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Observation of the Expression of Dazl and Vasa Suggests that Chinese Soft-shelled Turtle Use the Inductive Mode of Primordial Germ Cell Specification

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Abstract. The origin, migration and differentiation of primordial germ cells (PGCs) have always been an important issue in developmental biology. At present, studies on the specification of PGCs in amniotes mainly focus on birds and mammals. Turtles are reptiles known as "living fossils", and their sex determination is influenced by both genetic and environmental factors, making them an ideal model animal for the study of vertebrate sex differentiation. However, the study of PGCs specification in turtles is very limited. Here we reported using RNA probes for the germ cell-specific markers Dazl and Vasa to examine growing oocytes, early embryos and adult ovaries in the Chinese soft-shelled turtle. The results shows that the expression of Dazl and Vasa could not be detected in the growing oocytes and early embryos, and the specification of germline begins around E7, indicating that the Chinese soft-shelled turtle do not contain germ plasm and belongs to the inductive specification.

Keywords: Germ plasm; Oocytes; Chinese soft-shelled turtle; Specification

1. Introduction

There are two general modes of specification in animal primordial germ cells (PGCs): preformation and induction. In the former animal species, germ plasms are formed during early embryogenesis, with the division of the zygote, germ plasms are only allocated to certain cells, and these cells containing germ plasms will develop into PGCs. In the latter animal species, there is no germ plasm in the zygote and early embryo. When the embryo develops to a certain stage, PGCs is induced by special signals secreted by cells around the site where it occurs [1].

In recent years, the discovery and application of germline specific marker genes (such as Vasa, Dazl, Dnd, etc.) have greatly promoted the research on the specification of PGCs [2]. At present, PGCs specification patterns of many model animals from lower invertebrates to higher mammals have been defined. For example, the PGCs specifications of elegans, fruit flies, zebrafishes and African clawed frogs belong to the predetermined mode, whereas the PGCs specifications of crickets, salamanders, mice belong to the inductive mode [3].

Current studies on the origin of PGCs in amniotes mainly focus on birds, and some crocodilians and lizards in reptiles [4]. Turtles are reptiles with an evolutionary history of more than 200 million years and are known as "living fossils". At present, the research work on the occurrence and development of PGCs in tortoises and turtles is relatively lagging behind, and the relevant research is very scarce. So far, only the Red-eared Slider (Trachemys scripta) belonging to the hard-shelled turtle group has inferred that the specification of its PGCs belongs to the inductive mode by tracing and analyzing the expression of germline marker genes [5]. Therefore, the study of the PGCs specification of Chinese Soft-shelled Turtle (Pelodiscus sinensis) belonging to the soft-shelled turtle helps to fully understand the origin of turtle PGCs and evolution of vertebrate PGCs.

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2. Material and Methods

2.1 Total RNA Extraction

Total RNA was isolated from tissue samples using RNAiso plus kits (Takara, Dalian, China). The RNA quality was analyzed by 1.2% agarose gel electrophoresis and spectrophotometric absorption at 260 nm.

2.2 Reverse Transcription (RT) and Quantitative (q) PCR

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The qPCR was performed using SYBR Premix Ex TaqII (Takara, Dalian, China). Each reaction (in 20 μl) contained 10 μl of 2 × SYBR Premix Ex Taq™ II, 0.7 μl of each primer (Table 1), 1 μl template cDNA and 7.6 μl RNase-free water. The qPCR was performed as follows: an initial denaturation at 95 °C for 10 s followed by 40 cycles of amplification (denaturation at 95 °C for 5 s, annealing and extending at 60 °C for 30 s) and a dissociation stage with temperatures rising from 55 °C to 95 °C at a ramp rate of 0.1 °C/s. Allsamples were amplified in triplicates and the mean was used for further analysis using the $2^{-\Delta\Delta Ct}$ method.

Table 1. Primers list.

