嘉南藥理科技大學專題研究計畫成果報告

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計畫名稱：組織蛋白 H3B 與造精功能相關之研究分析

執行期間：94 年 1 月 1 日至 94 年 12 月 31 日

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組織蛋白 H3B 與造精功能相關之研究分析

The study of histone H3B in spermatogenesis

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中文摘要

組織蛋白是染色質中的基本組成蛋白質，組織蛋白與染色質規則纏繞成 nucleosome，成為染色質的最基本單位，包括 H2A、H2B、H3、H4 及當作為二單位 nucleosomes 間的 linker 蛋白 H1 組織蛋白。組織蛋白與染色質形成類似線上的珠串（beads-on-a-string）構造，可使染色質規律排列成纏繞緊密的染色體結構。

本研究是以過去我們在微陣分析（microarray）無精蟲症患者及正常生育者的睪丸組織表現的 mRNA 表現量分析，發現部分組織蛋白 H3/B 基因在睪丸組織表現的 mRNA 表現量有統計上顯著之差異，因此本研究希望針對此H3B基因參與造精過程做進一步深入研究。本研究以 bioinformatics search 方法進行染色體上序列的比對，檢索正確的基因所在位置及基因序列，並將組織蛋白進行分群結果，選定 H3d、H3h2ba、H3fa 及 H3fb 等為研究對象，其中 H3fa 及 H3fb 為正控制組的基因，進行此些 H3B 基因 mRNA 在老鼠睪丸發育不同階段，H3B 基因在睪丸表現情形。研究結果顯示，組織蛋白 H3d 是屬於造精過程中早期表現的基因；組織蛋白 H3h2ba 是屬於造精過程中每一階段均會表現的基因；組織蛋白 H3fa 及 H3fb 是屬於造精過程晚期表現的基因，本研究且同時分析 H1、H2 及 H4 等其他組織蛋白在造精過程中表現情形，以期對於組織蛋白的表現及其參與造精過程的功能與角色有更深入的瞭解。

關鍵字：組織蛋白，精子發育過程，染色質

Abstract

Mammalian spermatogenesis is a highly ordered process that occurs in mitotic, meiotic and post-meiotic phases. During spermatogenesis, chromatin is restructured, and dramatic changes in histone gene expression are observed. Histones are a major component of chromatin, the protein-DNA complex fundamental to genome packaging, function, and regulation in spermatogenesis . Indeed, during the post-meiotic maturation of male haploid germ cells, or spermiogenesis, histone are replaced by small basic proteins, which in mammals are transition proteins and protamines.

The genes of this study included by the following databank:

1.The meiotic or postmeiotic spermatogenic cells by Nikolaus S. et al.

2.Mouse spermatogenesis genes in SAGE

3.Genes of testis bank

4. the mouse UniGene library

Combining of the above mouse spermatogenesis genes banks by raw data, the 20 histone candidate genes were selected. Specific primers of 20 histone genes were designed by roche software. Total RNA from 5-d-old (5d) , 10-d-old (10d) , 15-d-old (15d) , 20-d-old (20d) ,35-d-old (35d) , and 47-d-old (47d) testes were isolated and determined by RT-PCR. The expression of these 20 histone genes were determined by semi-quantitative RT-PCR among normal, MA, and SCOS patients. The 20 candidate genes involved in spermatogenesis will determined by RNA immunoprecipitatin and EMSA. We hope to identify and characterize the histone genes expression patterns in spermatogenesis. The study may help to clarify the role of histone gene in human spermatogenesis.

Key words: histone, spermatogenesis, chromatin

Introduction

Spermatogenesis is the process of development of male germ cell from spermatogonia to highly differentiated spermatozoa. Type A spermatogonial stem cells undergo mitosis for either self-renewal or differentiation into later-stage spermatogonia that gradually become pachytene spermatocytes (PcSc). PcSc undergo 2 meiotic divisions to give rise to haploid round spermatids(RdSd), which eventually transform into spermatozoa.[1] The unique mechanisms responsible for this tightly regulated developmental process suggest the presence of an intrinsic genetic program composed of spermatogenic cell-specific genes. During spermatogenesis, histones are a major component of chromatin, the protein-DNA complex fundamental to genome packaging, function, and regulation. Histone genes are extremely conserved throughout evolution and includes five classes termed H1,H2A, H2B, H3,and H4 [2]. The basic unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped around an octamer of core histones, including two molecules of H2A, H2B, H3 and H4. A fifth histone, H1, protects additional DNA fragments linking neighbouring nucleosome.[3] These core histones all contain a conserved C-terminal histone fold domain and unique N-terminal tails. The four core histones interact in pairs via a “hand-shake motif” with two H3/H4 dimers interacting together to form a tetramer, while the two H2A/H2B dimmers associate with the H3/H4 tetramer in the presence of DNA.[4]

