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Report

Metal Responsive Transcription Factor-1 is Selectively Expressed among the Mouse Testicular Cells

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Metal responsive transcription factor-1 (MTF-1) is ubiquitously expressed in various tissues, and is thought to be an intracellular zinc sensor that regulates transcriptional activation of zinc responsive genes. We investigated the distribution of MTF-1 in the mouse testis, where zinc plays an essential role in spermatogenesis. By performing immunohistochemical analysis of the ddY mouse testis using the anti-MTF-1 antibody, we observed a donut-shaped staining pattern in the seminiferous tubules in the region proximal to the lumen, where spermatocytes primarily localize. A similar staining pattern was obtained using *in situ* hybridization to detect MTF-1 mRNA. Furthermore, we confirmed that MTF-1 could not be detected at both the protein and mRNA levels in the premature 20-d-old mouse testis, where spermatocytes are thought not to have been formed in the seminiferous tubules yet. These lines of evidence strongly indicated the selective expression of MTF-1 in mouse spermatocytes and suggested that MTF-1 played a role in a certain stage of spermatogenesis.

Key words zinc regulatory transcription factor, spermatogenesis, zinc-dependent regulation

INTRODUCTION

Zinc is an essential element for all living organisms, and the large number of enzymes and zinc finger transcription factors require zinc ions for their activity and maintenance of their protein tertiary structures.[1)] Additionally, zinc has now been recognized as an intracellular signaling molecule involved in the immune system[2)] and nerve transmission.[3)] It is probable that the fluctuation in intracellular free-zinc ion concentrations leads to activation of a certain group of genes, which are expected to be involved in unknown biological functions of zinc. In this context, we have focused on a metal responsive transcription factor (MTF-1)[4,5)] because it is one of the intracellular convergence points of the zinc signal leading to the expression of zinc regulatory genes.

MTF-1 was discovered as a protein factor, which binds to the metal responsive element (MRE) located upstream of mammalian metallothionein (MT) genes.[6,7)] The protein structure of MTF-1 is well conserved in organisms from insects to humans, and its structural hallmark is the six tandem repeats of the C_2H_2 type zinc finger motif. The MTF-1 gene harbors the TATA-less promoter region that is characteristic of housekeeping genes,[8)] and it is ubiquitously expressed in various organs.[8,9)] MTF-1 is indispensable for the activation of the MT genes by multiple heavy metal ions;[10)] however, direct activation of MTF-1 and binding to the MRE *in vitro* occur solely in a zinc-dependent manner.[11)] It is believed that the presence of heavy metals, other than zinc ions, increase the intracellular concentrations of free zinc ions to activate MTF-1. Although the mechanism of zinc response of MTF-1 remains unclear, MTF-1 is thought to be one of the intracellular zinc sensors,[12)] which lead to transcriptional activation of genes relevant to the cellular zinc response.

According to the gene knockout experiment reported by Gunes *et al*., MTF-1 null mice die at E13.5 of gestation due to hepatic decay.[13)] Therefore, MTF-1 is required for mouse embryonic development, apart from transcriptional activation of the MT genes. Plausible candidate genes involved in prenatal liver decay in the MTF-1 knockout mice are α-fetoprotein (Afp) and C/EBP-α (Cebpa), which are down regulated by MTF-1 gene knockout.[14)] Reportedly, various genes, such as ZnT1 (Slc30a1),[15)] placental growth factor (Pgf),[16)] and β-synuclein (Sncb),[17)] which is related to neurodegenerative disorders, are regulated by MTF-1. Considering the differences in the active genes in various cell species, it is likely that many novel genes regulated by MTF-1 remain yet to be discovered.

The testis is known to be one of the organs with high zinc contents, and depletion of zinc results in testicular atrophy and low sperm count.[1,18)] However, the role of zinc in the male reproductive system is poorly understood. It is often correlated with testosterone synthesis in Leydig cells, and it plays a protective role towards spermatozoa and shows a stabilizing effect on the cell membrane and chromatin.[19)] On the other hand, relatively high MTF-1 expression has been reported in the testis.[20)] However, MTF-1 function, other than the transcriptional activation of the MT genes, remains unknown. As the first step to investigate the role of MTF-1 in the testis, we performed analyses of the testicular distribution of MTF-1. At first, MTF-1 was anticipated to be expressed in almost all testicular cells because MTF-1 is ubiquitously expressed in various cells. Surprisingly, MTF-1 was selectively expressed in

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spermatocytes of seminiferous tubules, as compared to in the other testicular cell species, spermatogonia, Sertoli cells, and Leydig cells. This result suggested that MTF-1 played a role in zinc-dependent transcriptional regulation during a certain stage of spermatogenesis.

