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Nm23-M5 mediates round and elongated spermatid survival by regulating GPX-5 levels

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ABSTRACT

Nucleoside diphosphate (NDP) kinases are involved in numerous regulatory processes associated with proliferation, development, and differentiation. Previously, we cloned a new member of the NDPK family from mouse, Nm23-M5, which encodes a 211-amino acid protein and has 86% identity to the human Nm23-H5 [Hwang, K.C., Ok, D.W., Hong, J.C., Kim, M.O. and Kim, J.H. (2003) Cloning, sequencing, and characterization of the murine Nm23-M5 gene during mouse spermatogenesis and spermiogenesis. Biochem. Biophys. Res. Commun. 306, 198–207]. To better understand Nm23-M5 function, we generated transgenic mice with reduced Nm23-M5 levels in vivo using a short hairpin RNA (shRNA) knock-down system. Nm23-M5 expression was markedly reduced, as indicated by Northern and Western blot analysis. Nm23-M5 shRNA transgenic mice exhibited reduced numbers of haploid cells. Furthermore, the antioxidant enzyme glutathione peroxidase 5 (GPX-5) is regulated by Nm23-M5 at the level of both expression and activity. These results reveal that expression of Nm23-M5 plays a critical role in spermiogenesis by increasing the cellular levels of GPX-5 to eliminate reactive oxygen species.

1. Introduction

Nucleoside diphosphate kinases (NDPKs) (EC 2.7.4.6) are ubiquitous enzymes and catalyze the transfer of γ-phosphates between nucleoside and deoxynucleoside di- and tri-phosphates. A number of mammalian genes that encode NDPK-like molecules (denoted *non-metastatic 23* or *Nm23* genes) have been identified and cloned [1–6]. Among the human *Nm23* genes (*Nm23*-H1 to *Nm23*-H8), the *Nm23*-H1 gene product has been characterized as a human NDPK A isoform which is overexpressed in solid tumors as compared to normal tissues [7–9]. *Nm23*-H2 (a NDPK B isoform) encodes Puf, a protein which is a transcription factor for the c-myc proto-oncogene [2,10,11].

The overexpression of *Nm23*-H3 (a NDPK C isoform also called *DR-Nm23*) induces apoptosis of myeloid cells and suppresses granulocyte differentiation [2,12]. *Nm23*-H4 (a NDPK D isoform) is a mitochondrial specific NDPK isoform [4,13]. *Nm23*-H5 does not appear to possess NDPK activity and is predominantly expressed in testis, together with *Nm*23-H7 and *Nm*23-H8 [3,5]. *Nm23*-H6 (a NDPK F isoform) is expressed in most human tissues and overexpression of *Nm23*-H6 results in growth suppression and generation of multinucleated cells [13,14]. The *Nm23*-H7 gene is located in or near the hereditary prostate cancer susceptibility locus and has a duplicated NDP kinase domain sequence [13]. *Nm23*-H8 has a triplicated NDPK domain sequence and an N-terminal sequence that is homologous to thioredoxin [6]. Recently, we reported the cloning and characterization of the murine Nm23-M5 gene, which shares 86% identity with its human homolog *Nm23*-H5 [15].

Apart from their role in nucleotide synthesis, the NDPKs in mammalian cells are also involved in processes such as tumor metastasis, cell proliferation, transcriptional regulation, development, senescence, and apoptosis [3,10–12,16]. To better understand the function of the Nm23-M5 gene, we generated Nm23-M5 knock-down transgenic mice using a short hairpin RNA (shRNA) vector system. The shRNA leads to degradation of homologous mRNAs and may be used for the disruption of target gene expression to study the function of a specific gene [17]. Because not all shRNAs are effective, we verified knock-down of the Nm23-M5 gene by the shRNA Nm23-M5 vector in CHO cells

Abbreviations: NDPK, nucleoside diphosphate kinase; Nm23, non-metastatic 23; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR

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expressing Nm23-M5. Here, we show that Nm23-M5 gene expression is down-regulated in Nm23-M5 shRNA transgenic mice using RT-PCR and Western blot analysis. Nm23-M5 shRNA transgenic mice exhibit an abnormal testis phenotype and reduced cell numbers of haploid stage cells such as spermatids. These data clearly demonstrate that the expression of Nm23-M5 gene is a critical factor for spermiogenesis.

