Dynamic expression profile of DNA methyltransferases in rat testis development

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Abstract

DNA methyltransferases (Dnmts) are unique and perform specific functions during male germ cell development. To further characterize the significance of Dnmts in the events leading to production of spermatozoa, we investigated whether the expression patterns in Dnmt1, Dnmt3a, Dnmt3b and Dnmt3l were apparent in rat testes at different time points during development. The qRT-PCR results showed that expression levels of Dnmt3a and Dnmt3l were abundant before birth and were present at the highest levels in testes tissue at 18.5 days postcoitus (dpc), and gradually decreased from day 0 postpartum (dpp) to 90 dpp. Expression of Dnmt1 and Dnmt3b reached a peak after birth (P <0.01), and then gradually reduced until adulthood. Western blotting and immunolocalization analysis of Dnmt3a and Dnmt3b further confirmed the differential expression and localization of the two proteins during rat testis development. The dynamic expression profile of Dnmts implies specific and potentially nonredundant roles for each of these enzymes in the developing rat testis.

Key words: DNA methyltransferases, testis development, spermatogenesis, rat

Introduction

Spermatogenesis is a dynamic, complex process, tightly regulated by the precise control of a variety of factors, which occurs within the seminiferous tubules in the testis. This process involves not only the spermatogenic cells but also several types of somatic cells in testicular tissue, such as the Sertoli cell and Leydig cell (Costa et al. 2013, Urriola-Munoz et al. 2014). Sertoli cells play a key role in protection, support and nutrition of germ cells, while Leydig cells secrete testosterone, stimulate testis development, and regulate germ cell differentiation and spermatogenesis. Spermatogenesis depends not only on unique processes such as specialized transcription, meiosis and histone-to-protamine replacement, but also on various epigenetic events, for instance DNA methylation, which affects turn-on and turn-off of gene expression. Methylation of the mammalian genomic DNA at the 5 loci in cytosine residues via DNA methyltransferases (Dnmts) is an important epigenetic modification, which plays central regulatory roles in the control of cellular physiology, including embryonic development, cellular reprogramming, spermatogenesis,
X chromosome inactivation, genomic imprinting and transposon silencing (Li 2002, Pontier and Gribnau 2011, Cedar and Bergman 2012, Smith and Meissner 2013). DNA methylation patterns which established in the germ line during gametogenesis are largely erased at early stage of embryogenesis and are reset after implantation (Cedar and Bergman 2012). The Dnmts mainly includes the Dnmt3 family and Dnmt1. The Dnmt3 consists of two catalytically active members (Dnmt3a and Dnmt3b), and a catalytically inactive member called Dnmt3-like (Dnmt3l) (Jurkowska et al. 2011, Neri et al. 2013). Previous research showed that Dnmt3a and Dnmt3b mainly take part in catalyzing de novo methylation at the cytosine of unmethylated CpG sites during embryonic development; both of them have similar biochemical properties but distinct functions (Okano et al. 1998, Okano et al. 1999, Chen et al. 2003). In addition, the Dnmt3a genomic locus produces two transcripts giving rise to two proteins, the longer Dnmt3a1 and the shorter Dnmt3a2 (here collectively referred to as Dnmt3a), which differ in that a 219-amino-acid (aa) amino (N)-terminal tail is only present in Dnmt3a1. Dnmt3l may not possess DNA methylation activity, but plays a regulatory role in DNA methylation by enhancing or inhibiting the activity of Dnmt3a and Dnmt3b in different chromatin contexts (Bourc’his et al. 2001, Hata et al. 2002, Neri et al. 2013). Dnmt1 functions as a major maintenance methyltransferase in vivo, has a preference for hemimethylated CpGs and is critical for the maintenance of methylation patterns during DNA replication (Schaefer et al. 2007). In male mice, Dnmt1 is not detected in fetal gonocytes when methylation patterns are initially set up, but is detected in proliferating spermatogonia, as well as spermatocytes and round spermatids (Sakai et al. 2001, Goossens et al. 2011).

