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Expression of Dazla Gene in the Development of Mouse Ovary*

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생쥐 난소의 발달 중 Dazla 유전자의 발현 양상*

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서창석[1,2] · 김용범[1] · 구승엽[1] · 지병철[3] · 최영민[1,2] · 김정구[1] · 문신용[1,2]
이진용[1] · 김석현[1,2,*]

연구목적: 본 연구에서는 생쥐 Dazla 유전자의 난소내 발현 위치를 확인하고, 배아의 발달에 따른 Dazla 유전자의 발현 양상을 관찰하고자 하였다.

연구재료 및 방법: 임신 제 7일, 10일, 11일, 14일의 태자 (각각 n=9)와 생후 27일된 암컷 미성숙 생쥐 (n=32), 8주령의 암컷 성숙 생쥐 (n=9)로 부터 난자, 과립막세포, 난소 조직을 획득하였으며, 9주령의 수컷 성숙 생쥐 (n=3)로부터 고환 조직을 획득하였다. 각각의 획득된 조직과 세포에서 Dazla mRNA의 발현 여부를 RT-PCR, in situ hybridization (ISH) 방법 등으로 확인하였다. 성선자극호르몬을 투여하지 않은 미성숙 및 성숙 생쥐에서 획득한 미성숙 난자 (GV)와 PMSG와 hCG를 투여한 미성숙 및 성숙 생쥐에서 획득한 성숙 난자 (MII)에서 RT-PCR로 Dazla 유전자의 발현 여부를 확인하였다. 미성숙 및 성숙 생쥐의 난소 조직과 성숙 생쥐의 고환 조직에서 RT-PCR 및 ISH 방법으로 Dazla 유전자의 발현 여부를 확인하였다.

결 과: 난소의 과립막세포에서는 미성숙 및 성숙 생쥐, PMSG와 hCG 투여 여부 등과 상관없이 모두 Dazla 유전자의 발현은 음성으로 판정되었다. PMSG 및 hCG를 투여하지 않은 난소에서 획득한 미성숙난자 (germinal vesicle, GV) 또는 PMSG 및 hCG 투여 후 채취한 성숙 난자 (metaphase II, MII) 모두 Dazla 유전자가 발현되었다. Dazla 유전자의 발현은 수정 직후 (2PN) 음성으로 전환되었으며, 착상 직전의 배반포 시기까지 유전자 발현이 음성으로 지속되었다. Dazla 유전자는 임신 제 7일 (PCD 7), 10일 (PCD 10), 11일 (PCD 11)의 태자에서도 유전자 발현이 계속 음성으로 관찰되었으나, 성 분화가 일어나기 시작하는 임신 제 12~14일 (PCD 12~14)의 태자에서 유전자 발현이 다시 관찰되었다.

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결 론: Dazla 유전자는 난소 내 난자에서만 특이적으로 발현하는 난자 특이 유전자 (oocyte specific factor)로서 Dazla 유전자가 난소 내 난포 생성과 관련성이 있음을 제시하고 있다. 향후 조기 폐경 환자에서의 연관성 등을 확인한다면 임상적으로 유용한 지표가 될 수 있을 것으로 사료된다.

Key Words: Dazla, in situ hybridization, RT-PCR

Ten to twelve percent of married couples are suffering from infertility. On the contrary to the initial belief on infertility as primarily of female problem, now it is accepted that half of them are entirely or in part due to male factor. Significant portion of these are infertile either as a result of not producing enough sperm (oligospermia), or not producing sperm at all (azoospermia). Some of these patients show microdeletion of Yq (distal long arm), suggesting that underlying genetic factor is responsible for spermatogenetic abnormalities in certain portions. This led to the postulation that a locus encoding azoospermic factor (AZF) is located in distal long arm of Y-chromosome, subsequently found three cluster of genes (AZFa, AZFb, and AZFc).[1]

