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RESEARCH ARTICLE

Dlk1-Dio3 Locus-Derived lncRNA *Dio3os* Affects Growth, Development and Reproductive Activity in Mice

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A B S T R A C T

Dio3os is a significant lncRNA gene located in the *Dlk1-Dio3* imprinted locus, and plays an important role in the body growth and development. However, there is relatively little information regarding the effects of this gene on reproductive activity in mice. Thus, the aim of this study was to investigate the effects of *Dio3os* on growth, development and reproductive activity in mice. Moreover, expression levels of associated genes were also monitored, using mouse endometrial stromal cells (ESCs). Firstly, qPCR and Western Blot were used to determine the expression levels of genes associated with *Dio3os*, using mouse ESCs. Then, RNA-seq was utilized to classify and annotate the activity of differentially expressed genes affected by *Dio3os*. Moreover, RNA pull-down and mass spectrometry were used to identify and analyze *Dio3os*-binding proteins. Additionally, 24 female mice were randomly divided into 4 equal groups (n=6 each), including LV3-*Dio3os*, LV3-NC, LV5-*Dio3os* and LV5-NC. Data of body weight, body length, tail length, occurrence rate of estrous cycle and organ coefficients for these mice were recorded. Results of qPCR and Western Blot showed that *Dio3os* affected the expression levels of *Dlk1*, *Gtl2*, *Dio3* and genes related to development and apoptosis. Besides, *Dio3os* was highly expressed in mouse uterus and ovary. This gene also increased body weight and body length and affected the organ coefficients. Moreover, *Dio3os* increased the occurrence rate of estrus and correspondingly decreased the occurrence rate of diestrus. Together, these findings indicate that *Dio3os* affects growth, development and reproductive activity in mice.

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INTRODUCTION

Dio3os (os, opposite strand) lies at the distal end of *Dlk1-Dio3* imprinted cluster (Edwards *et al.*, 2008; Dietz *et al.*, 2012) and the exon-intron structure of *Dio3os* is similar in mouse and human (Hernandez *et al.*, 2004). Unlike the mass of ncRNAs within *Dlk1-Dio3* cluster which are maternally expressed, *Dio3os* is biallelic, rather than imprinted (Tierling *et al.*, 2006; da Rocha *et al.*, 2008). *Dlk1-Dio3* imprinted region has great research value for the study of growth and development mechanisms. *Dio3* is an important paternal gene in *Dlk1-Dio3* region, which plays a key role in protecting body tissues from the adverse effects of excessive thyroid hormones during development (Stevenson and Prendergast, 2013).

Previous studies (Hagan *et al.*, 2009; Sigova *et al.*, 2013) have shown that *Dio3os* is immediately adjacent to *Dio3*, and partially overlaps the *Dio3* promoter (Hernandez, 2005; Labialle *et al.*, 2008). Additionally, *Dio3os* is highly expressed in uterus and placenta. In a recent study by Xie *et al.* (2019), only the characteristics of *Dio3os* sequence were analyzed and it was proved to be a cytoplasmic lncRNA. However, there is relatively little information regarding the role of *Dio3os* in growth, development and reproductive activity.

Therefore, the present study was planned to investigate the effects of *Dio3os* gene on growth, development and reproductive activity, using mice as model. Moreover, mouse endometrial stromal cells (ESCs) were used to study expression levels of associated

genes for the investigation of *Dio3os* activity at cellular level.

MATERIALS AND METHODS

The present study consisted of two series of experiments. In the first series, mouse endometrial stromal cells were used to study expression levels of associated genes for the investigation of *Dio3os* activity at cellular level, while intact mice were used in the second series to investigate the effects of *Dio3os* gene on growth, development and reproductive activity. All animal experiments were performed in accordance with relevant guidelines and regulations, and the experimental protocol was approved by Regulations for the Administration of Affairs Concerning Experimental Animals in Nanjing Agricultural University. Experimental mice were purchased from Qing Longshan Experimental Animal Breeding Ground (Nanjing, China).