Primer name	Primer sequence
Dazl563-F	5'-AGAACACCCAGTTGTCG-3'
Dazl563-R	5'-GTAATTCCTTTGCTCCC-3'
Vasa490-F	5'-AAGACAGGCGTCAAACA-3'
Vasa490-R	5'-CCACAGCGTCCAGTTCG-3'
Gapdh-F	5'-TCCTGCTAACATCAAGTGG-3'
Gapdh-R	5'-GGTCCTCCGTGTATCCC-3'
QDazl-F	5'-AGTCTGTGGACCGAAGC-3'
QDazl-R	5'-TTTAAGCACTGCCCTAC-3'
QVasa-F	5'-GGTACGCAAATAGGTCA-3'
QVasa-R	5'-ATCAAGCATACGGTCAG-3'
QGapdh-F	5'-AATGGCTTTCCGTGTTC-3'
QGapdh-R	5'-ACCTGGTCCTCCGTGTAT-3'

2.3 Fluorescence in Situ Hybridization (FISH)

Samples were fixed in 4% paraformaldehyde for 4 h and then the slides were rinsed with 2× SSC (saline sodium citrate) prior to hybridization, and the appropriate amount of probe was applied in a hybridization solution containing 10% formamide, 2 × SSC, and 10% dextran sulfate (w/v). Hybridization was allowed to occur overnight in a humid chamber at 37°C. Slides were then washed twice for 30 min at 37°C with 10% formamide in 2 × SSC. DAPI was applied during the second wash. The probe sequences are listed here.

Dazl:tgcttcggtccacagacttcttttgtggactgtttccagatgattgtgcaggctctggaacagaacattctgcttggataacttctggctct acttcactgcagtgatagtttattgaagtataagccggaggtaaaacgtaattcctttgctccccaggaggccactgtggtgggacctgatagttgt aggtgggctggtatcct

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Vasa:tatacttctgcaccggcgtcagttttgaatatccagctttagcaatattcttgattaacgtttgacaaagattagcttcttcaaaagtcagtattg ctggaggaggatcaagtcctgagacctccacaaggattgtgtcatatttgtcaaaattaattcctgtctggtagtgtgcaaatatggcttcttcattgt caggt ggaggtggaggcacatag

3. Results and Discussion

3.1 Verification of the Germ Cell Specificity of Dazl and Vasa Probes

We have successfully cloned the full-length cDNA sequence of the Dazl/Vasa gene of Chinese soft-shelled turtle, and extracted the total RNA of the heart, spleen, lung, muscle, skin, blood, testicle and ovarian tissues of 8-month-old female and male turtle respectively. The results of RT-PCR showed that, Dazl/Vasa were only expressed in testicle and ovarian tissues containing germ cells, but not in other tissues (Figure.1A). The results of Real-time PCR further showed that Dazl/Vasa was only highly expressed in the testicle and ovarian tissues, and the expression level of Vasa and Dazl in female ovarian was about 13 times and about 6 times of that in male testicle, respectively (Figure.1B). These results verified that both Dazl and Vasa could be used as molecular markers for the germ cells of Chinese soft-shelled turtle.