There are two AZF candidate genes isolated. One is RBM (RNA binding motif) gene, the other one is DAZ (deleted in azoospermia)/SPGY (spermatogenesis gene on the Y) gene. RBM encodes a nuclear protein, which has a single RNA recognition motif (RRM) and an internally repeated sequence called the SRGY box. Its expression is restricted to the male germ line in man and mouse. In human, it is located on AZFb,[2] while it is located on the short arm of Y-chromosome between Sry and the centromere in the mouse.[3] DAZ and SPGY were originally considered as different gene, but it is now clear that both are same gene located on AZFc in the human.[4]

One distinctive thing between DAZ/SPGY and RBM genes is that DAZ/SPGY do not exist in other species except human and primate (old world monkey). Species other than human and old world monkey have DAZ/SPGY homology in autosomal site. Interestingly, there is also DAZ homologue in monkey and human autosome. The autosomal homologue has been cloned from many species. Mouse homologue of DAZ mapped to chromosome 17 band 1. Human autosomal homologue of DAZ would be on the short arm of human chromosome 6, based on homology between the mouse and human gene maps.[5]

The genomic structure of the DAZLA (or Dazla, DAZ like autosome), which gene is autosomal homologue of DAZ, was elucidated and found to be consisting of 11 exons spanning 19 kb.[6] The exon/intron boundaries are conserved between DAZ and Dazla. The homology between human DAZ and mouse Dazla is not strong, and Dazla contains only one of the seven DAZ repeats found in DAZ. Instead, there is more homology between human and mouse Dazla. Human Dazla shows 88% homology with mouse Dazla whereas 76% homology with human DAZ and Dazla protein sequence.[7]

The role of Dazla gene regarding female folliculogenesis paid little attention by most researchers until recently because the intensity of Dazla expression in the female is 1/100 of the male, albeit it is expressed both in male and female.[8] The function of Dazla was identified by mutational study of Dazla gene in the mouse.[9] In homozygous female, killed 6~9 weeks after birth, had tiny ovaries compared to littermate controls. Histologic analysis of the ovaries showed that follicles and ova were absent. However, there is no universal consensus among researchers on the exact location of the Dazla gene expression in the ovary. Thus, this study was performed to investigate the location of Dazla gene expression and to observe temporal expression patterns of Dazla mRNA during embryogenesis in the mouse ovary.

MATERIALS AND METHODS

1. Source of ovary tissues, oocytes, and embryos

Pregnant mice with PCD 7, 11, 12, 14 (n=9 respectively), 27 days old immature female mice (n=32), 8 weeks old female mice (n=9), and 9 weeks old male mice (n=3) were sacrificed to retrieve embryos, fetuses and ovaries respectively. Oocytes and granulosa cells were collected without injecting gonadotropin or after injecting PMSG (10 IU), which is followed by hCG injection 48 hours later (10 IU). Oocyte and granulosa cells were separated under dissecting microscope after puncture of ovary follicles. Animal experiment was performed under observance of AFSG (animal facility service guidelines).