2. Materials and methods

2.1. Cloning and expression of murine Nm23-M5 in CHO cells

The Nm23-M5 cDNA was inserted into the EcoRI and BglII digested pCXN2 expression vector (pCXN2-Nm23-M5). The pCXN2-Nm23-M5 vector was digested with PvuI, purified using a gel extraction kit (Solgent, Daejeon, Korea), and then used for cell transfection. CHO cells were transfected with 10 μg of pCXN2-Nm23-M5 using Effectene (Qiagen, Valencia, CA, USA) and selected in the presence of G418 (700 μg/ml) for 30 days. Stable cell lines harboring the Nm23-M5 gene were chosen by limiting dilution and were confirmed by Western blot analysis. Stable CHO cell lines which expressed the Nm23-M5 gene were plated at a density of 2×10^5 cells per 35-mm dish and grown with McCoy's 5A medium containing 10% FBS.

2.2. Design and cloning of shRNA against murine Nm23-M5

Oligonucleotides which target nucleotides 256–277 of the Nm23-M5 transcript (NCBI accession no: AF343565) were synthesized. The complementary oligonucleotides were annealed and ligated into the pU6shx vector (Vector corea, Seoul, Korea), which contains the human U6 RNA polymerase III promoter and a neomycin resistance gene (pU6shx-Nm23-M5). The sequences of the oligonucleotides were 5′-GACATAAGGCCATCTCCTACTTTCGAGTAG- GAGATGGCCTTATGTC-3′ (sense) and 5′-GACATAAGGCCATCTCCTA- CTCGAAAGTAGGAGATGGCCTTATGTC-3′ (antisense). The pU6shx-Nm23-M5 vector was digested with XbaI and EcoRI, purified using a gel extraction kit (Solgent, Daejeon, Korea) and then used for cell transfection and/or production of transgenic mice.

2.3. Production of Nm23-M5 polyclonal and monoclonal antibodies

For polyclonal antibody production, rabbits were injected with affinity-purified Nm23-M5 protein, initially with complete Freund's adjuvant and subsequently with incomplete Freund's adjuvant. Polyclonal antibodies from serum were purified using a Protein G Sepharose column (Amersham Pharmacia, Orsay, France) and tested for Nm23-M5 recognition by ELISA. The method of Kohler and Milstein [18] was adapted to produce monoclonal antibodies. The isotypes of the selected monoclonal antibodies were determined using a commercially available isotyping kit (Roche Diagnostics, Mannheim, Germany).

2.4. Real time RT-PCR, Northern blot, Western blot, and immunohistochemical analysis

Total RNA was isolated from adult testis tissues using the TRIZOL reagent (Gibco BRL). In order to specifically detect the expression of Nm23-M5 cDNA transcripts, RT-PCR was performed with specific primers using a Peltier Thermal Cycler 200 (MJ Research, Nevada, USA). Nm23-M5 cDNA detection was performed using the following primers: (forward) 5′- ATGGGGGTGCACGAATGTCC-3′ and (reverse) 5′-TCATCTGTCCCCTGTCCTGC-3′. Northern blot, Western blot, and immunohistochemical analyses were designed as described previously [15,20,21].

2.5. Generation of heterozygous and homozygous transgenic mice

Transgenic mice were produced as described previously [20]. All experiments were performed under the Animal Care guidelines of the Konkuk University. Heterozygous transgenic mice were confirmed by PCR using the following primers: forward, 5′-TTTATGCTTCCGGCTCGTAT-3′, and reverse, 5′-CGTAATACGACTCACTATAGG-3′. For the production of homozygous transgenic mice, heterozygous male and female transgenic mice were mated. Homozygous transgenic mice were selected and confirmed by PCR, and the level of Nm23-M5 gene expression was determined.

2.6. Nm23-M5 and mGPX-5 modeling

The comparative modeling program MODELLER v8 [29] was used to generate structural models for Nm23-M5 and mGPX-5. The available crystal structures of the human NDPK A (PDB ID: 1JXV, sequence identity 28%) and the human GPX-5 (PDB ID: 2I3Y, sequence identity 30%) were used as the templates during the homology building process. A first set of twenty crude structures was automatically generated with MODELLER using the default options. The best model with the lowest MODELLER restraint energy was kept and further refined by energy minimization using the CNS program [30]. The stereochemistry of the final model was validated by PROCHECK [31]. A binding model structure (NM23-M5 and mGPX-5) was built using the HEX 4.5 program. The iterative steps of model building by the "O" program [32] and energy minimization using CNS yielded a final model, displayed by PyMOL [33].