Although DNA methylation and Dnmts expression during murine germ cell development have been demonstrated (Reik and Dean 2001, La Salle and Trasler 2006, Schaefer et al. 2007), such information in rats is largely unknown. Sprague-Dawley rats have served as an important animal model for research in reproduction with excellent reproductive performance and maternal characteristics. Therefore, the goal of this study was to describe the dynamic expression of the Dnmts for identifying candidate methyltransferases which are likely to be involved in the DNA methylation process during testis development in rats.

Materials and Methods

Isolation of testes tissue

Sprague-Dawley rats were obtained from the Fourth Military Medical University (Xian, P. R. China). The animals were allowed to mate naturally. In the morning of a day when vaginal plugs were observed were considered to be 0.5 dpc and the day of delivery as 0 dpp. Testes were collected in fetal (15.5 dpc and 18.5 dpc) and postnatal (0 dpp, 7 dpp, 14 dpp, 21 dpp and 90 dpp) stages, three of them from each stage from three different individuals were fixed in carbazotic acid buffer solutions (saturation picric acid: acetic acid: methanol = 75 : 20 : 5) for 8-12 hours and the others were decapsulated and rinsed in sterile DEPC-treated saline, pooled, and frozen in liquid nitrogen for RNA extraction. Among them thirty male rats were collected at embryonic stage, and ten at each postnatal time point. The entire experimental procedure was approved by the Animal Care Commission of the College of Animal Science, Northwest A&F University, China.

RNA isolation, cDNA synthesis and qRT-PCR analysis

Total-RNA extractions were carried out from pooled testes from different developing stages (57 testes from each embryonic stages, and 27 testes from each postnatal time point) using Trizol (Takara, Japan). The first strand cDNA was synthesized by reverse transcription of 1-5 μg total RNA using M-MLV Reverse Transcriptase and Oligo(dT)15 (Invitrogen, USA). qRT-PCR was performed on the IQ5 (Bio-Rad, USA) using the GoTaq® qPCR Master Mix (Promega, USA). Gene-specific primers (including each transcript) were used to determine the relative expression levels of Dnmt1, Dnmt3a, Dnmt3l and Dnmt3l according to the standard curve method, the primer sequences that were used are listed in Table 1. Gene expression levels were quantified relative to the expression of the β-actin gene, using Gene Expression Macro software (Bio-Rad, USA) by employing an optimized comparative Ct (ΔΔCt) value method. The expression level was calculated as 2^(-ΔΔCt) to compare the relative expression, and one-way ANOVA was conducted to identify genes differing in expression; P <0.05 was considered as significant. Data are presented as mean±SEM.

Western Blotting

Rat testes were prepared by homogenization in 0.15 M NaCl, 0.05M Tris.HCl (pH 7.5), 2 mg/ml leupeptin, 2 mg/ml aprotinin and 100 mg/ml PMSF. 30 fg proteins were denatured by heating at 100°C, separated by electrophoresis on 10% SDS polyacrylamide gels, and transferred to PVDF membranes. The primary antibodies used were polyclonal rabbit
Table 1. Primer sequences for the specific genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Primer length</th>
<th>Product size</th>
<th>GeneBank sequence No.</th>
</tr>
</thead>
</table>
| Dnmt3a | F: GGTTGTTGTGCTGAGAAGCTCA  
R: CCAAGGGCCCCACCTCACAATCAT | 20 bp  
20 bp | 222 bp | NM_001003958.1 |
| Dnmt3b | F:GGGCGGACCTACACGGTCAAGG  
R:AGGGCCGTTGCTGCTCAAGT | 20 bp  
20 bp | 178 bp | NM_001003959.1 |
| Dnmt3l | F:GTATGCCCGCCTGCAGCAAG  
R:CAGGTCCGCTGCTGCTCAAGT | 20 bp  
20 bp | 208 bp | NM_001003964.1 |
| Dnmt1 | F:GGTTCTGCGCGGGGACAGAC  
R:CCGGCAACATGGCCTCAGGG | 20 bp  
20 bp | 183 bp | NM_053354.3 |
| β-actin | F:GCCTCACCACCGCAGATACAA  
R:ACATGCCGAGACCGTTTCTGCG | 20 bp  
20 bp | 118 bp | NM_031144.3 |