2. Semiquantitative RT-PCR

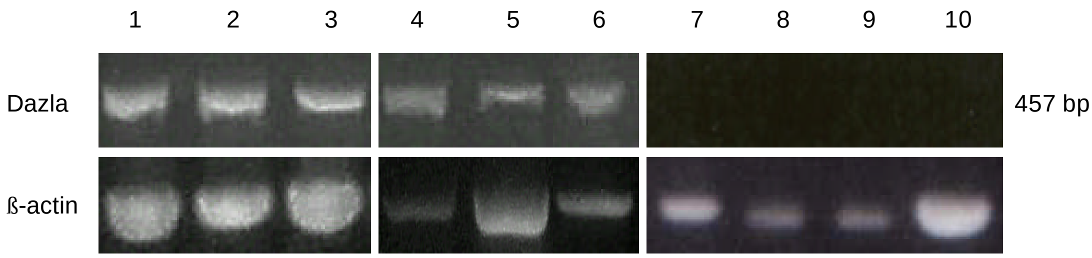
Primer design was based on the most homologous mRNA sequences among human, mouse, and rat within exonal region. The forward primer was TCA CTG ATC GAA CTG GTG TG, and reverse primer was TCA GCT CCT GGA TCA ACT TC. RT-PCR was performed as previously described (10). In brief, 500 ng of extracted RNA was subjected to the RT reaction using first strand cDNA synthesis system (Life Technologies Inc.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (dNTP, 0.5 mM) at 42℃ for 50 min, 70℃ for 10 min. Serial diluation of cDNA (1/2, 1/4, 1/8, 1/16) corresponding to the different experimental time points (20, 25, 30, 35 cycles of amplification) was made to determine the log phase of amplification reaction. After resuspending first strand cDNA in 50 μl water, PCR was performed under following conditions: 30 cycles of denaturation at 94℃ for 30 sec, annealing temperature at 55℃ for 30 sec and extension at 72℃ for 30 sec.

3. In situ hybridization

Paraffin-embedded mouse ovarian tissue sections (10 μm) were used for these experiments. The sections were taken randomly from the ovary pieces, and no effort was made to standardize where the sections were taken (i.e. maximal cross-sections or whole-thickness slices). The relevant in situ hybridization procedures have been described previously.[11] In brief, after dewaxing, sections were treated with proteinase K, acetylated, washed, dehydrated, and hybridized with radioactive probe (10^6 cpm/μl). Dazla probes were designed to contain homologous region for mouse, rat, and human. Plasmids constructed for the probe preparation were as follows: Dazla-457 for Dazla, a DNA segment (457 bp) of mouse Dazla exonal region was amplified by RT-PCR from ovarian total RNA and cloned into a pCR II vector, (Invitrogen, Carlsbad, CA). Antisense and sense RNA probes were synthesized by transcription using 35^S-UTP and T3 or SP6 polymerase after digestion with BamHI and XhoI restriction enzymes. After hybridization, sections were treated with ribonuclease A, washed in decreasing concentrations of saline sodium citrate (SSC), and finally in 0.1X SSC at 65℃ for 40 min. After dehydration in graded ethanol solutions, slides were exposed X-ray film for 3 days. After obtaining adequate X-ray film images, the sections were treated with xylene, rinsed in 100% ethanol, air-dried, and then coated with Kodak NTB-2 liquid autoradiographic emulsion. Slides were exposed for 2 weeks at 4℃ in a desiccated, dark box. After exposure, the slides were developed (Kodak D19, 3.5 min at 14℃), rinsed briefly in distilled water, and fixed. After washing, slides were lightly stained with hematoxylin and eosin. In each experiment, duplicate slides from all ovaries studied were processed in the same in situ hybridization run using the same probe, conditions of hybridization, and length of autoradiographic exposure. The sections from all ovaries in all experiments were scored and the data were repeated at least three times with similar results.

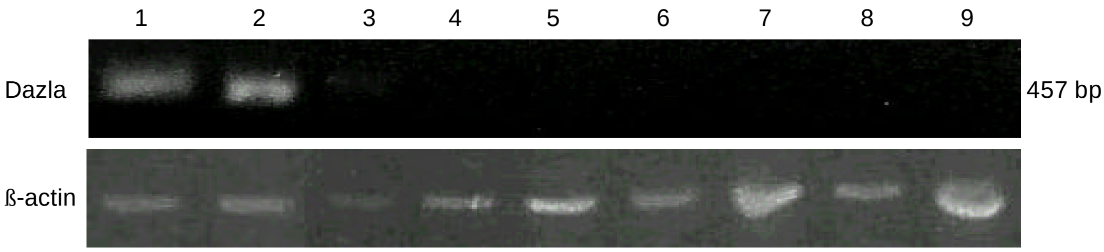
RESULTS

Dazla mRNA was detected in adult testis, immature ovary, and adult ovary tissues by RT-PCR (Figure 1). RT-PCR of Dazla was positive in oocytes from immature and adult mice regardless of their maturity (GV, MII), while granulosa cells (GCs) from immature and mature mice were negative. Granulosa cells treated by PMSG/hCG were also showed negative RT-PCR. Human luteinized granulosa cells from IVF-ET patients and granu-