Endometrial stromal cell treatment: Mouse ESCs were prepared, as described previously (Tan *et al.*, 2004; Cora *et al.*, 2015). Then, these cells were transfected with pcDNA3.1-*Dio3os* and siRNA-*Dio3os* for next experiment. Total mRNA and proteins were extracted from these cells at 24 h and 48 h, following the protocol of Yan *et al.* (2020).

QPCR and Western Blot: All primers used for qPCR have been listed in Tables 1 and 2. For the Western Blot assay, DIO3 (1:1000, Affinity Biosciences, Cat# DF8532) and Tubulin beta chain (1:2000, Servicebio, Cat# GB11017B) were used as primary antibody and internal reference, respectively. The HRP-labeled goat anti-rabbit IgG (1:3000, Servicebio, Cat# GB23303) was used as secondary antibody. Quantity-One and Image J software was used to measure and compare the density of protein bands among all the protein samples.

RNA-seq assay: RNA-seq assay was used to examine the differentially expressed genes (DEGs) in mouse ESCs and analyze their functional enrichment. Library construction and data processing were performed by SHBIO, Shanghai, China.

Identification of *Dio3os*-binding proteins: RNA pull-down and mass spectrometry (MS) were used to identify and classify the *Dio3os*-binding proteins. Briefly, the target RNA was used to pull down RAN-binding proteins; then the binding proteins were isolated for MS assay, as described earlier (Bierhoff, 2018). Finally, the identified proteins were submitted to STRING database (https://string-db.org/) for protein-protein interaction (PPI) network analysis.

Experimental mice: A total of 24 female mice (aged 4-5 weeks) were kept at room temperature (18-23°C) under 40-60% humidity and 14:10h light: dark cycle. These mice were randomly divided into 4 equal groups (n=6 each), including LV3-*Dio3os* group, LV3-NC group, LV5-*Dio3os* group and LV5-NC group. Lentivirus carrying sh-*Dio3os* was given to mice of LV3-*Dio3os* group; lentivirus carrying negative control of sh-*Dio3os* was given to mice of LV3-NC group; lentivirus carrying *Dio3os* was given to mice of LV5-*Dio3os* group; lentivirus carrying negative control of *Dio3os* was given to mice of LV5-NC group. The dose of lentivirus was 10^9 TU/kg body weight, and all the lentiviruses used in this study were purchased from GenePharma, Shanghai, China.

Before drug administration, mice were kept off-feed for 12h but were allowed to drink water freely. The day of the first injection was taken as day 0. After that, the injection was given every 5 days for a total of 5 times. Thus, the drug was given on day 0, 5, 10, 15 and 20, respectively.

Record of estrous cycle: Each stage of estrous cycle in mice was determined at 9 am every day by observation of the vulva and smear of vaginal exfoliated cells (Tsiligianni *et al.*, 2004; Goldman *et al.*, 2007; Cora *et al.*, 2015); the recording of estrous cycle was started after initiation of treatments. Different stages of estrus cycle were differentiated by the condition of vulva and the type and amount of vaginal exfoliated cells (Bekyurek *et al.*, 2002). Besides, in order to facilitate analysis, the stage of metestrus was merged into diestrus. The data of estrous cycle was continuously collected for 25 days, and the occurrence rate was calculated through dividing duration time of each phase (proestrus, estrus, diestrus) by duration time of the entire estrous cycle and multiplying by 100.

Measurement of body growth parameters and organs coefficients: For all experimental mice, body growth parameters, including body weight, body length and tail length were measured every 5 days for 25 days. Increase in the growth data was calculated by subtracting the data taken on day 0 from the data recorded on different days.

Organ coefficients for mice of different groups were determined to access the effects of *Dio3os* on organs development. All the mice were killed at the end of 26 days after the first measurement. A total of 8 organs, including heart, liver, spleen, lung, kidney, stomach, uterus and ovary, were taken after killing each mouse. The organ coefficients were calculated through dividing organ weight with body weight and multiplying by 100 (Zhang *et al.*, 2014).

Statistical analysis: Results were analyzed by using SPSS Statistics 17.0 and GraphPad Prism 7.0 software. Data were presented as mean±SD. One-way ANOVA and Dunnett's multiple comparison post-test were applied to analyze data and draw diagrams. A value of $P<0.05$ was considered significant.

