and HTLV-I infection (Supplementary Table S9), while down-regulated DEGs under SMAD4-silencing were mainly involved in TGF- β signaling pathway, regulation of actin cytoskeleton, and focal adhesion (Supplementary Table S10).

Figure 4 DEGs involved in cell-cycle related processes. (A) Heat map for DEGs enriched only in cell cycle. (B) Heat map for DEGs enriched in both cell cycle and mitotic cell cycle. (C) Heat map for DEGs enriched only in mitotic cell cycle. (D) Five genes (*CDK1*, *CCNA2*, *TGFB1*, *CCNB1*, and *CCNB2*) detected by qRT-PCR were consistent with the RNA-Seq results. Data were represented as the mean $\pm$ S.E.M. of $n=3$ per group. * $P < 0.05$, ** $P < 0.01$.

Pathway-Act-Network analysis

To further understand interactions among the important pathways and obtain the key pathways for the role of *SMAD4* gene in porcine follicular GCs, a Pathway-Act-Network was built using KEGG database. As shown in Fig. 6 and Supplementary Table S11, most of DEGs involved in key pathways including cell cycle, TGF- β signaling pathway, pathway in cancer, regulation of actin cytoskeleton, antigen processing and presentation, focal adhesion, viral carcinogenesis, and ECM-receptor

interaction. Of these, cell cycle and TGF- β signaling pathway were identified as the most important pathways for upregulated and downregulated pathways, respectively.

Discussion

In the present study, a global view on the role of *SMAD4* gene in porcine follicular GCs was demonstrated. The RNA-based silencing of *SMAD4* changed

Figure 5 DEGs involved in extracellular matrix (ECM) related processes and TGF- β /SMAD signaling pathway. (A) Heat map for DEGs enriched in ECM related processes. (B) Heat map for DEGs enriched in TGF- β /SMAD signaling pathway. (C) Five genes (*FN1*, *FBN1*, *MMP9*, *SMAD6*, and *SMAD9*) detected by qRT-PCR were consistent with the RNA-Seq results. Three genes including *THBS1*, *TGFB3* and *LOC100737374* were common genes of both ECM related processes and TGF- β /SMAD signaling pathway. Data was represented as the mean $\pm$ S.E.M. of $n=3$ per group. * $P < 0.05$, ** $P < 0.01$.

Figure 6 Pathway-Act-Network analysis. The Pathway-Act-Network was built according to the interactions with pathways from the KEGG database. Cycle nodes represent pathways, while the arrow between two nodes represents an interaction target between pathways. The power of the interaction among the pathways is showed by the size of nodes. Red nodes represent significantly upregulated pathways, green nodes represent significantly downregulated pathways, and yellow nodes represent nonsignificant pathways.

the expression levels of 1025 DEGs, which were mainly associated with cell cycle, type I interferon signaling pathway, mitotic nuclear division, immune system process, response to virus, TGF- β /SMAD signaling pathway, and ECM organization, suggesting that *SMAD4* played a core role in multiple biological processes in porcine follicular GCs.

Basically, as a co-SMAD, *SMAD4* has a critical function in mediating TGF- β /SMAD signaling pathway, but its role on other SMADs is unclear. A previous study showed that silencing of *SMAD4* did not affect the expression of *SMAD1*, *SMAD3* and *SMAD5* in porcine GCs (Wang *et al.* 2011). In this study, *SMAD1* and *SMAD5* were not detected, and *SMAD2* and *SMAD3* expressions were not significant changes under *SMAD4*-silencing (Supplementary Tables S3 and S4), suggesting that these genes might not play important roles in *SMAD4*-mediated pathway. Interestingly, other three inhibitory SMADs, including *SMAD6*, *SMAD7*, and *SMAD9*, were significantly downregulated by silencing of *SMAD4* (Fig. 5 and Supplementary Table S4). In general, *SMAD6* and *SMAD7* play negative feedback loop in the TGF- β /SMAD signaling pathway and are considered as potential regulators of ovarian function (Nakao *et al.* 1997, Uchida *et al.* 2000, Tsukamoto *et al.* 2014, Li 2015), while *SMAD9* suppresses intracellular bone morphogenetic protein (BMP) signaling (Tsukamoto *et al.* 2014). By decreasing the levels of these inhibitory SMADs, *SMAD4*-silencing might reduce inhibitory SMAD-related pathways or ovarian function in porcine follicular GCs.