1. Adult testis
2. Immature ovary
3. Adult ovary
4. Oocytes from adult ovary (GV: 48 hrs after PMSG)
5. Oocytes from adult ovary (MII: 48 hrs PMSG/14 hrs after hCG)
6. Oocytes from immature ovary without treatment
7. GCs from immature mouse ovary without treatment
8. GCs from mature mouse ovary without treatment
9. GCs from mature mouse ovary with 48 hrs after PMSG
10. GCs from mature mouse ovary with 48 hr PMSG/14 hrs after hCG

Figure 1. RT-PCR of Dazla in the mouse testis, ovary, oocyte and granulosa cells



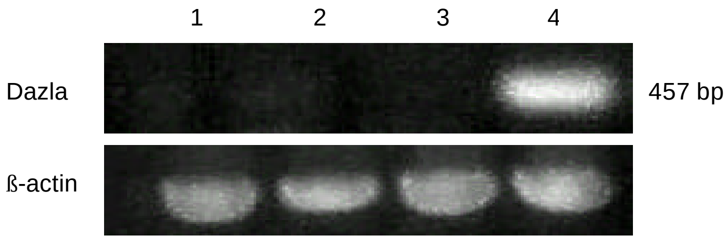
1. Mouse oocytes (GV)
2. Mouse oocytes (MII)
3. 2 pronucleus (2PN)
4. 2 cell embryos
5. 4 cell embryos
6. 5-7 cell embryos
7. 8 cell embryos
8. Morula
9. Blastocyst

**3-9: In vivo development*

Figure 2. RT-PCR of Dazla in the mouse oocytes and various stages of embryos

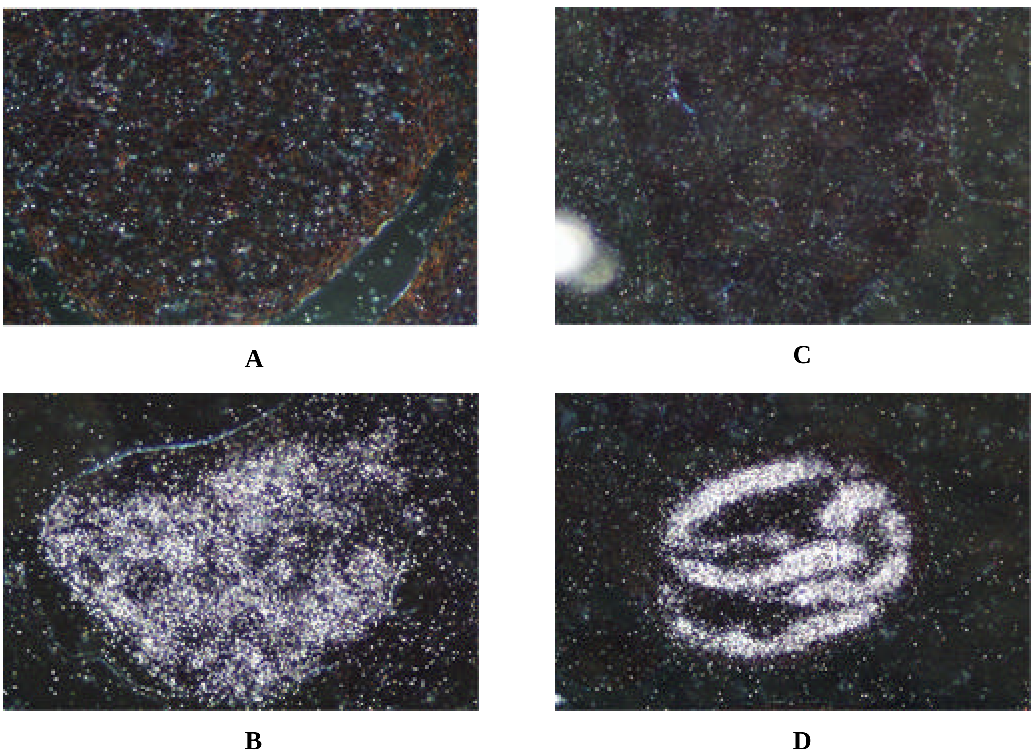
losa cells from granulosa cell line also showed negative signal RT-PCR (data not shown).