RESULTS

Expression levels of *Dlk1*, *Gtl2* and *Dio3*: As shown in Fig. 1, the expression level of *Dio3os* was significantly higher in pcDNA-3.1-*Dio3os* than that of pcDNA3.1 ($P<0.05$). However, it was lower in siRNA-*Dio3os* than that for siRNA-NC. Fig. 2A shows that the expression levels of *Dlk1*, *Gtl2* and *Dio3* were significantly reduced by overexpressed *Dio3os*, while they were increased by inhibited *Dio3os* activity. Overall, the data demonstrated that *Dio3os* affected the expression levels of imprinted genes- *Dlk1*, *Gtl2* and *Dio3*.

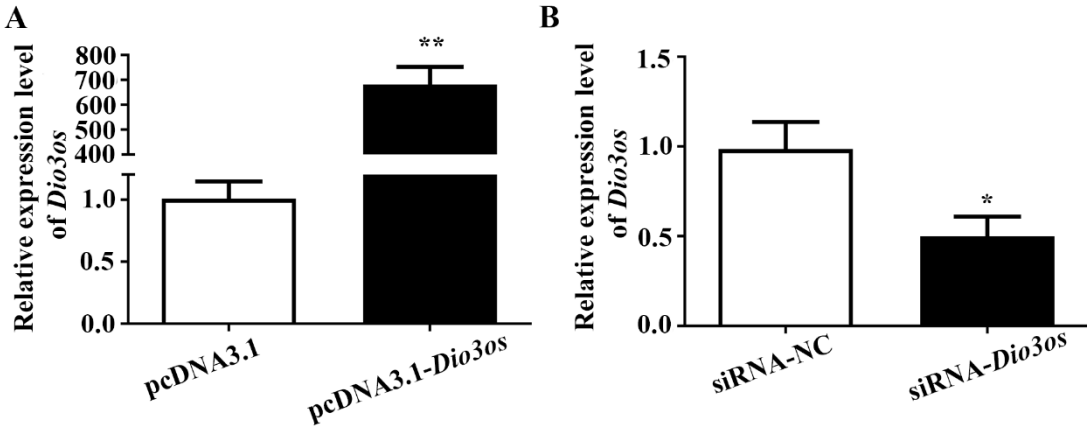

Fig. 1: (A) Expression level of *Dio3os* in mouse ESCs transfected with pcDNA3.1-*Dio3os* and pcDNA3.1. (B) Expression level of *Dio3os* in mouse ESCs transfected with siRNA-*Dio3os* and siRNA-NC. **P*<0.05, ***P*<0.01.