Interestingly, interrupted *SMAD4* acted on cell-cycle related processes via increasing the levels of many key markers of cell cycle. From our current knowledge,

cyclins and cyclin-dependent kinases (CDKs) play the key role in cell cycle (Sanchez & Dynlacht 2005, Lim & Kaldis 2013). In this work, our data showed that *SMAD4*-silencing upregulated the expression levels of *CDK1*, *CCNA2*, *CCNB1*, and *CCNB2*, while there were no significant differences under *SMAD4*-silencing in *LOC100154715* (*CDK2*), *LOC100518921* (*CDK6*), *CCND2*, *CCND3*, and *CCNE1*, with *CDK4* and *CCND1* expressions undetected (Fig. 4 and Supplementary Tables S3 and S4). Actually, cyclin A2/B-CDK1 complex mainly functions to control G2/M phase (Pagano *et al.* 1992, Pines 1999, Lim & Kaldis 2013); for this reason, our results suggested that interrupted *SMAD4* might promote cell cycle progression of G2/M. Among cyclins, *CCNA2* is shown to bind CDK1 via forming cyclin A2-CDK1 complex and has been required at S and G2 phases in Hela cells as well as G2/M transition (Pagano *et al.* 1992, Rosenblatt *et al.* 1992), playing an important role in both mitosis and meiosis (Persson *et al.* 2005). *CCNB1*, as a regulatory protein involved in mitosis, can bind CDK1 to form cyclin B1-CDK1 complex, which is activated by *CCNA2* for promoting the early mitotic events (Gong & Ferrell 2010). Our data indicated that the expression levels of *CDK1*, *CCNA2*, and *CCNB1* were upregulated under *SMAD4*-silencing, further providing strong evidence that silencing of *SMAD4* might impact on both mitosis and meiosis in porcine follicular GCs by improving the progression of cell cycle. More interestingly, we found *CCNB2* was induced by *SMAD4*-silencing. A major TGF- β receptor in TGF- β /SMAD signaling pathway, *TGFB2*, could bind *CCNB2* for indirectly interacting with CDK1, which lead to CDK1 inactivation and cell cycle arrest in the G1/S for preventing the cells entry into the G2/M phase

(Liu *et al.* 1999). Our results showed that silencing of *SMAD4* increased the expression of *CDK1* and *CCNB2* but decreased the levels of *LOC100038019* (*TGFBR2*), suggested that downregulated *TGFBR2* levels might not cause cell-cycle arrest in the G1/S phase, thus confirming that the progression of cell cycle induced by silencing of *SMAD4* mainly occurred in the G2/M phase. In addition, some important genes related with other checkpoints of cell cycle, such as *CDC20*, *CDC45*, *BUB1*, *BUB1B*, and *CKS1B* (Fig. 4 and Supplementary Table S6), were also induced by silencing of *SMAD4*, strongly suggesting that silencing of *SMAD4* promoted cell cycle in porcine follicular GCs.

In the previous study, interrupted *SMAD4* enhanced apoptosis levels by reducing the expression of *BCL2* but not *BAX* in porcine GCs (Wang *et al.* 2010c). Moreover, our recent data also showed *SMAD4*-silencing promoted the rate of apoptosis in porcine follicular GCs and downregulated the anti-apoptotic genes of *BCL2* instead of *BAX* (Liu *et al.* 2014). These data confirmed that the levels of apoptosis were increased under *SMAD4*-silencing in porcine follicular GCs. Consistent with the above results, we found interrupted *SMAD4* reduced anti-apoptotic genes of *BCL2* (−1.56-fold decreased but did not reach a significant level) but not *BAX* and significantly increased the levels of the important proapoptosis genes such as *CASP1* and *PTEN* (Supplementary Table S4). Interestingly, we did not detect the expression changes of *TP53*, *MYC*, *FAS*, *RB1*, *CDKN1A* (*p21*), and *BCL2L2* (*BCLW*) (Supplementary Table S3), indicating that these genes might not have important functions in *SMAD4*-mediated apoptosis. Taken together, our data provided a comprehensive clue on the role of *SMAD4* gene in apoptosis of follicular GCs.

As expected, our data found that *SMAD4*-silencing regulated the expressions of steroidogenesis-related genes including *HSD17B7*, *HSD3B1*, and *STAR* (+2.27-fold increased but did not reach a significant level), whereas the expressions of *CYP11A1*, *CYP17A1*, and *CYP19A1* genes were not detectable (Supplementary Table S3). In fact, the role of *SMAD4* gene in steroidogenesis is still controversial. In ovarian-specific *Smad4*-knockout mouse GCs, *Smad4* loss increased the expression levels of *STAR*, *Cyp11a1*, *Hsd3b1*, and *Hsd17b7* and resulted in disrupting regulation of steroidogenesis (Pangas *et al.* 2006), but *SMAD4* knockdown in porcine GCs was shown to downregulate the expression levels of *CYP19A1* and *HSD3B*, without any significant changes in expressions of *CYP11A1* and *STAR* genes (Wang *et al.* 2010c, 2011). In the present study, upregulated expressions of *HSD3B1* and *STAR* under *SMAD4*-silencing were consistent with the results discovered by Pangas *et al.* (2006), indicating *SMAD4*-silencing might enhance the synthesis of steroid. However, we also found that silencing of *SMAD4* decreased the levels of *HSD17B7*, illustrating that the exact role of *SMAD4* gene in steroid metabolism needs further investigation.