2.7. Statistical analysis

Values are reported as means ± standard deviation. Comparisons were performed by ANOVA. At least three independent experiments were performed. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Nm23-M5 shRNA knock-down of Nm23-M5 expression at the transcriptional and translational levels in CHO cells expressing Nm23-M5

A stable CHO cell line harboring the Nm23-M5 gene (a testis specific NDPK isoform) was established. To verify the constitutive expression of Nm23-M5, we examined the presence of Nm23-M5 transcripts and protein through Northern blot and Western blot analysis, respectively (Fig. 1A). Fig. 1B shows the protein levels of Nm23-M5 in CHO cells after transfection, indicating that the CHO cells stably expressed the Nm23-M5 gene.

Nm23-M5 gene knock-down was achieved using a vector-based shRNA approach. A Nm23-M5 shRNA corresponding to nucleotides 256–277 of the murine Nm23-M5 cDNA sequence was designed and confirmed by BLAST similarity search to minimize off-target effects. To examine whether the Nm23-M5 shRNA expression vector was effective in knocking-down Nm23-M5 expression, different concentrations (0, 1, 5 and 10 nM) of Nm23-M5 shRNA were transfected into a CHO cell line stably expressing Nm23-M5. A treatment of approximately 10 nM Nm23-M5 shRNA was sufficient to completely down-regulate the Nm23-M5 gene, indicating that the Nm23-M5 shRNA expression vector efficiently knocks-down the Nm23-M5 gene (Fig. 1).

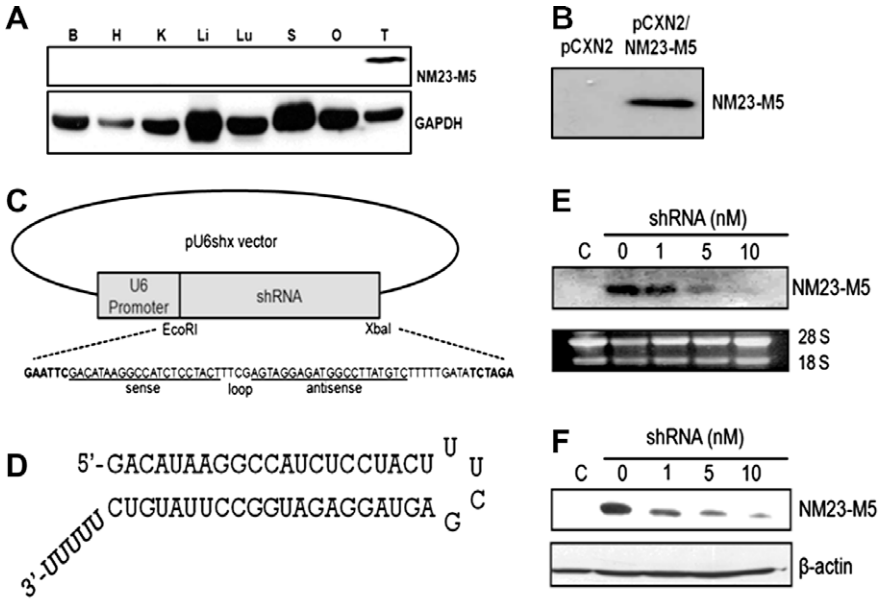

Fig. 1. Nm23-M5 expression pattern in mouse and shRNA silencing of Nm23-M5 expression. (A) Expression pattern of Nm23-M5 in various murine tissues by Western blot. B, brain; H, heart; K, kidney; Li, liver; Lu, lung; S, spleen; O, ovary; and T, testis. (B) Establishment of a stable CHO cell line for Nm23-M5 expression. (C) Construction of a Nm23-M5 shRNA expression vector for the production of transgenic mice. (D) Secondary structure prediction for the Nm23-M5 shRNA. (E and F) Nm23-M5 down-regulation by treatment with various Nm23-M5 shRNA concentrations was detected by Northern or Western blot analysis of CHO cells stably expressing Nm23-M5.

3.2. Nm23-M5 protein expression is localized into round and elongated spermatids in the mouse testis

To knock-down Nm23-M5 expression in haploid stages, we produced transgenic mice. Heterozygotes were produced by microinjection and confirmed by PCR (Fig. 2). Homozygotes were produced by mating female and male transgenic mice. To confirm the levels of Nm23-M5 gene expression in transgenic mouse testes, we performed Western blots (Fig. 3A) and real-time RT-PCR (Fig. 3B). Nm23-M5 gene expression was significantly down-regulated in the Nm23-M5 shRNA transgenic mouse testes compared to those of control mouse testes. The expression of Nm23-M5 in homozygous shRNA transgenic mice was approximately half of that detected in heterozygotes (Fig. 3).