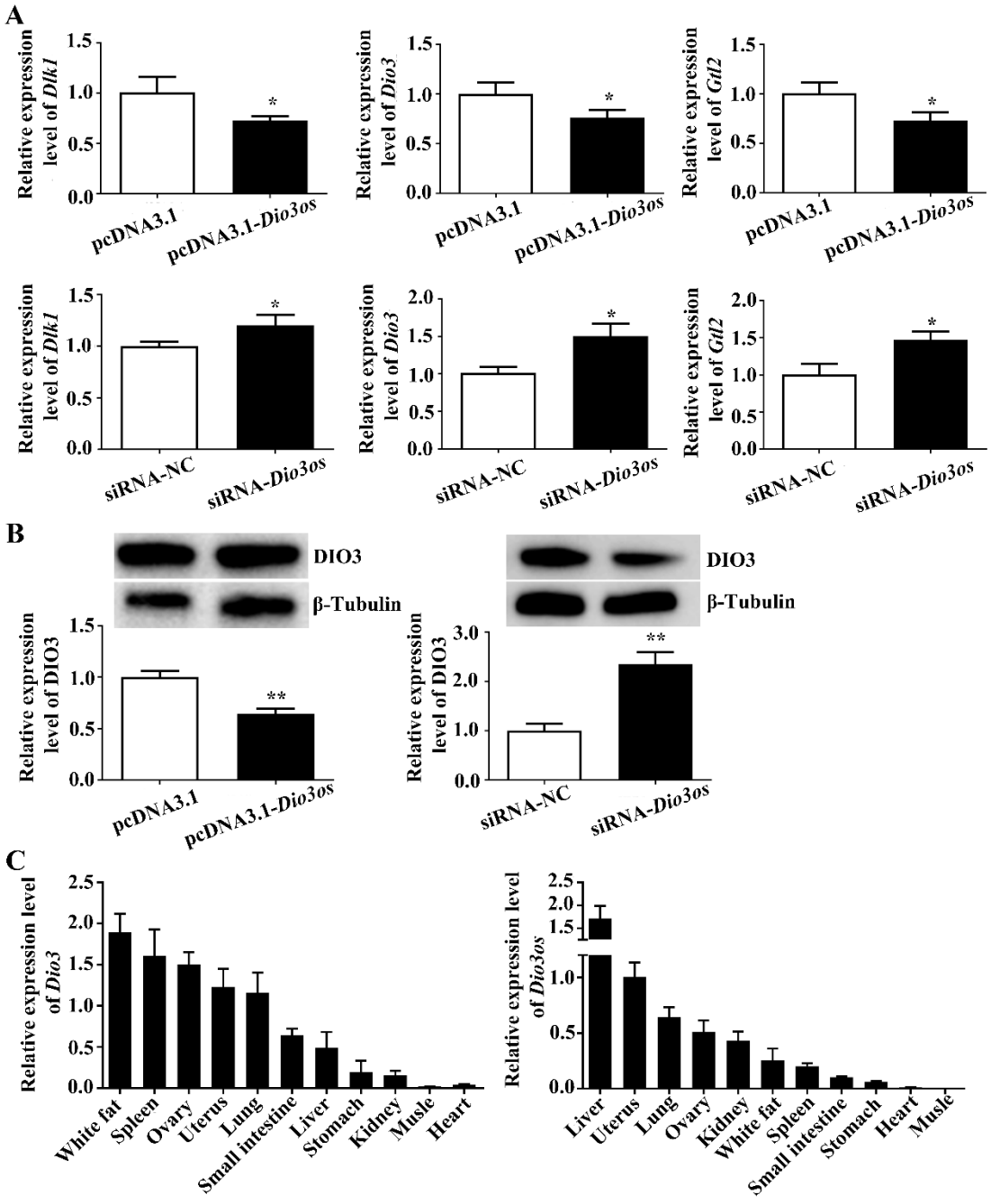

Fig. 2: (A) Effects of *Dio3os* on *Dlk1*, *Gtl2* and *Dio3* in mouse ESCs. (B) Effects of *Dio3os* on DIO3 protein in mouse ESCs. (C) Relative mRNA expression levels of *Dio3os* and *Dio3* in different mouse tissues. *P<0.05, **P<0.01.

***Dio3os* expression in body organs:** The results of Western Blot revealed that an increase or decrease of *Dio3os* correspondingly reduced or promoted the protein level of *Dio3* (Fig. 2B). Meanwhile, *Dio3os* was highly expressed in liver, uterus, lung, ovary and kidney, but was low in heart and muscle (Fig. 2C). Simultaneously, *Dio3* was highly expressed in fat, spleen, ovary, uterus and lung, but was low in heart and muscle. On the whole, both *Dio3os* and *Dio3* showed high mRNA expression in mouse uterus and ovary.

Expression levels of genes of development and apoptosis: Results of RNA-seq showed that most of DEGs were enriched in terms and pathways related to development and apoptosis. Furthermore, the results of qPCR for the validation of RNA-seq revealed that not only the expression levels of *EsR*, *Peg10* and *Igf2* but also those of *Caspase-3* and *Caspase-9* were significantly affected by *Dio3os* (Fig. 3A, B). Moreover, the apoptosis rate of cells was significantly increased or decreased by up-regulating or down-regulating the expression level of *Dio3os* (Fig. 3C). These observations indicate that *Dio3os* affects genes related to development and apoptosis in mouse ESCs.