In addition, our results demonstrated that *SMAD4* mediated ECM-related processes. It is not surprising because ECM plays a marked role similar as *SMAD4* in follicle growth and oocyte maturation by affecting GCs morphology, communication, survival, and steroidogenesis (Berkholtz *et al.* 2006), suggesting that an interactive regulatory mechanism might link *SMAD4* with ECM-related genes. Actually in other cell types, many evidences for *SMAD4* functions were also discovered in regulating ECM-related processes. For example, *SMAD4* has a discriminate effect in murine mesangial cells for regulating ECM molecules stimulated by TGF- β (Tsuchida *et al.* 2003); *SMAD4* reexpression in *SMAD4*-deficient human C4-II cervical carcinoma cells was associated with the ECM-related genes (Klein-Scory *et al.* 2007); mutation of *SMAD4* led to Myhre syndrome and resulted in disorganization of ECM (Piccolo *et al.* 2014). But until now, little is known about regulatory mechanism that *SMAD4* mediated ECM-related processes in porcine follicular GCs. In the present study, we found that silencing of *SMAD4* mainly downregulated the genes involved in ECM organization/disassembly, particularly some important ECM genes, such as *FN1*, *FBN1*, *LTBP2*, and *MMP9* (Fig. 5 and Supplementary Table S4). As one of fibronectins, FN1 participates in cell adhesion, migration, and differentiation processes, but its role in GCs is controversy. A study of bovine GCs from superstimulated follicles showed that the expression of *FN1* was increased and resulted in delayed maturation and differentiation of GCs compared with untreated controls (Dias *et al.* 2013), but anti-inhibin alpha subunit treatment of porcine GCs increased their proliferation and the levels of *FN1* (Cai *et al.* 2015). Here our results showed that silencing of *SMAD4* attenuated the levels of *FN1* in porcine GCs, provided an evidence that downregulated *FN1* might influence the differentiation of porcine GCs. Meanwhile, as main components of ECM in connective tissue, fibrillin (FBN)-latent transforming growth factor beta binding protein (LTBP) family members, including *FBN1* and *LTBP2*, were reduced by silencing of *SMAD4*. FBN1 is a major component of microfibrils and has an impact on the bioactivity of TGF- β via interacting with LTBP, thus regulating TGF- β mediated processes such as production of collagen, while LTBP is discovered as part of the TGF- β large latent complex and function to direct isolation of latent TGF- β to ECM microfibrils (Kanzaki *et al.* 1990, Saharinen *et al.* 1999, Hatzirodos *et al.* 2011, Davis & Summers 2012). In adult bovine ovaries, FBN1 and LTBP2 were found to be associated with stromal tissue including cortical stroma and follicular thecal layers (Prodoehl *et al.* 2009), while FBN1 was considered as a major component of ECM of ovarian stromal compartments during ovarian development and high mRNA levels of *LTBP2* were discovered in the stroma (Hatzirodos *et al.* 2011). These data indicated that *FBN1* and *LTBP2* might affect the development of follicular or ovary by regulating

TGF- β bioavailability. Here our studies showed the expression of *FBN1* and *LTBP2* were reduced by silencing of *SMAD4*, suggesting that these genes were associated with *SMAD4*-mediated ovarian development processes in porcine follicular GCs. Interestingly, as the largest and most complex member of the key enzymes involved in tissue remodeling and proteolysis of ECM, *MMP9* was induced by silencing of *SMAD4*. Previously, *MMP9* has been shown to be expressed in porcine GCs (Basini *et al.* 2011). In chicken ovary, *MMP9* mRNA levels in GCs were discovered significantly higher than in theca cells and its protein expression was found in both the conditioned medium and the GC lysate, showing GCs could be considered as important cellular origin of *MMP9* protein (Zhu *et al.* 2014). Here our data was consistent with the study by Costello *et al.* (2009), who found that the expression of *MMP9* was decreased in *SMAD4* mutant embryonic stem cells and embryoid bodies, indicating that *SMAD4* functional loss led to decrease the expression of *MMP9*. Overall, our data provides a novel clue for the role of *SMAD4* gene in ECM-related processes.

Conclusion

As a key regulator in multiple biological processes related with follicular development, the role of *SMAD4* gene in porcine follicular GCs is still limited. In this study, our transcriptomic data indicated that silencing of *SMAD4* resulted in increasing a series of genes mainly involved in cell-cycle related processes, interferon signaling pathway, cytokine-mediated signaling, and immune system process, while decreasing other genes mainly associated with ECM organization/disassembly, TGF- β /SMAD signaling pathway, pathogenesis, and cell adhesion, strongly suggesting that *SMAD4* has extensive roles in follicular development. As such, these findings provide novel insights into the role of *SMAD4* gene in porcine follicular GCs.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-16-0034>.

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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