RT-PCR of Dazla showed that Dazla expression was disappeared shortly after fertilization in the pronuclear stage (2PN), which pattern of expression was persisted through embryo stages before implantation (2 cell, 4 cell, 8 cell, morula, and blastocyst) (Figure 2).



1. Mouse fetus PCD 7
2. Mouse fetus PCD 11
3. Mouse fetus PCD 12
4. Day 27 ovary after birth

Figure 3. RT-PCR of Dazla in the fetal stage of mice



A: Sense, PCD 14 female fetal ovary
B: Antisense, PCD 14 female fetal ovary
C: Sense, PCD 14 male fetal testis
D: Antisense, PCD 14 male fetal testis

Figure 4. In situ hybridization in PC D 14 mice fetal gonads

RT-PCR of Dazla showed that it did not reappear until PCD 12 of fetal stage, which corresponds to the stage of differentiation into male or female gonads (Figure 3).

However, In situ hybridization of Dazla in the PCD 14 mice showed strong positive signals both in testis and ovary (Figure 4).

DISCUSSION

Dazla is autosomal homologue of DAZ, which is related with male spermatogenesis in the human and primate only. In other species, its autosomal homologue Dazla is regarded as having function of DAZ instead. Dazla is expressed in both male and female gonad with lesser extent of expression in the female. The function of Dazla was identified by mutational study of Dazla gene in the mouse.[9] After replacing region of the gene with neomycin resistant marker by homologous recombination in embryonic stem (ES) cells, which truncates the Dazla gene at nucleotide 309, leaving the RNA recognition motif intact. In homozygous female, killed 6 ~9 wks after birth, had tiny ovaries compared to littermate controls. In homzygous male, size of testis was estimated as 1/3 of the control. Histologic analysis of the ovaries showed that follicles and ova were absent. Testis sections also showed complete absence of germ cells beyond spermatogonial stage. Thus, it seems clear that Dazla mRNA is related gametogenesis both in male and female.

Interestingly, in the absence of Xdazla (Xenopus Dazla), premordial germ cells (PGCs) do not successfully migrate from the ventral to the dorsal endoderm and do not reach the dorsal mesentery.[12] Germ plasm aggregation and intracellular movements are normal indicating that the defect occurs after PGC formation. It is, therefore, important to know when the expression of Dazla gene starts? The first expression of Dazla by Northern blot analysis appears in embryonic gonads at PCD 12.5, and even earlier at PCD 11.5 by RT-PCR in male and female gonads.[8] In PCD 11.5, the only germ cells present in the gonad are PGCs. PGCs are first recognized in the extraembryonic mesoderm at PCD 7.5, subsequently migrate to the developmental genital ridges at PCD 11.5. From PCD 8.5 to PCD 13.5, PGCs replicate by mitosis at a uniform rate with a doubling time of about 16 hours.[13] However, in our experiment, we could not detect Dazla mRNA expression by RT-PCR at PCD 7, 11 and 12, which is contradictory result from previous report (Figure 3). We performed RT-PCR several times after cutting abdominal portion to get concentrated amounts of mRNA from gonads as well as collecting supposed gonads directly under dissecting microscope. It is possible that total amounts of mRNA from gonad may not be enough to get positive signal in our experiment. However, we tried to avoid dilution effect from surrounding tissues by taking gonads directly under microscope. And also we have seen persistent positive signals from 5 to 10 cells of oocytes, which suggests less chances of technical failure in our experiment. Thus, it should be elucidated further to confirm this conflicting result.

