

行政院國家科學委員會專題研究計畫 期中進度報告

小鼠睪丸專一性同源箱基因 Tex1 之功能分析(1/3)

計畫類別：個別型計畫

計畫編號：NSC91-2320-B-038-023-

執行期間：91 年 08 月 01 日至 92 年 07 月 31 日

執行單位：臺北醫學大學醫學研究所

計畫主持人：謝秀梅

報告類型：精簡報告

報告附件：出席國際會議研究心得報告及發表論文

處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 92 年 6 月 2 日

一、中文摘要

我們在提出計畫時，已完成 *Tex1* 基因剔除質體(A)之構築。在過去的一年中，已將剔除質體(A)利用 electroporation 送到 D129 的胚幹細胞中兩次，共計以 PCR 及 Southern blot analysis 篩選了約 300 個細胞的 DNA，均沒有得到正確同源重組的標的細胞。檢討之結果，認為可能是(1)此基因位在 X 染色體；(2)質體上的 *Tex1* 基因片段沒有包含有轉譯蛋白區，可能是基因上下游一些重覆序列較高的區域造成同源重組機率過低。因此，我們又另行構築了一個包含有 3'端轉譯區的質體(B)。這個質體也經過兩次 electroporation 並篩選了近 450 個細胞，亦仍然沒有得到標的細胞，我們推論，X 染色體可能仍是一個主要的關鍵，但同時我們也考慮到，在(B)質體中，我們剔除的片段較大，可能也是造成同源重組的機率偏低的原因，因此我們又構築了剔除片段較小的質體(C)。在經過 PCR 篩選進 400 個細胞 DNA 後，我們初步看到有 4 個可能是正確的同源重組細胞株，目前正以 Southern analysis 方法加以證實這個結果。如果這幾個細胞的確是正確的標的細胞。則我們將會進行顯微注射將細胞打入小鼠的囊胚，以建立 chimeric 小鼠。再經過一代的交配，即可取得 X 染色體 *Tex1* 基因剔除的公鼠，再加以分析此基因對睪丸及造精功能之影響。其次關於融合蛋白方面，我們以將 *Tex1* 基因-His6 融合蛋白質體構築完成並送至細菌宿主中，並可引誘及純化足夠量的融合蛋白。利用這些融合蛋白我們一方面已去免疫兔子取得抗體。另外也進行 DNA 結合序列的篩選。初步尚未看到有較一致的結合序列。我們將進一步多做 PCR 循環及 EMSA 確認，以了解這個同源蛋白的結合區，以便找出這個蛋白的下游基因。

關鍵詞：睪丸、精子成熟、同源箱基因、不孕症

Abstract

This is our first year's progress report of this 3-year grant. We have generated the knock-out construct (A) when we were applying this grant. This construct was electroporated into the mouse embryonic stem (ES) cells twice. Almost 300 ES cell DNAs were screened by PCR and Southern analyses but no targeted ES cell were identified. We suspected that the X-chromosomal localization of this gene and no coding region was included in this construct (A) were the reasons to have a rare homologous recombination event happened. We thus construct the second targeting vector (B) with the 3' partial coding region included in the short arm. However, we still did not identify any positive clones after around 450 ES cell screening. The X-chromosomal localization might be a key factor to control the frequency of homologous recombination. On the other hand, we suspected that the big deleted fragment (~ 6 kb) might be another reason to cause the low frequency of homologous recombination. We thus generate the third targeting vector (C) with a smaller fragment deleted (~ 2 kb) in this construct. In the primary screening, we got 4 candidate targeted clones from around 400 ES clones. We are now using Southern analysis to confirm

the preliminary PCR results. Positive ES clones will then be injected into blastocyst and used to generate chimeric mice. Male mice with *Tex1* knock-out on the X chromosome will be obtained from the intercrossing of germ line chimera with wild-type mice. The *Tex1* function in the testis development and spermatogenesis will be elucidated from these targeted mutants. To identify the *Tex1* DNA binding sites, we have generated the *E. coli* expression construct and purified the *Tex1*-his6 fusion protein from the host. We have used the fusion protein to immunize the rabbit to raise the antibody. On the other hand, we are in progress to identify the DNA binding site by using the fusion protein in an approach combining random DNA selection with recycled PCR amplification. We have not got any conservative DNA binding sequence through the preliminary study. We will further performed more rounds of selection and PCR amplification and the consensus sites will be confirmed by electrophoresis mobility shift assay (EMSA). The conserved binding sites selected will then be used to search for the potential target genes of *Tex1*.

Keywords: Testis; Spermatogenesis; Homeobox gene; Infertility

二、緣由與目的

Male infertility, affecting as many as 10% of the adult population, is an extremely prevalent disorder. In most cases, these men are infertile either because of insufficient sperm (oligozoospermia) or lack of sperm (azoospermia). The cause of these defects in sperm production is unclear, but recent work points to both potential environmental and genetic causes. Genetic factors that might affect male infertility, other than some sequences on the Y chromosome, have not been identified.

