The mechanisms whereby *nanog* gene functions in male germ cells' development in the Chinese soft shelled turtle (*Pelodiscus sinensis*)

Xu Jianfei, Tan Zhimin, Chen Kaili, Xu Hongyan*

Key Laboratory of Freshwater Fish Reproduction and Development, Ministry of Education, Key Laboratory of Aquatic Sciences of Chongqing, College of Fisheries, Southwest University, Chongqing 402460, China. xuhyzqh@163.com

INTRODUCTION

- Nanog, a homeodomain-containing transcription factor, has been well studied in regulating the development of stem cells in mammals. However, due to the limitation of animal manipulation and the lack of stem cell lines in reptiles, especially in turtles, the study on the development of stem cells and function analysis of NANOG in turtles is rare.
- Germ cells are the unique cells, being able to differentiate into gametes, i.e. eggs or sperms in the female and male, respectively. At present, there are the increasing problems of genetic diversity reduction, germplasm degradation, and being short of aquatic animal species with excellent traits, it is necessary to establish the germ stem cell lines and related techniques of genetic modifications to improve breeding efficiency.
- Turtles, one of the main members of reptiles, provide a unique model for the studies on evolutionary and developmental biology due to their shared ancestry with fish and mammals, especially *Pelodiscus sinensis*. Now, *P. sinensis* is over-exploited because of their highly medicinal, nutritional and ornamental values, resulting in some of species from wild field underwent extinction, as well the germplasm of farmed species became deteriorated. Therefore, it is essential to develope the related manipulation technologies of germ stem cells and investigate the development and reproduction biology aimed at preserving the genetic resources of endagered turtles and improve the germplasm of farmed species.

RESULTS

1. Isolation and characterization of testicular cells in *Pelodiscus sinensis*.

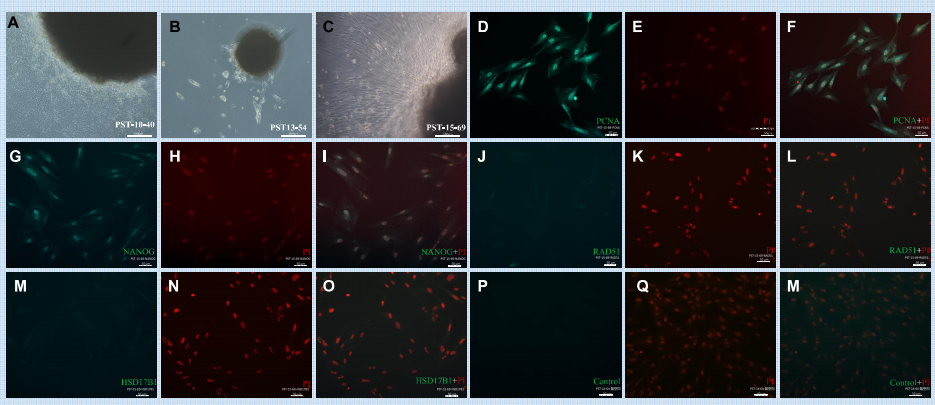

Figure 1. Primary culture and subculture of testis cells of *Pelodiscus sinensis*
Characterizing the isolated and cultured cell *via* fluorescence immunostaining for the protein of related to germ stem cells, germ cells and proliferation in males. Testicular cells of *P. sinensis* were from primary culture of adult testis and were passaged over 15 times (**A-C**). The cells at passage 15 were used for fluorescent immunostaining analysis, and examined with the antibodies, including PCNA, NANOG, RAD51, HSD17B1 and blank mouse serum as a control (green in **D, G, J, M and P**), and nuclei were counterstained with propidium iodide (PI; red in **E, H, K, N and Q**). (**F, I, L, O and M**) Merged images.