During spermatogenesis, chromatin is restructured, and dramatic changes in histone gene expression are observed. In elongating and elongated spermatids, major restructuring of the somatic chromatin occurs; this process involves the replacement of somatic histones and H1t by transition nuclear proteins(TNP1 and TNP2), which are subsequently replaced by protamines (PRM1 and PRM2)[5].

To classify histone gene with respect to their expression, into three groups: (1) replication-dependent genes transcribed during the S phase of the cell cycle which produce the bulk of the somatic histones; (2) replication-independent genes transcribed at a low constant level throughout the cell cycle and in nondividing cells which supply the so-celled “replacement histones”, and (3) genes transcribed only in certain types of the differentiated tissue such as the testes. The tissue-specific genes can be expressed either in

a replication-dependent or –independent mode.[6]

Until recently, little was well known about the role of histone genes in the spermatogenic failure. To gain a better understanding the expression profile of histone genes in human spermatogenesis. In this study, we determined the mRNA transcript of histone genes in the different developmental stage of mouse spermatogenesis .By comparision the mRNA expression of selected 20 histone genes among normal, MA, and SCOS patients, thus, it will help to identify the role of these histone genes involved in spermatogenesis.

Materials and Methods

Patients and record of phenotypes

1. gene bank search and candidate genes determined:

Used UniGene bank[14] from NCBI[13] to find out all histone genes of mouse, based on previous 4 databanks to arrange these gene and search for their mRNA.

2. Animals and testis collected：

Male and female C57BL/6 mice, were obtained from Laboratory Center NCKU and maintained in a temperature- and humidity-controlled room on a 12-hr light/dark cycle. [7]

testis collected:

To test if transcripts are expressed at particular stage of spermatogenesis, from prepubertal to adult male mice (age range 1-47 days) were sacrificed, and total RNA isolated from their testes was used for reverse transcription.[8]

3. Isolation of spermatogenic cells:

Based on the majority of germ cell types, different ages of male C57BL/6 mice were used for the isolation of different spermatogenic cell types (1 days old for spermatogonia, 5 days old for type A spermatogonia, 10 days old for type B spermatogonia, 15 days old for preleptotene spermatocytes, 20 days old for pachytene spermatocytes,35 days old for round spermatids, 47 days old for elongated spermatids.) [7]

4. RNA preparation:

The testis of differential stage were harvested from C57BL/6 mice. Total RNA were extracted using RNase Mini kit (Qiagen) according to the manufacture's protocol. The RNA quality and concentration were assessed using spectrophotometric reading (ng/μl) [7]

5. cDNA (complementary DNA) preparation:

The concentration of total RNA 10μl (500 ng/μl), 1μl oligo-dT (500μg/ml), and heated to 65℃ for 5 minutes ,500 ng/μl of oligo-dT12-18, 10mM dNTP Mix(10mM each dATP ,dGTP,dCTP, dTTP), 5X first-strand buffer, 0.1M DTT, 40unit/μl RNaseOUT Remcombinant ribonuclease inhibitor, superscript II RT(200 unit). The cDNA synthesis was allowed to proceed for 1 hour at 42℃ 50min, 72℃ for 15 min, after which the product was used immediately for PCR amplification or was stored at -20℃.[9]

6. RT-PCR(reverse transcription –polymerase chain reaction):

The gene-specific primer were designed by Roche soft, and synthesized by invitrogen (table 1). Add the following to a PCR reaction tube for a final reaction volume of 10μl , each cycle consisted of 1 min at 95℃, 1 minute at 60℃ and 1 minute at 72℃, except that the temperature for the annealing

stage was 55.5℃ in the some gene (H1t, H2afy, H2afz, H2h2aa1, H2ae, H2bb, H3f3b, H3d). to visualize the amplified products, 10μl of each reaction mixture was electrophoresed through a 1.8% agarose gel containing ethidium bromide using TBE as the running buffer.[9]