Activity of *Dio3os*-binding proteins: The results obtained through RNA pull-down and mass spectrometry indicated that 32 proteins interacted with *Dio3os* (Fig. 4A, B). Moreover, the activity of these proteins was mainly associated with cell life cycle and growth and development-related hormones. Additionally, the statistical analysis of PPI network showed that these proteins had more interactions among themselves than that with a random group of proteins of similar size selected from the genome (Table 3).

Body growth parameters: Compared with LV3-NC group, the body weight increment was significantly lower in LV3-*Dio3os* group ($P<0.05$) from day 5, while the body weight increment in LV5-*Dio3os* group was higher than in LV5-NC group from day 15 to the end of this study ($P<0.05$, Fig. 5A). Moreover, the body size of mice in LV3-*Dio3os* group was smaller than that in LV3-NC group; likewise, the body size of LV5-*Dio3os* group was larger than that in LV5-NC group (Fig. 5B). Almost similar trend was seen for body length (Fig. 5C).

However, the tail length increment was dramatically higher in LV3-*Dio3os* group compared with LV3-NC group only on day 5 ($P<0.05$). Meanwhile, there was no difference between LV5-*Dio3os* group and LV5-NC group in tail length increment (data not shown).

Coefficients of body organs: As shown in Fig. 5D, only organ coefficient of stomach was higher, and that of uterus was lower in LV3-*Dio3os* group compared to LV3-NC group ($P<0.05$). For LV5-*Dio3os* group, organ coefficient for ovary was significantly higher than that in LV5-NC group ($P<0.05$).

Estrous cycle: The occurrence rate of estrus was significantly lower in LV3-*Dio3os* group ($P<0.05$) compared with LV3-NC group, with non-significant difference in occurrence rates of proestrus and diestrus. On the contrary, the occurrence rate of diestrus was significantly lower in LV5-*Dio3os* group compared with LV5-NC group ($P<0.01$, Fig. 5E), with non-significant difference in occurrence rates of proestrus and estrus.

DISCUSSION

Significance of lncRNAs in scientific research has long been recognized (Gong *et al.*, 2017). It is well known that lncRNAs cannot encode functional peptides like mRNA, so the research on lncRNAs makes slow progress. However, lncRNAs can rely on other biological macromolecules to exert their biological functions (Yuan *et al.*, 2014). In a previous study, *Dio3os* was found to be a cytoplasmic lncRNA (Xie *et al.*, 2019). However, the regulation activity of *Dio3os* is still unidentified.

Since the cytoplasmic lncRNA activity is usually accomplished by binding biological macromolecules, such as protein, DNA or RNA (Wang *et al.*, 2014; Li *et al.*, 2015; Wang *et al.*, 2015), *Dio3os* activity was studied by a series of associated biological macromolecules. The results revealed that *Dio3os* not only regulated *Dio3* expression but also affected the expression levels of *Dlk1*

Table 1: Primers of genes related to development

Gene	Primer sequence (5'→3')	Length (bp)
GAPDH	F: CGTGTTCCTACCCCCAATGT R:TGTCATCATACTTGGCAGGTTTCT	73
PgR	F:TGACCAGATAACCCTGATTC R: GGTAAGGCACAGCGAGTAGA	166
EsR	F:ATGCCTCTGGCTACCATT R: GCTTCAACATTCTCCCTCC	228
cAMP	F: GCTGTGGCGGTCACTATCA R: GAAGGCACATTGCTCAGGTA	256
Peg10	F: CATCCTTCGTGGCATCGCAGAG R: GGTGTTGTTGTTGTTGTTGTTGTTGG	213
Fam150a	F:GATGTGCTAGATTGCTGACAAGATTAG R: GTCCTCTTCCTGTAGCCACTCAT	211
Hck	F:ACAGAGCCAAGTGCCAATCAGAAG R:AGCCTCCTCCAGAACCACCATC	213
Igf2	F:AGGGGAGCTTGTTGACACG R: GGGTATCTGGGGAAGTCGTC	225