MATERIALS AND METHODS

Animals All experiments involving animals were conducted according to protocols approved by the Committee on Animal Experimentation of Teikyo University. Male ddY mice (Nihon SLC, Shizuoka, Japan), aged 20-56 d and weighing 20-40 g, were used in this study.

Immunohistochemical (IHC) Analysis The MTF-1 antibody used for IHC analyses had been raised against a synthetic peptide corresponding to amino acid residues 430-450 of the human MTF-1,[21] and the cross-reactivity to human and murine MTF-1 was confirmed by western blot analysis using recombinant human and mouse MTF-1 (data not shown).

Mice were killed by cervical dislocation, and the testes were isolated, weighed, and immersed successively in Bouin's solution, 70% (v/v) ethanol, 100% ethanol, and xylene before they were embedded with paraffin. Testis sections of 7-12 µm thickness were cut via microtomy. The sections were deparaffinized using xylene and ethanol, stained with Mayer's hematoxylin, and examined by microscopy to observe the progress of spermatogenesis. Testicular cell types were identified by their location and morphological characteristics such as shape of the nuclei.

Testis sections obtained via microtomy were placed on glass slides, deparaffinized, rehydrated, and rinsed with x1/20 fetal bovine serum diluted with 1x phosphate-buffered saline (PBS) to block non-specific antibody binding. Sections were treated with 2.5 ng/mL of anti-MTF-1 antibody dissolved in a solution of 0.05% Tween 80, 1% bovine serum albumin (BSA), and PBS and maintained overnight at 4°C. Sections treated with primary antibody were subsequently stained with Alexa Fluor 488 conjugated goat anti-rabbit IgG2b secondary antibody (A-11034, Thermo Fisher Scientific, Waltham, MA, USA). Sections were also stained with rabbit IgG (I5006, Merck, Branchburg, NJ, USA) as a negative control. Antibody-stained sections were imaged using a BX53 microscope and DP26 camera (Olympus, Tokyo, Japan).

***In situ* Hybridization (ISH)** ISH analysis for MTF-1 mRNA expression in mouse testis was performed using the ViewRNA™ ISH Cell Assay Kit (QVC0001, Thermo Fisher Scientific, Waltham, MA, USA) for fluorescent RNA *in situ* hybridization, according to the manufacturer's instructions. Mouse testis sections were prepared by the same method as that used for IHC analysis. Samples were boiled in a 1x pretreatment solution for 20 min, digested with protease at 40°C for 20 min, and refixed with 4% neutral-buffered paraformaldehyde at room temperature for 5 min. Target probe sets (MTF-1 antisense, VB1-20981; MTF-1 sense, VB1-6000227) were diluted x1/40 with Probe Set Diluent QT and hybridized to the tissue by incubating at 40°C for 2 h. The hybridization probes were fluorescently labeled, and the signal was amplified through sequential hybridizations using the kit-supplied reagents. The fluorescence signal was imaged and captured using the BX53 microscope and DP26 camera system.

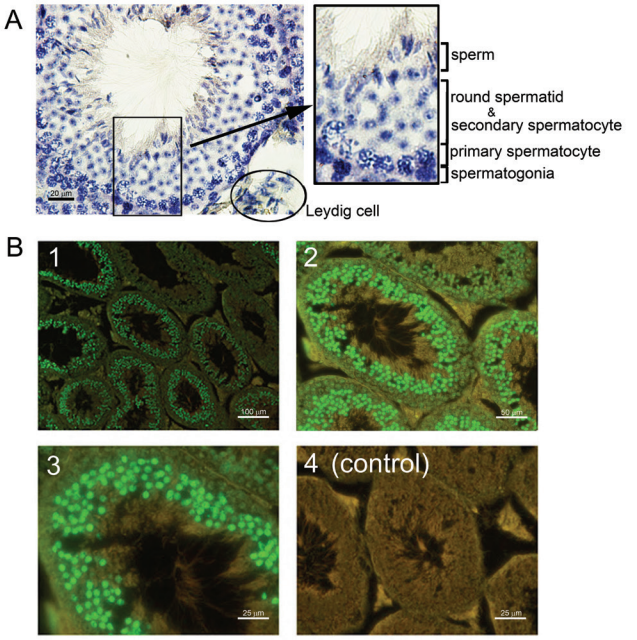

Fig. 1. IHC Analysis for MTF-1 in the Testis

A) Hematoxylin staining for nuclei in the testis of 56-d-old mouse. The approximate area where each cell species at the various differentiation stage is indicated on the right. B) Immunofluorescent staining for MTF-1. The testis section on the slide glass was treated with anti-MTF-1 antibody and successively with the Alexa Fluor 488 conjugated second antibody. The microscopic observations at 100- (A and B1), 200- (B2), 400- (B3 and B4) fold magnification, respectively are shown. In the case of control, rabbit IgG was used instead of the anti-MTF-1 antibody.