2. Construction of *nanog* overexpression and knockdown virus cell lines.

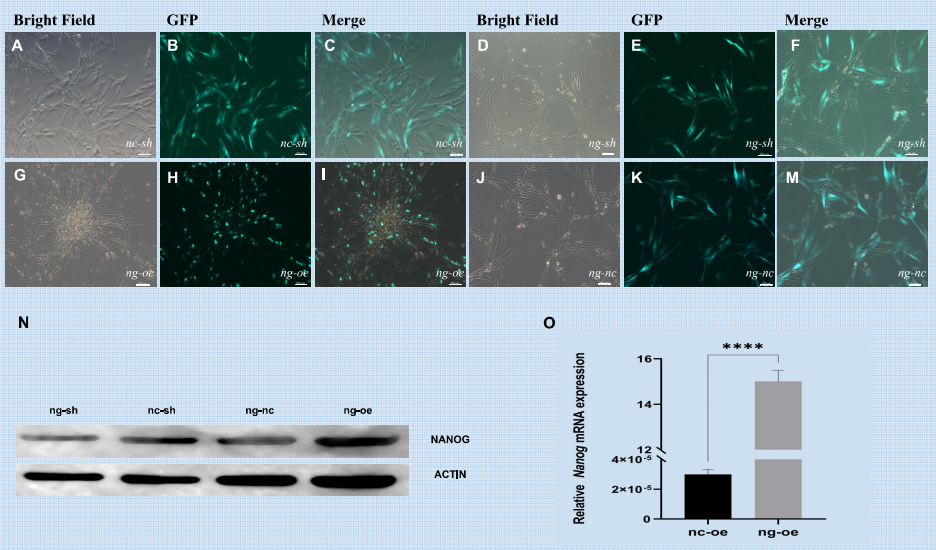

Figure 2. Overexpression and konckdown of *nanog* in the cultured cells.
(**A-C**), (**D-F**), (**G-I**) and (**J-M**) were infected with the *nc-sh*, *ng-sh*, *ng-oe* as well as *nc-oe* viruses respectively. *Nanog* expression was detected by western bloting (**N**) and qRT-PCR(**O**). *nc-sh*， *Non-targeting Control shRNA; ng-sh, Nanog shRNA; nc-oe, Non-targeting Control overexpression; ng-oe, Nanog overexpression.*

3. Effect of *Nanog* expression on the proliferation of testicular cells.

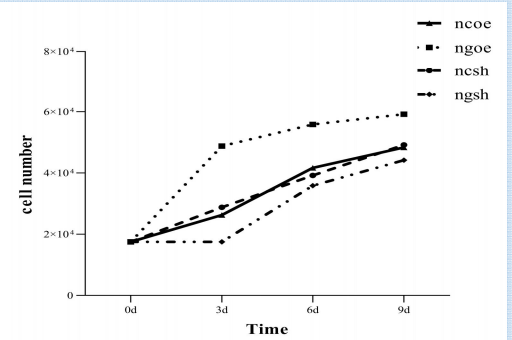

Figure 3. The cells' proliferation affected by *Nanog* expression.
The cultured cell were infected with *Nanog* overexpession (*ngoe*) or knockdown (*ngsh*) virus, the results showed that the cells infected with *ngoe* virus proliferated fastest and with *ng-sh* the slowest during 9 days culturing.

