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-conditioned 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 Torren 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 Torren Proton Sequencers (Life Technologies).

Gene mapping

The raw sequencing data were evaluated by FAST-QC 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⁻ΔΔCt 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 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 ± 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 significantly 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, 28.49 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
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, 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 downregulated DEGs under SMAD4-silencing were mainly involved in TGF-β signaling pathway, regulation of actin cytoskeleton, and focal adhesion (Supplementary Table S10).

**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.

**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 −Log2 (P-value) of the significant terms.
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 interaction.
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). 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.
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 LTBPs, thus regulating TGF-β mediated processes such as production of collagen, while LTBPs are 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, Hatzioridos 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 (Hatziorodos 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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References


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Rosenblatt J, Gu Y & Morgan DO 1992 Human cyclin-dependent kinase 2 is activated during the S and G2 phases of the cell cycle and associates with cyclin A. *PNAS* 89, 2824–2828. (doi:10.1073/pnas.89.7.2824)


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