RESULTS AND DISCUSSION

IHC Analysis of MTF-1 in the Testis As seminiferous tubules are folded and packed in the testis, multiple cross-sections of the tubules characterized by the presence of an inner cavity were observed in the sections obtained via microtomy (Fig. 1A). In each cross-section, cells in varying stages of differentiation were lined up in layers from the outer side of the tubule towards the luminal side. Spermatogonia were found next to the basement membrane, and further differentiation was seen in the form of spermatocytes, round spermatids, and spermatozoa. Other than these germ cells, two types of somatic cells, Sertoli cells and Leydig cells, were found inside and outside of the seminiferous tubule, respectively, and they are known to support the growth and differentiation of germ cells. the nuclei of Sertoli cells were localized at the peripheral regions of the seminiferous tubules, but their whole-cell shape could not be specified (Fig. 1A).

The IHC experiments showed that strong fluorescence signals derived from the antibody against MTF-1 were observed in a donut-shaped pattern (Fig. 1B). We found that the signal emanating from the area of the seminiferous tubule corresponded to the location of spermatocytes and round spermatids, and the signals localized in the nuclei of each cell. A low-intensity signal was also detected in the nuclei of the cells near the basement membrane of the seminiferous tubule, where spermatogonia and Sertoli cells are commonly located. Additionally, a negligible signal could be detected in the interstitial space, but this signal was non-reproducible and also observed in the control samples, indicating that expression of the MTF-1 protein in Leydig cells was relatively low. These results suggested that MTF-1 was spatially localized within

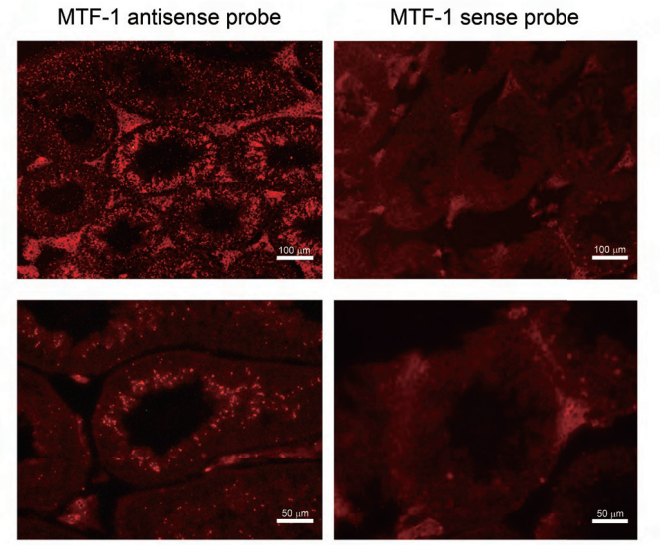

Fig. 2. ISH Analysis for MTF-1 mRNA in Cells of the Seminiferous Tubules

MTF-1 mRNA was stained with ViewRNA™ (affymetrix) on testicular paraffin sections of 56-d-old mouse. The microscopic observations at 100- (left panels) and 200-fold (right panels) magnifications are shown. The dot signals obtained by the antisense probe represent MTF-1 mRNA, and those obtained by the sense probe are negative control.

the seminiferous tubules and preferentially expressed in spermatocytes and, to lesser extent, spermatogonia.

ISH Analysis for MTF-1 mRNA in Cells of the Seminiferous Tubules To confirm whether the signals detected by IHC analysis were intrinsically derived from MTF-1, we investigated the localization of MTF-1 mRNA using ISH. We observed that the signal from ISH was consistent with the results obtained by the IHC analysis and it displayed a donut-shaped pattern proximal to the lumen (Fig. 2). The signal detected in the interstitial space outside of the seminiferous tubules was non-reproducible and non-specific since it could also be detected using an MTF-1 sense probe. Thus, the results obtained from the IHC and ISH experiments indicated that the MTF-1 protein distribution detected by the anti-MTF-1 antibody was reliable and MTF-1 was highly expressed at both the protein and mRNA levels primarily in spermatocytes.