After sexual differentiation at PCD 13.5 in the mouse, oogonia enter meiotic prophase, while prospermatogonia continues to divide by mitosis until PCD 14.5 and then prospermatogonia remain quiescent until after birth. Thus, after PCD 15 it shows sexual dimorphism in the expression of Dazla. Dazla transcription level increases and reaches similar levels in gonads of both sexes until PCD 15, which is the time of first oogonia enter meiosis in the mouse. After this, in the female gonad, Dazla transcription level decreases to half and remains on that level while oogonia proceeds meiosis and arrests in prophase I, shortly before birth. In contrast to female gonad, in the male gonad, steady levels of transcription were detected through embryonic development. The number of spermatogonia per testis increased significantly from 0.5×10^5 at birth to about 6×10^5 cells per testis after day 25,[14] while the number of oocytes per ovary decreased significantly from about 0.1×10^5 at birth to about 4,500 when female reaches puberty. Thus, total transcription level of Dazla between testis and ovary reflects these differences in germ cell numbers. In our experiment, Dazla expression was strong enough both in testis and ovary by In situ hybridization. Thus, it seems to be clear that Dazla is expressed both in testis and ovary after sexual development.

Then, where is the location of expression in the ovary follicle? Dazla expression in the growing follicles or embryos as well as the exact location of mRNA expression was not reported clearly. It is reported that Dazla mRNA is expressed in the cytoplasm of oocyte during embryonic periods, while it can be detected in the zona pellucida, and eripheral cytoplasm in the adult ovary follicle.[9, 15~17] Recently, experiment performed from human adult and embryo ovaries, Dazla expression was detected in the granulosa cells as well as cytoplasm of the oocytes performed by immunohistochemistry, and in situ hybridization.[15~17] However, most studies regarding location of Dazla expression were made from immunohistochemistry, which method can give too many non-specific false positive signals. Thus, the exact location of Dazla expression in the ovary is still controversial, which urges further studies on the exact location of Dazla mRNA expression. Thus, we designed our experiment to get oocytes and granulosa cells separately in order to avoid false positive signals (Figure 1). Of course, there is a chance that cells can be contaminated each other. But, there is a scarce chance to be contaminated each other, because separation of single oocyte and granulosa cells was made under microscopy. Moreover, even though granulosa cells have more chances of contamination by oocytes potentially, we did not observe any signal of Dazla in the granulosa cells consistently, while oocytes showed positive signals regardless of their maturity (Figure 1). This is exciting result because Dazla can be another candidate gene as a new oocyte specific growth factor that can control ovarian folliculogenesis if our result is true.

Another interesting question is that when does the Dazla expression turn off. No one tried to make an experiment to respond this question until now. In our experiment, we confirmed that Dazla mRNA expression is turned off shortly after fertilization at the stage of pronucleus (2PN). Negative signal persists through preimplantation stage embryos, that is, blastocyst (Figure 2). Taken together, Dazla mRNA expression is down regulated after fertilization and reappears first in the germ cell of male and female at least from the stage of gonadal sex differentiation.

The implications of Dazla in the clinical practice are obvious. There is one report related with the Dazla used for prognostic value in male infertility.[18] In the azoospermic infertile male, if Dazla gene is detected, there is a chance to find spermatogenesis in the testis after biopsy before pathological report.[18] If not, there is little chance of spermatogenesis. Even though Dazla potentially can be a marker of presence of oocytes in premature ovarian failure likewise azoospermia, however, there is no report on the clinical application of Dazla in the female. And there is no human report on the Dazla single gene mutation in the primary amenorrhea or premature ovarian failure patients. Thus, it may be necessary to explore whether one or both copies of Dazla genes are deleted or mutated in the primary amenorrhea or premature ovarian failure.

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