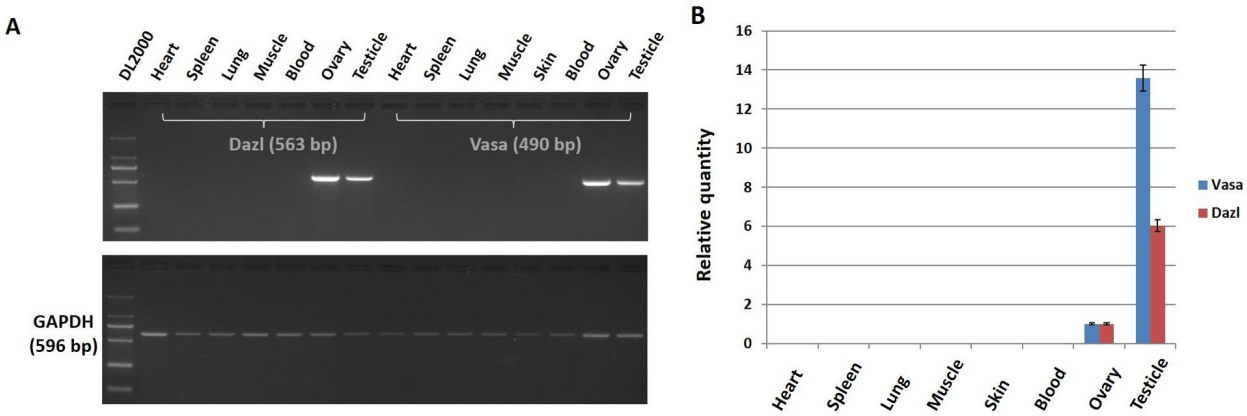

Figure 1. Expression of Dazl and Vasa in different tissues of 8-month-old Chinese soft-shelled turtle.

A: RT-PCR was used to detect the expression of Dazl andVasa in different tissues of 8-month-old soft-shelled turtle; B: The relative expression levels of Dazl andVasa in different tissues of 8-month-old Chinese soft-shelled turtles were detected by Real-time PCR, and the expression level of Dazl/Vasa in the testicle was set as 1.

3.2 Expression of Dazl and Vasa RNA in Growing Oocytes

The expression of Dazl and Vasa was not found in the developing oocytes of the 3-year-old turtle ovary, but it was found in the surrounding cells (Figure.2). This suggests that the Dazl and Vasa RNAs, which specific for germ cells, are expressed in the ovary of Chinese soft-shelled turtle, whereas germ plasm is not found in the cytoplasm of growing oocytes.

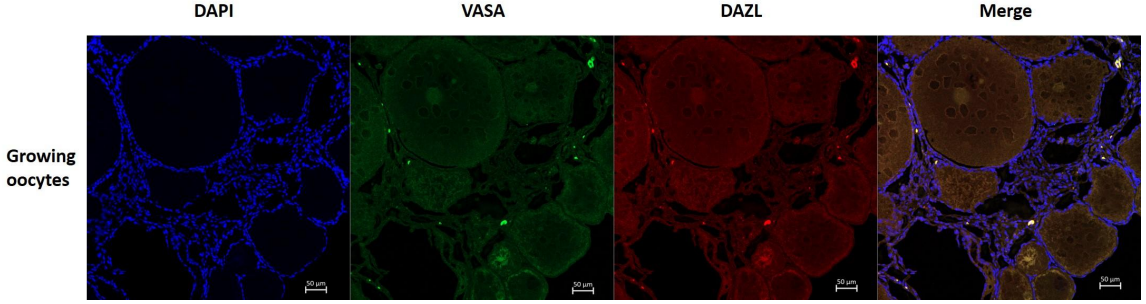

Figure 2. Fluorescence micrographs of RNA FISH assays of growing oocytes

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3.3 Expression of Dazl and Vasa RNA in Early Embryos

Expression of Dazl and Vasa was not detected in embryos from soon-to-be-laid eggs (E0) of the oviduct of five-year-old adult female Chinese soft-shelled turtle at peak laying time (Figure.3). There was also no detectable expression of Dazl and Vasa in the embryos (E1-E6) of the laid eggs. Strong Dazl and Vasa could be observed in the E7 embryos, indicating that the primordial germ cells of the Chinese soft-shelled turtle were induced to generate (Figure.3). Different species have different sites and times of initial occurrence of PGCs. Mouse PGCs originated from the base of the embryonic allantoic sac at E7.5 and migrated at E8.5 [6]. Chick PGCs first appeared in the blastoplast, clustered in the reproductive crescent at 18 h, and migrated into the blood system and along the vascular network at 33 h [7].