Sperm production (spermatogenesis) is one of the most dramatic processes of differentiation. In the initial stages, spermatogonia undergo two mitotic divisions, giving rise to primary and secondary spermatocytes, which undergo two meiotic divisions to produce the haploid round spermatids. During the spermiogenesis, the round spermatids elongate, compact their chromatin into the sperm head and produce the other sperm components (1-2). This progression from pro-spermatogonia to mature spermatozoa has been well defined and makes spermatogenesis an attractive system to study the regulation of a developmental process in an adult organism. An important facet of spermatogenesis occurs at the level of transcriptional regulation, and the characterization of transcription factor genes, such as homeobox genes, expressed during spermatogenesis constitutes a necessary step toward an understanding of this level of control.

Homeobox genes encode transcription factors which containing a 60-amino acid DNA binding motif, term the homeodomain. The tertiary structure of which conforms to a helix-turn-helix motif conserved in species as evolutionarily distant

from yeast to human (3). Homeobox genes are known to be intricately involved in the embryo development (4-5), but less is known about their function in the differentiation processes of the adult organism. As previously stated, spermatogenesis represents such a process, and interestingly, several homeobox genes have been shown to be expressed in murine male reproductive systems, suggesting the possibility that they may regulate developmental events during male gametogenesis (6).

To identify additional developmental regulators of the homeobox gene family, we employed a PCR strategy using degenerate oligonucleotides and DNA from several cDNA libraries. One of the additional mouse homeobox genes identified by this approach, *Tex1*, showed testis-specific expression. Sequence comparison indicated it is localized on X chromosome and belongs to the pair-type homeobox gene subfamily. To further characterize the function of *Tex1*, we proposed to establish the knock-out mice and identify the downstream target genes.

三、結果與討論

To establish the *Tex1* knock-out mice, we have generated three different targeting constructs (Fig. 1). The homologous recombination frequencies after electroporation of these constructs into ES cells were summarized in Table 1. We suspected that X chromosomal localization is the major cause for the low frequency of the targeting events. However, we isolated 4 possible knock-out ES clones from the second electroporation of construct (C). This result seems to rule out the factor of X chromosome since the successful rate reached 1.3% which is significantly higher than the targeting rates from the constructs (A) and (B). On the other hand, we think the construct structure might be critical since the deleted size was changed from 6 kb in construct (B) to 2 kb in construct (C), which is a compatible size to the Neo resistance gene cassette. We are now using the Southern analysis to check these 4 candidate clone DNA since artifact results arise from PCR quite often. If any one of these 4 clones was confirmed, the ES cells will be cultured and microinjected into the blastocysts to make the chimeric mice.

The other project we are making progress is to purify the *Tex1* protein which can be further used to raise antibody and fish out the *Tex1* binding site and associate proteins. We have used the purified protein to screen random DNA sequences but have not got any conclusive results yet.

四、計畫成果自評

The major goal of this grant is to understand the molecular function of *Tex1* gene. We have constructed the *Tex1* targeting vector and electroporated it into ES cell line

and identified 4 potential targeted ES cells. The further confirmed targeted ES cells will be used to make the chimeric mice and obtain the male mutants from the breeding of chimeric and wild type mice. We should be able to characterize the in vivo function of *tex1* in the testis through the phenotype analyses in the mutant male mice. For the other specific aims, we also make a good progress since we are able to obtain Tex1 protein to raise antibody, identify binding sites and associate proteins.

五、参考文献

1. Russell LD, Ettlin RA, Hikim APS, Clegg ED (1990) Histological and Histopathological Evaluation of the Testis. Cache River Press, Clearwater.
2. Poccia D (1994) Molecular Aspects of Spermatogenesis. R. G. Landes, Austin, Molecular Biology Intelligence Unit.
3. Manak RJ, Scott MP (1994) A class act: Conservation of homeodomain protein functions. Development, supplement: 61-71.
4. McGinnis W, Krumlauf R (1992) Homeobox genes and axial patterning. Cell 68:283-302.
5. Krumlauf R (1994) Hox genes in vertebrate development. Cell 78: 191- 201.
6. Lindsey S, Wilkinson MF (1996) Homeobox genes and male reproductive development. J Assist Reprod Genet 13:182-192.

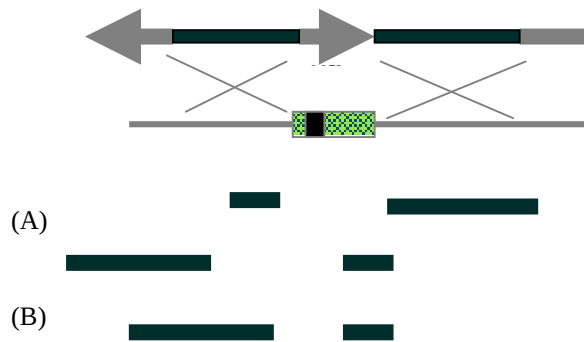


Fig. 1 *Tex1* knock-out constructs

The two homologous recombination take place between the targeting vector and *Tex1* are shown on the top. MCLneo and PGK-TK in the targeting vector represent positive and negative selection marker individually. The localization of the two fragments along the *Tex1* gene on the three targeting constructs are shown on the bottom. HD, homeodomain.

Table1. The targeting frequency of the three constructs.

	(A)		(B)		(C)	
	screened	+	screened	+	screened	+
1	190	0	154	0	105	0
2	98	0	293	0	303	4