4. Transcriptomic profiles in the testicular cells infected with *ng-oe and nc-oe* viruses.

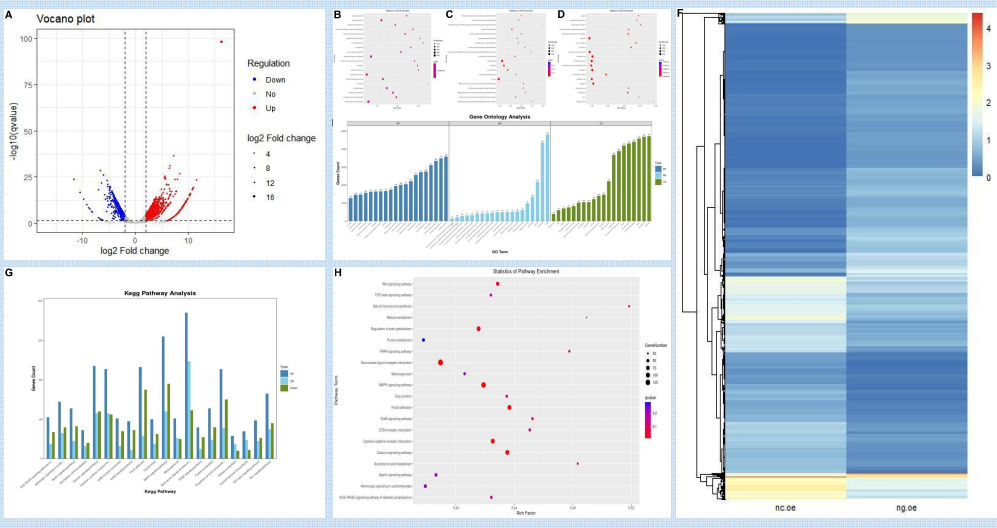

Figure 4. Analysis of differentially expressed genes.
The distribution of visualised differential genes is shown by volcano plots (**A**).The distribution of the number of differentially significant genes on Term enriched in bp, cc, and mf is demonstrated by the GO enrichment classification histogram (**E**) and each ontologys by sctatterplot individually (**B-D**), and in KEGG Pathway by the KEGG enrichment classification histogram (**G**) and sctatterplot (**H**). The genes were clustered and analysed to visualise the different expression of the genes in different treatments by heatmap (**F**).

5. *Nanog* overexpression affected the expression of genes related to male germ cells in *Pelodiscus sinensis.*

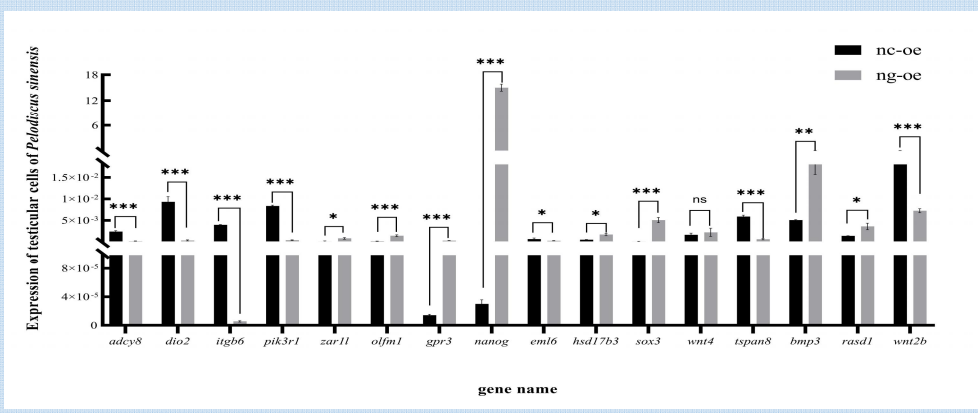

Figure 5. Differential gene expression levels were tested by RT-qPCR.
The results showed that the *nanog* gene overexpression could significantly modulated a serial of genes, including up-regulating the genes, *zar1l, olfm1, gpr3, hsd17b3, sox3, bmp3* and *rasd1*, while down-regulating the genes, *adcy8, dio2, itgb6, pik3r1, eml6, tspan8, wnt2b*, and so on.

CONCLUSION

- ✓ In this study, a testicular cells line were established in *P. sinensis*.
- ✓ The overexpression and knockdown of *nanog* gene were carried out in the testicular cells of *P. sinensis*.
- ✓ The overexpression of *nanog* gene could promote the proliferation of the cultured testicular cells of *P. sinensis*.
- ✓ Overexpressing *nanog* gene could significantly regulate a serial of genes, including the up-regulated genes, such as *zar1l, olfm1, gpr3, hsd17b3, sox3, bmp3* and *rasd1*, and the down-regulated genes: *adcy8, dio2, itgb6, pik3r1, eml6, tspan8, wnt2b*.