anti-Dnmt3a (sc-20703) and polyclonal goat anti-Dnmt3b (sc-10236) (Santa Cruz, USA). The membrane was incubated in the primary antibody solution (1:1000) overnight at 4°C with agitation. Anti-rabbit IgG and anti-goat IgG were used as secondary antibodies (Santa Cruz, USA). The membrane was incubated in the secondary antibody reagent (1:1000) for 2 hours at room temperature. The membrane was washed 3 times for 5 min each time with TBS containing 0.05% Tween 20. The detection of proteins was performed using enhanced chemiluminescence (ECL), and then the blot was imaged using the FluorChem M multicolor fluorescence Western blot imaging system (ALPHA, USA).

Immunocytochemistry

The cross-sections were deparaffinized in xylene for 10 min and rehydrated in a descending alcohol series. Sections were washed for 5 min in phosphate-buffered saline (PBS). Next, tissue sections were performed antigen retrieval step by keeping them in boiling Tris-EDTA Buffer for 10 minutes, and then were pretreated after being cooled to room temperature, incubated with 3% H₂O₂ for blocking of endogenous peroxidases, the followed is the blocking step with 5% BSA for blocking non-specific binding. The specific primary antibodies for Dnmt3a and Dnmt3b were added and incubated overnight at 4°C. For the negative controls, PBS was added instead of primary antibody. The next day, sections were washed three times with PBS after which the biotin labeling secondary antibody (Beyotime, China) was added for one hour at room temperature, and then with HRP-Avidin working liquid (Beyotime, China). The sections were again washed three times with PBS. The visualization of the staining was done with Diaminobenzidine (DAB) (Beyotime, China). The preparations were immersed in PBS and counterstained with hematoxylin. The sections were dehydrated in a mounting series of alcohol and finally in xylene. Glycerol jelly mounting medium (Beyotime, China) was added before placing the cover slip. The images were visualized using Nikon 80i with NIS-Elements software (Nikon, Japan).

Results

Dynamic expression of DNA methyltransferases in the developing testes

As the establishment and maintenance of DNA methylation pattern are ongoing during gametogenesis, we examined the dynamic gene expression of Dnmts in the prenatal (15.5, 18.5 dpc) and postnatal (0, 7, 14, 21 and 90 dpp) testes using qRT-PCR. Expression of Dnmt3a was abundant before birth and was present at the highest levels at 18.5 dpc, which was much higher than that at any other time points (P <0.01), and gradually decreased from 0 dpp to 90 dpp (Fig. 1). Dnmt3l had a similar expression pattern with Dnmt3a, but also with minor differences, such as a dramatic drop of Dnmt3l expression detected after birth, about 54 times lower at 14 dpp than that at 18.5 dpc (Fig. 1). However, the expression pattern of Dnmt3b was different. Before birth, Dnmt3b expression was low, only increased most notably on 7 dpp and 14 dpp, whereas it was 5 times higher than that at the embryonic period. Compared with the Dnmt3 family, the expression of Dnmt1 was relatively low from 15.5 dpc to 90 dpp, while the expression pattern was very similar to Dnmt3b (Fig. 1). In general, the four genes were all present at the lowest levels at 90 dpp.
Fig. 1. Expression pattern of DNA methyltransferases in rat developing testes. Relative quantification of Dnmt1, Dnmt3a, Dnmt3b and Dnmt3l in testis mRNA populations was done in triplicate from pooled testes at the indicated developmental stages via qRT-PCR. For each DNA methyltransferase, the expression level was normalized to β-actin and measured with $2^{-\Delta\Delta T}$ value. Results were averaged from three independent replicates during all stages. Data are shown as the mean±SE of 3 independent replicates. Day postcoitus (dpc); day postpartum (dpp).