Table 2: Primers of genes related to apoptosis

Gene	Primer sequence (5'→3')	Length (bp)
Bax	F: CAGGATGCGTCCACCAAGA R: GGTGAGGACTCCAGCCACA	389
Bcl-2	F: GGATGACTGAGTACCTGAACC R:AGCCAGGAGAAATCAAACAG	118
Caspase-3	F:ACAGCACCTGGTTACTATTC R: CAGTTCTTTCGTGAGCAT	225
Caspase-9	F: GGGAAGCCCAAGCTCTTCTT R: CCTGGGAAGGTGGAGTAGGA	221
c-Myc	F: CCCAGCGAGGAATCTGGAAGAA R: GAGAAGCCGCTCCACATCAGTC	286
P53	F:ATGGAGGAGTCACAGTCGGATA R: GACTTCTTGTAGATGGCCATGG	479

Table 3: Statistical table of PPI network of 32 RNA pull-down proteins

Item	Data
Number of nodes	31
Number of edges	37
Expected number of edges	13
Average node degree	2.39
Avg. local clustering coefficient	0.346
PPI enrichment p-value	1.81e-08
Analysis result	Your network has significantly more interactions than expected

Note: PPI represents protein-protein interaction.

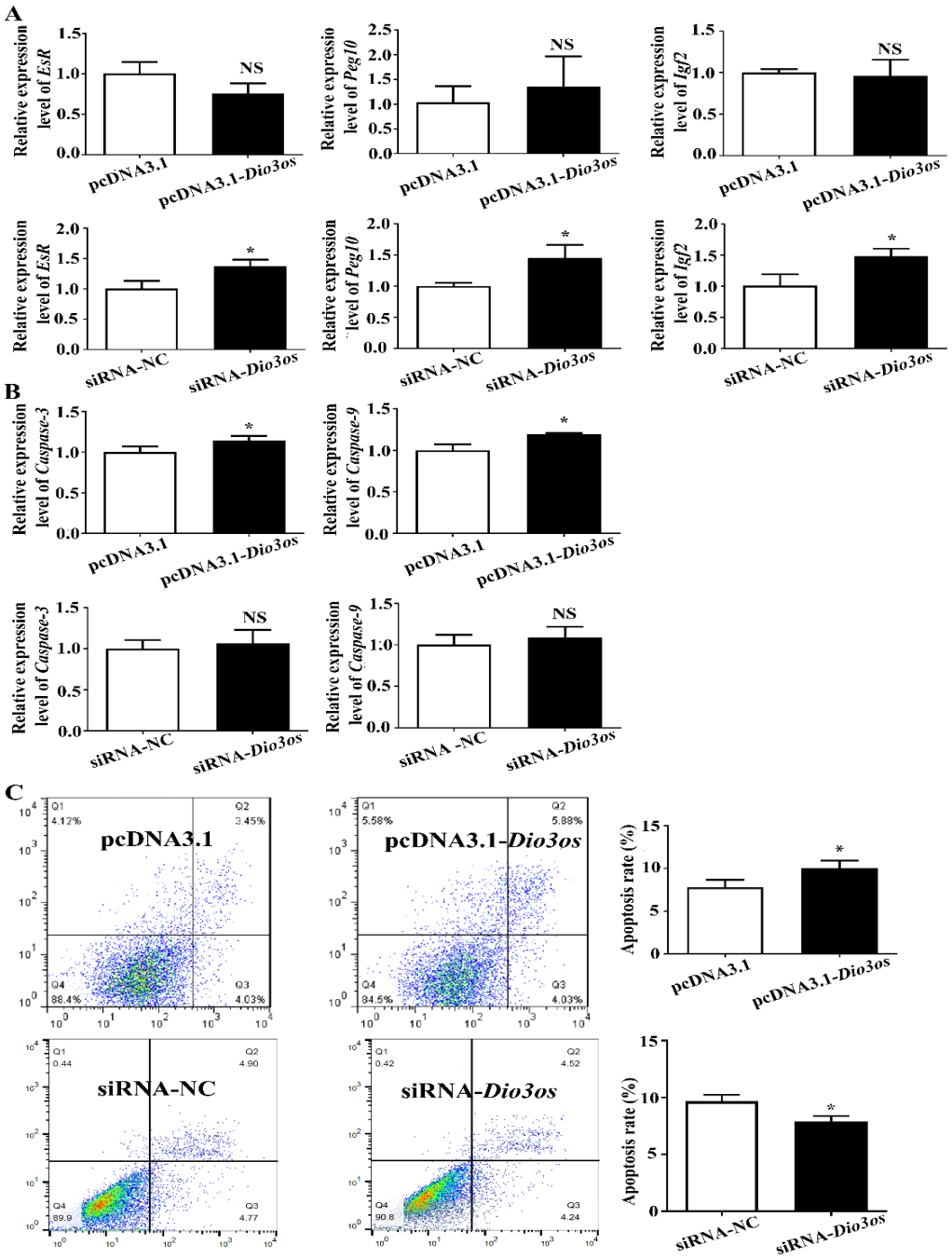

Fig. 3: (A) Effects of *Dio3os* on genes related to development. (B) Effects of *Dio3os* on genes related to apoptosis. (C) Effects of *Dio3os* on the apoptosis rate of mouse ESCs. *P<0.05.

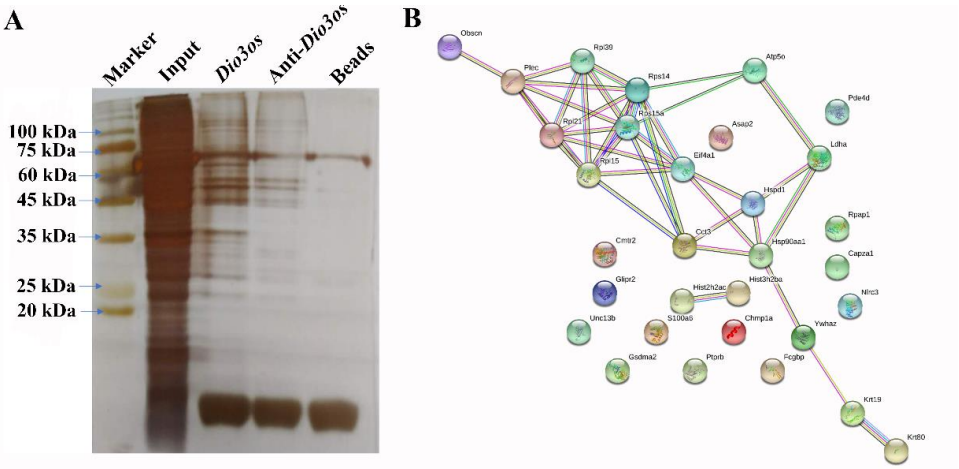

Fig. 4: Identification and analysis of RNA pull-down. (A) Silver staining map of RNA pull-down. (B) PPI network analysis of 32 proteins.