Results

Expression pattern of the mouse histone genes by RT-PCR analysis

To determine the tissue distribution of the mouse histones gene from some reference, we performed PCR analysis using mouse cDNA from differential stage of spermatogenesis. We carried out RT-PCR using mouse testis obtained at different days after birth. Spermatogenesis occurs in seminiferous tubules containing a mixture of spermatogenic cells and somatic cells such as Sertoli cells. In the first round of spermatogenesis in prepubertal mouse, stem cells proliferate and differentiate gradually to yield the sequence of spermatogonia, spermatocytes, spermatids, and spermatozoa.[8]

We were chosen five histone genes for positive control group, they are H2afx, H3f3a, H3f3b, H1t, and Hils1, the expression pattern conform to the published pattern (figure 1). In addition, based on the expression pattern, some genes, H3d, H2bg, H1f0, H1c, H4i, H1d, H2ae, and H2bb, highly expressed in the early spermatogenesis (from spermatogonia to spermatocytes) (Fig 2), some, H2h2aa1,H2bp and H3h2ba, are expressed rich in late spermatogenesis (in spermatids) (Fig 3 A), and the others, H2afz, H2h2bb, H4j, H2afy, are expressed in all stage of spermatogenesis(from spermatogonia to spermatozoa)[6] (Fig 3B).

Discussion

Mammalian spermatogenesis appears to be regulated by the organized stage- and cell-specific gene expression in both germ cells and the supporting somatic cells. The molecular mechanisms of this process are believed to involve the selective modulation of defined sets of genes that facilitate spermatogenic cell differentiation. Most previous reports regarding gene expression during spermatogenesis are limited to the late stages of spermatogenesis. Gene expression patterns in spermatogonia or early spermatocytes are rarely reported.[7]

In this study, we have isolated high purity of different stages of spermatogenic cells from C57BL/6 mice testis and performed the RT-PCR analyses for histone genes. The histone genes, expressed in early spermatogenesis, indicate that these genes may be necessary for chromatin assemble of early spermatogenesis, so these genes will has some significance in spermatogenesis, The genes were highly expressed in late stage during spermatogenesis, these genes may participate in chromatin remodeling during spermiogenesis (from spermatid to spermatozoa). The genes expressed in the all stage during spermatogenesis, they are possibly participate the all processes of chromatin remodeling or regulation the mechanism of gene transcription in spermatogenesis.

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Electronic-Database Information：

1. testisBank: http://medweb.uni-muenster.de/TestisBank/
2. NCBI (National Center for Biotechnology Information) http://www.ncbi.nlm.nih.gov/
3. UniGene: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene&cmd=search&term=

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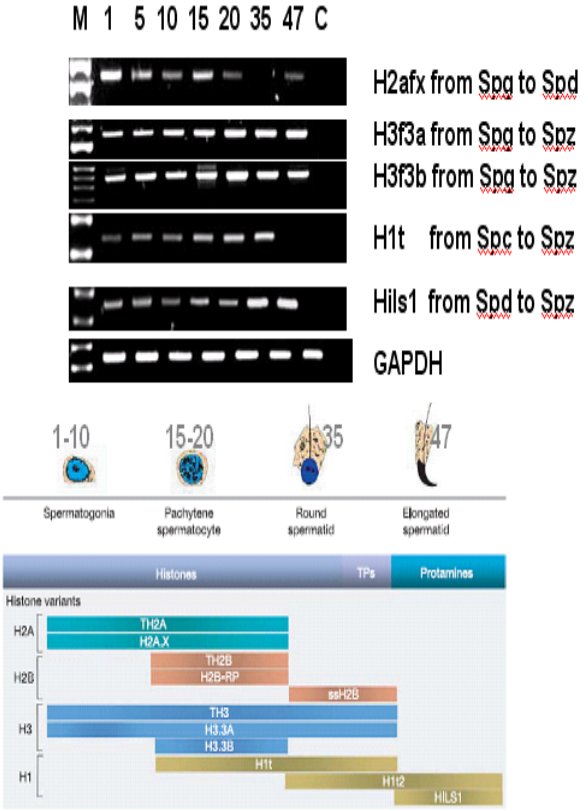

Fig 1. The positive control group of histone genes. Spg, spermatogonia; Spc, spermatocyte; Spz, spermatozoa.