The full-length Nm23-M5 protein fused to a hexahistidine tag at the NH2 terminus was expressed [19] and purified as described by Yu et al. [17] and used as immunogen to raise polyclonal antibodies in rabbits. These affinity-purified antibodies specifically recognize Nm23-M5, as shown by Western blotting (Fig. 1A). Our previous study showed that Nm23-M5 mRNA expression was first detected at day 15 after birth, when primary spermatocytes in the early pachytene stage of meiosis are the most advanced germ cells present in the testis. In this study, we further characterized the cellular localization of Nm23-M5 in testes sections of adult testis. Nm23-M5 protein expression was strongly localized in round and elongated spermatids, with lesser staining on spermatocytes. Somatic cells, such as the Sertoli and Leydig cells, were negative (Fig. 3C, left panels). In elongating spermatids, Nm23-M5 is

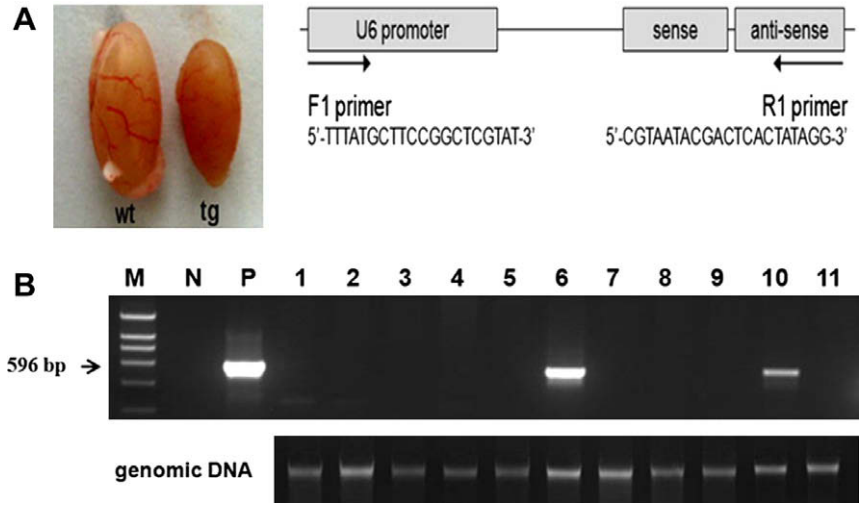

Fig. 2. Production of transgenic mice expressing the Nm23-M5 shRNA. (A) Comparison of testis phenotype between wild type and transgenic mice harboring the Nm23-M5 shRNA. (B) Primer design for identification of transgenic mice harboring the Nm23-M5 shRNA by PCR analysis. (C) Identification of transgenic mice (596 bp size) by PCR analysis (M: size marker, N: negative control, P: positive control, lanes 1–11: individual pups tested).