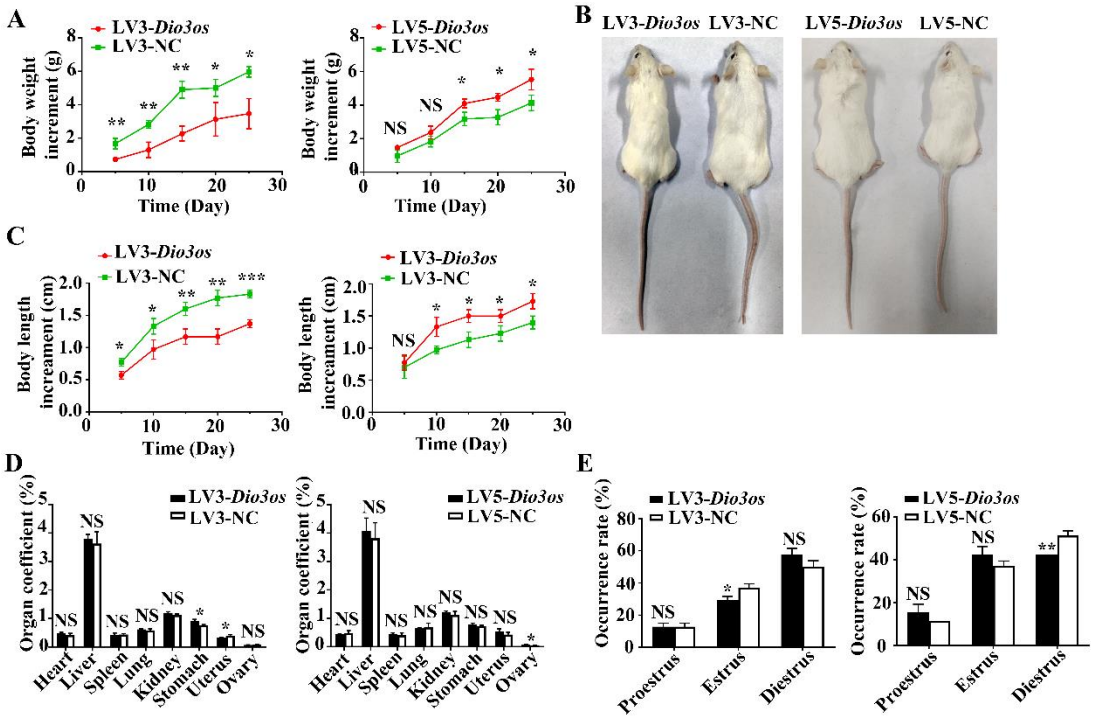

Fig. 5: (A) Mean body weight increment of mice injected with different lentiviruses. (B) Photograph of mice injected with different lentiviruses. (C) Mean body length increment of mice injected with different lentiviruses. (D) Organ coefficient of mice transfected with different lentiviruses. (E) Occurrence rate of each phase of estrous cycle in mice transfected with different lentiviruses. Diestrus represents the stages of metestrus and diestrus. *$P<0.05$, **$P<0.01$.

and *Gtl2*, which suggests that *Dio3os* activity is associated with *Dlk1-Dio3* imprinted locus. Meanwhile, *Dio3os* and *Dio3* were highly expressed in mouse uterus and ovary, which is supported by the fact that *Dlk1-Dio3* imprinted locus was related to development and reproduction.

The examination of RNA-seq indicated that *Caspase-3* and *Caspase-9* were significantly affected by *Dio3os*. It is well known that *Caspase-9* is usually the initiator of apoptosis, while *Caspase-3* is the executor of apoptosis (Yang *et al.*, 2017; Ahn *et al.*, 2018; Ge *et al.*, 2018). Activation of *Caspase-9* by external action can lead to the activation of *Caspase-3*, which eventually leads to apoptosis. The activity of proteins interacting with *Dio3os* was related to development and growth, suggesting that *Dio3os* probably regulates development and growth by affecting these proteins. From these results, it is obvious that *Dio3os* activity is related to reproduction, development and apoptosis at the cellular level.

The data on the increment of body weight and body length showed that *Dio3os* promoted not only body weight but also body length. However, tail length growth was not affected by *Dio3os* in mice. This parameter is mainly used to maintain body balance, and there was no concrete evidence showing that tail length was related to growth, development or reproduction. These results suggest that *Dio3os* can promote physical growth and development in the mice.

The results of the present study also showed that organ coefficients of uterus and ovary were significantly affected by LV3-*Dio3os* and LV5-*Dio3os*. It implies that *Dio3os* can regulate reproduction by affecting growth of reproductive organs. Furthermore, the changes of estrous cycle in mice can reflect the growth status of ovarian follicles and the secretion of estrogens or related hormones in vivo. The data of estrous cycle indicated that *Dio3os* affected the occurrence rate of estrus and diestrus, which further supports the idea that *Dio3os* may affect the reproductive activity in mice.

Conclusions: Results of this study showed that *Dio3os* can affect growth, development and reproductive activity in mice. It may be noted that this study fails to elaborate the exact mechanism behind this activity of *Dio3os*. However, this study does suggest that *Dlk1-Dio3* locus-derived lncRNA *Dio3os* affects growth, development and reproductive activity in mice.

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Authors contribution: YX and JC conceived the idea and designed the study. HX, YH and XL executed the experimental work. HX and GX analyzed the data. All authors interpreted the data, critically reviewed the manuscript for important intellectual contents and approved the final version.

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