Identification of MTF-1 Expression Patterns during Spermatogenesis From previous studies, it is known that spermatocytes start to appear postnatally at around 23 d;[22] accordingly, there should be no spermatocytes in the seminiferous tubules of 20-d-old mice and spermatocytes should be evident in the seminiferous tubules at 28 d of age. To identify the types of cell that express MTF-1, we examined the localization of MTF-1 in the cells of the seminiferous tubules from the testes of mice aged 20 and 28-d using IHC and ISH (Fig. 3). In 20-d-old mice, neither the MTF-1 protein nor mRNA was detectable in the cells of the seminiferous tubule. On the other hand, a strong positive signal for both the MTF-1 protein and mRNA was detected in the seminiferous tubules of 28-d-old mice, with staining patterns similar to those seen in the adult mouse. Thus, the primary site of MTF-1 expression was found to be temporally regulated to occur selectively in spermatocytes. We observed that the spermatocyte-derived GC-2spd[23] cells preserved a high MTF-1 expression level as compared with other testicular cell lines (manuscript in preparation).

Spermatocytes further undergo meiosis. Therefore, they were found to be comprised of two groups of cells, i.e., diploid and haploid types. However, it was not possible to compare the expression level of MTF-1 in the two spermatocyte types during the IHC and ISH experiments described in this study, and MTF-1 may be expressed in both groups of cells. The high expression may be maintained in round spermatids, but at present, it is unclear whether MTF-1 plays a role in meiosis. Interestingly, it has been reported that a zinc signal is essential for meiotic maturation of mouse oocytes.[24] Since MTF-1 is the transcription factor directly activated by sensing the increase in intracellular free zinc ions, it is probable that the zinc signal is necessary also for the spermatogenic meiosis. In this context, it is extremely interesting to know the zinc dynamics during spermatogenesis.

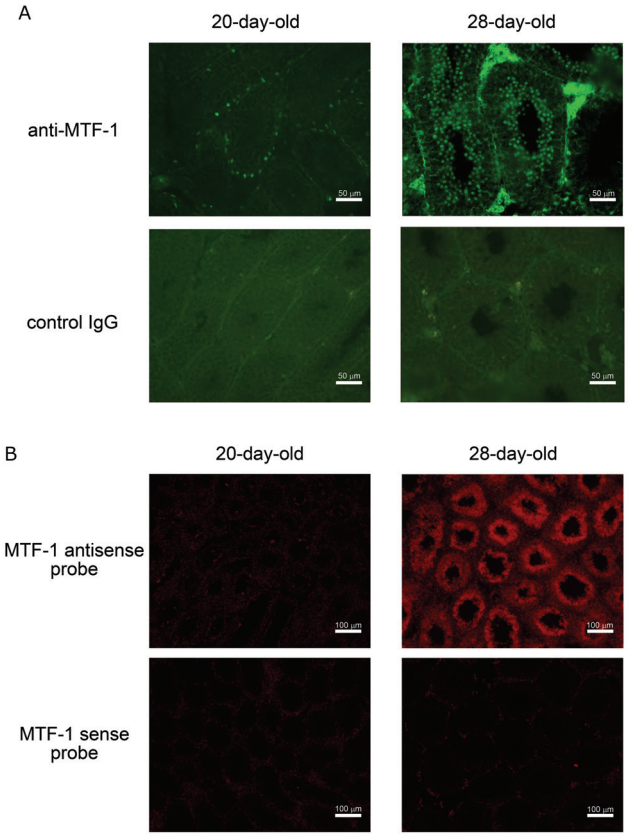

Fig. 3. Identification of MTF-1 Expression Patterns during Spermatogenesis

The testis of a 20- or 28-d-old ddY mouse was fixed with Bouin's solution, embedded in paraffin, and sliced 7-12 mm-thick sections.The slice on a slide glass was subjected to immunostaining using anti-MTF-1 antibody (A) or *in situ* hybridization with ViewRNA™ (B). The microscopic observations at 100 (A)-and 200 (B)- fold magnification.

To date, the gene expression is considered to be stochastic, because regulatory proteins for transcription in a single cell are limited in their copy number.[25] Therefore, the high expression of MTF-1 in spermatocytes probably contributes to make sure the expression of important genes associated with spermatogenesis. To clarify the role of MTF-1 during the spermatogenic progression, investigations to determine the zinc regulon under the control of MTF-1 are now in progress using GC-2spd cells.

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Conflict of interest The authors declare no conflict of interest.

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