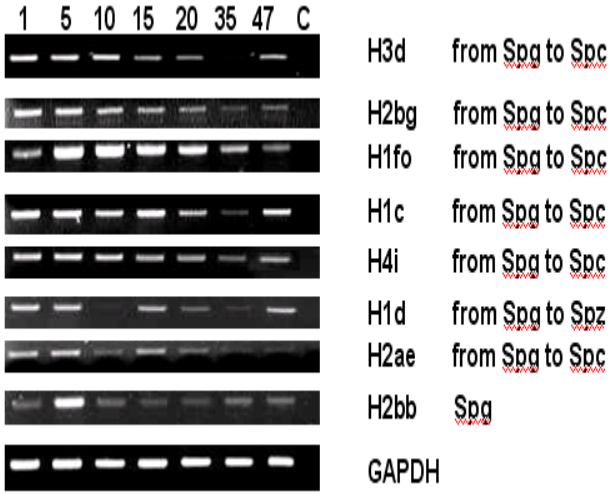

Fig 2.The histone gene are expressed relatively early during spermatogenesis. Spg, spermatogonia; Spc, spermatocyte; Spz, spermatozoa.

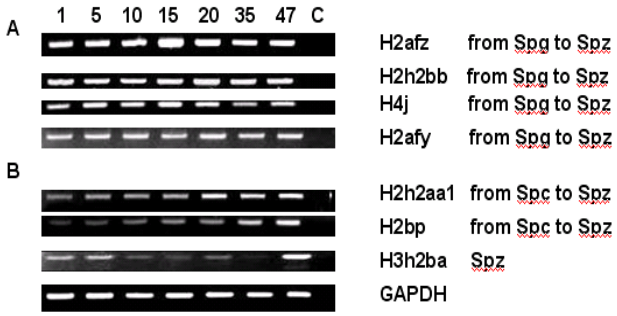

Fig 3. The histone gene are expressed relatively late (A) or all (B) during spermatogenesis. Spg, spermatogonia; Spc, spermatocyte; Spz, spermatozoa.

Table 1 The gene-specific primer for RT-PCR

NO.	uniGene	Gene	Forward primers	Reverse primers
NM_145713	Mm.222733	Hist1h1d	GTTCCGAAGCAGATCAAT	GTCTACTGACCAAATAAGGG
NM_008197	Mm.24350	H1f0	GCCCATTCTATTCTGACTT	CGCAATCCATCAATCGT
NM_015786	Mm.193539	Hist1h1c	CAAACCCCAGGCTAAGA	GTCCAATACGAACTAGCG
NM_018792	Mm.30482	Hils1	GAGGGAAGCAGAAGCC	CAAGGTAGCAAGGGACA
NM_010377	Mm.8048	H1t	TGCGGCTGGTTACGAC	GTCTTGCTACTCTTGGG
NM_012015	Mm.283802	H2afy	TTGCCGAATATCCATCCT	CATCGTGGAGACAAAGT
NM_010436	Mm.245931	H2afx	CCCAAGAAGAGCAGCG	CCTACGAATGGCGACA
NM_016750	Mm.117541	H2afz	AAAGCGTATCACCCCT	AGCTTATCCACCAGAGT
NM_013549	Mm.261670	H2h2aa1	CCGTTCGTCTGTTTGC	ATCGTCACTTTGCCCA
NM_178187	Mm.261665	H2ae	CCACTTCCATGTACGAG	CTCCGAGTAGTTGCCT
NM_175666	Mm.5220	H2h2bb	CGGTCTACAATCACATCG	GCGGTAATCTGGCACT
NM_178196	Mm.261676	H2bg	TTCTACCATGCCCGAG	TGTGTCATTTCCCAGCG
NM_030082	Mm.28022	H3h2ba	AAGAAACGCAAACGGG	GGTCCAGTACAACACCC
NM_175664	Mm.371766	H2bb	TGCACCAGCCCCTAAG	TGAGCCCTACGAGCTCACTT
NM_178202	Mm.264645	H2bp	CCTGTTAAGTCCGTTCCC	TGCGCTCGAAGATGTC
NM_008211	Mm.371563	H3f3b	GAAGAAGCCTCACCGCTACA	ATGACAAGACTCCCCACCAC
NM_008210	Mm.352429	H3f3a	GAAAGCCTCGGTGTCAG	CGTTCTCCGCGTATGC
NM_178204	Mm.15595	H3d	TGAAGAAGCCTCACCG	CCTCTCCCCACGAATG
NM_175656	Mm.14775	H4i	GGACAACATCCAGGGC	ACTCGGGAAGCAAAGG
NM_178210	Mm.144300	H4j	CTGGCAGAGGTAAGGG	GCTCCGTGTAGGTGAC