Fig. 2. Protein expression of Dnmt3a and Dnmt3b during rat testis development. Expression was analyzed by western blotting, and GAPDH expression levels were used as controls. Day postcoitus (dpc); day postpartum (dpp).
Western blotting analysis of Dnmt3a and Dnmt3b expression during testis development

Having established that the mRNA expression profiles of the Dnmts, Dnmt3a and Dnmt3b have distinct patterns in developing rat testes, they can be chosen as the two representative proteins for further analysis. The immunoreactive results showed that Dnmt3a was detected in all stages of testes development with its expression peaking at newborn, and then dropped gradually after birth (Fig. 2). Dnmt3b was expressed faintly in newborn rat testes, and was higher at 7 dpp and 14 dpp, then decreased in testes at 90 dpp. Most surprisingly, Dnmt3b was not detec-
Fig. 5. Quantitative results from germ cells expressing Dnmt3a and Dnmt3b in rat testes. For each stage (15.5 dpc, 18.5 dpc, 0 dpp, 7 dpp, 14 dpp, 21 dpp, 90 dpp), the percentage of germ cells expressing Dnmt3a or Dnmt3b was determined by choosing several sections from three testes of each developing stage and counting randomly the numbers of each cell type (gonocyte, spermatogonia, spermatocytes) to 200 as well as how many of these cells are positive for Dnmt3a or Dnmt3b expression.

Immunolocalization of Dnmt3a and Dnmt3b in testis cells

We further investigated the expression and localization of Dnmt3a and Dnmt3b in specific cell types in fetal and postnatal developing testes by immunohistochemistry. Dnmt3a was already present in the testis, but did not express intensely in prepuberty germ cells; not only was the percentage of gonocytes expressing Dnmt3a small, but the positive staining was also weaker than the Sertoli cells and Leydig cells that surround them (Fig. 3 A-C, Fig. 5). In addition, a positive reaction could not be detected in spermatogonia and spermatocyte from 7 dpp, 14 dpp and 21 dpp (Fig. 3 D-F, Fig. 5). Compared with Dnmt3a, except for somatic cells, Dnmt3b showed a weak but distinct positive signal in gonocytes at 15.5 dpc, and the signal of Dnmt3b staining was decreased at 18.5 dpc (Fig. 4 A-B, Fig. 5), but after birth, the staining signals became relatively stronger again in spermatogonia and spermatocytes (Fig. 4 C-F, Fig. 5). It was noted that in the adult testes Dnmt3a could be detected in spermatogonia and spermatocytes, but Dnmt3b was expressed only in spermatogonia (Fig. 3 G-H, 4 G-H, Fig. 5).

Discussion

Spermatogenesis is an intricate and highly specialized process, and proper establishment of DNA methylation patterns in the dinucleotide sequence CpG during male germ cell development are crucial for gamete integrity and transmission of epigenetic information to the next generation (Bourc’his and Bestor 2004, Kaneda et al., 2004, Neri et al. 2013). Recent research in mice has clearly shown that Dnmts are involved in these important processes, and play a vital role in establishment and maintenance of DNA methylation patterns (Kaneda et al., 2004, La Salle and Trasler 2006, Showlin et al. 2007). In rats, an earlier research reported that DNA MTase expression was developmentally regulated during the initiation of spermatogenesis (Jue et al. 1995). However, the four members of Dnmts were not distinguished. So it is still unclear if all Dnmts contribute equally to the establishment and the maintenance of DNA methylation patterns throughout germ cell development and spermatogenesis in rats. Rat gonocytes occur about 13 dpc-5 dpp (Culty 2013). At 6-7 dpp, the testis contains more spermatogonia cells; by 13-14 dpp, leptotene spermatocytes appear; by 19-23 dpp, pachytene spermatocytes are seen, and adult testes tissue contains multiple types of germ cells; development of any one generation of spermatogonia, spermatocytes or spermatids are closely integrated with that of other generations present in the same area of the seminiferous