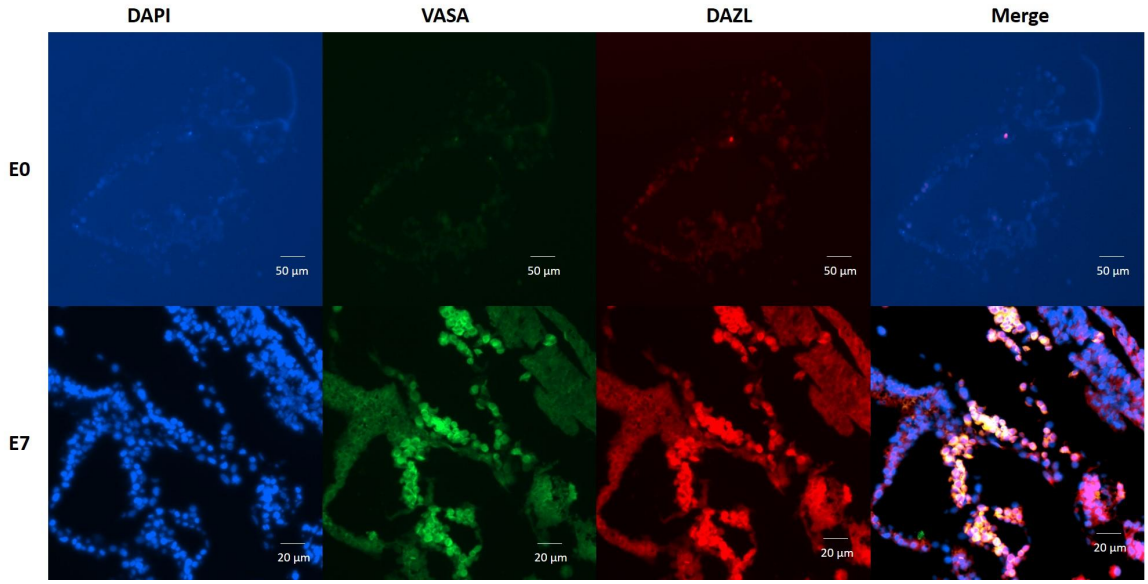

Figure 3. Fluorescence micrographs of RNA FISH assays of early embryos

3.4 Expression of Dazl and Vasa RNA in Adult Ovaries

Large amounts of Dazl and Vasa can be observed to be expressed in mature ovaries of five-year-old adult female Chinese soft-shelled turtle at peak laying time (Figure.4). Dazl and Vasa signals are concentrated in the cells surrounding mature oocytes, but absent in the cytoplasm of the oocytes. This result is consistent with that has been reported previously for the hard-shelled turtle (Trachemys scripta), whose PGCs specialization mode is also induction [5].

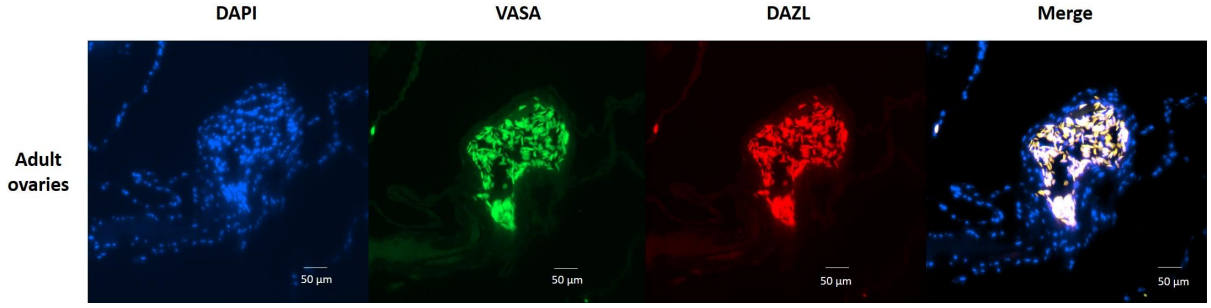

Figure 4. Fluorescence micrographs of RNA FISH assays of adult ovaries

Conclusion

In conclusion, germline specific marker Dazl and Vasa can be detected in mature ovaries and E7 embryos, but absent in growing oocytes and early embryos E0-E6, indicating that there is no germ plasm in the embryos of Chinese soft-shelled turtle. These data support that Chinese soft-shelled turtle use the inductive mode of germ cell specification.

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