This study was the first to reveal that the expression levels of the Dnmts varies with the developmental stage undergoing transition of germ cell types in rats through qRT-PCR. The trends of Dnmts expression are similar between rat and mouse although a slight deviation was observed (La Salle et al. 2004, La Salle and Trasler 2006, Shovlin et al. 2007). However, western blotting and immunohistochemistry detection produced discrepancies from the qRT-PCR results, although the general trend was consistent. For example, Dnmt3a had relatively abundant expression in mRNA levels, but did not express, or had weak expression, in prepuberty germ cells under immunohistochemistry detection. This disagreement may be explained by the specific primer including Dnmt3a1 and Dnmt3a2 used in qRT-PCR, and the commercial Dnmt3a antibody against the amino-terminal portion of Dnmt3a only recognizing Dnmt3a1. Studies have reported that Dnmt3a2 may be the de novo DNA methyltransferase responsible for the global methylation of the genome, and strongly stains the germ cells (Sakai et al. 2004), and that Dnmt3a1 positively affects transcription of specific genes at the promoter level and targets chromosomal domains to play silence (Kotini et al. 2011). However, the specific roles for either Dnmt3a1 or Dnmt3a2 in de novo DNA methylation and transcription regulation from epigenetics are not yet known, and further investigations are urgently needed.

From the overall expression trend of Dnmts, we showed here that Dnmt3a and Dnmt3l have a high expression during the perinatal period (15.5 dpc-0 dpp), at which time a global methylation occurs in male germ cells. De novo methylation begins in the male germ line before birth at the gonocytes stage and finishes after birth and before the end of pachytene. Once established, methylation patterns must be maintained during DNA replication that takes place in spermatogonia and spermatocytes (Lees-Murdock et al. 2003, La Salle et al. 2004). Previous studies have identified that Dnmt3l cooperates with Dnmt3a to establish maternal imprints in mice, and the two members interact with each other to stimulate DNA methylation activity of Dnmt3a (Chedin et al. 2002, Hata et al. 2002). A crystallography study also showed that Dnmt3l forms a heterotetrameric complex with Dnmt3a, and this tetramerization prevents Dnmt3a oligomerization and localization in heterochromatin (Jia et al. 2007, Jurkowska et al. 2011). More interestingly, Dnmt3a-deficient mice have similar phenotypes with Dnmt3l-deficient mice, which exhibit infertility associated with abnormal chromosomal structures in germ cells (Kaneda et al. 2004, Oakes et al. 2007). These findings suggest that Dnmt3a may have a synergistic effect with Dnmt3l in the de novo methylation process during prenatal testis development. Dnmt3b, another de novo methylation member of the Dnmt3 family, had different expression patterns compared to Dnmt3a and Dnmt3l, but very similar to Dnmt1. Dnmt3b and Dnmt1 had the highest expressed levels in postnatal rat testes, at times when the predominant cells are either mitotic spermatagonia (7 dpp) or spermatocytes entering meiotic differentiation (14 dpp) (Malkov et al. 1998, Lagos-Cabre and Moreno 2008). It is possible that Dnmt3b and Dnmt1 plays a key role at these important stages undergoing transition of germ cell types in rats to ensure proper pairing and recombination between homologous chromosomes. However, these results have some variance with the previous research that Dnmt3a and Dnmt3b have similar biochemical properties, and mainly play a key role in catalyzing de novo methylation at the cytosine of unmethylated CpG sites during embryonic development (Okano et al. 1998, Okano et al. 1999, Chen et al. 2003). Further investigations are needed to evaluate whether the different expression levels of Dnmt3a and Dnmt3b in rat testis development are associated with their role in spermatogenesis.

The results presented in this study showed that Dnmts are expressed in a defined, spatiotemporal manner during rat testis development. Testicular levels of specific transcripts vary according to the rat developmental stages. Our data support the idea that each DNA methyltransferase is expressed in a specific manner at different times during spermatogenesis, which implies specific and potentially nonredundant roles for each of these enzymes.

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