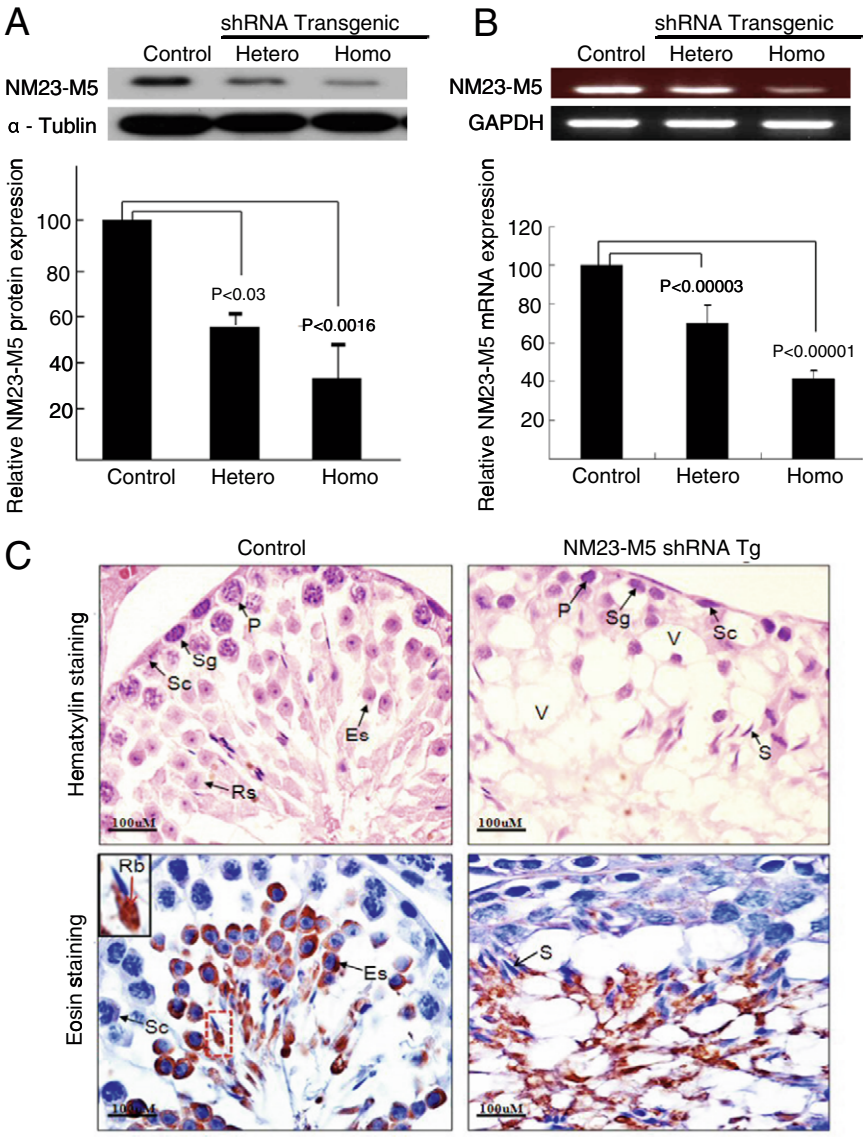

Fig. 3. Disruption of Nm23-M5 gene expression results in defects in spermiogenesis. (A) RT-PCR analysis shows the down-regulation of Nm23-M5 gene expression in the Nm23-M5 shRNA transgenic mouse testis. (B) Western blot analysis shows the down-regulation of Nm23-M5 gene expression in the Nm23-M5 shRNA transgenic mouse testis. (C) Abnormal testis phenotype in Nm23-M5 shRNA transgenic mice. Scale bars = 20 μm. S, Sperm; Es, elongating spermatids; P, pachytene spermatocyte; Rs, round spermatids; Sc, Sertoli cell; Sg, spermatogonium; and V, vacuoles.

localized primarily to the residual body, but is more faintly detected in the head and the flagellum. Additionally, Nm23-M5 in ejaculated spermatozoa is present primarily at the base of the mid-piece and along the flagellum, and in decreasing amounts in the sperm head. However, Nm23-M5 shRNA heterozygous and homozygous transgenic mice exhibited an abnormal testis phenotype and reduced cell numbers, especially of round and elongated spermatids (Fig. 3C, right panels).

3.3. Nm23-m5 silencing results in the down-regulation of GPX-5 and depletion of round and elongated spermatids

Bax induces apoptosis due to the generation of ROS. We hypothesized that Nm23-M5 might serve to protect the testis from oxidative stress. To test this hypothesis, we examined whether overexpression of this gene in CHO cells, which do not normally express Nm23-M5, would enhance antioxidant activity in order to suppress ROS-induced apoptosis. A Nm23-M5-expressing CHO cell line was established by transfection with an expression vector containing a mouse Nm23-M5 cDNA under the control of the cytomegalovirus promoter (CMV/*Nm23*-M5) (Fig. 4C). A control CHO cell line was transfected with the CMV-neo expression vector alone. We assessed the transcript levels of nine antioxidant enzymes, including those belonging to the superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPX) families. CMV/Nm23-M5-transfected cells showed altered expression profiles of several antioxidant enzymes. Expression of GPX-5, which catalyzes conversion of hydrogen peroxide to water, was highly increased (4-fold) in the CMV/*Nm23*-M5-transfected cells. Therefore, we examined apoptosis in CMV/*Nm23*-M5-transfected cells and control cells by adding H_2O_2 to the culture medium. As shown in Fig. 4D, CMV/*Nm23*-M5-transfected cells were resistant to cell death, but control cells were not.

We further explored whether Nm23-M5 silencing resulted in in vivo changes in expression of GPX-2 and -5. A real-time RT-PCR assay showed that GPX-2 and -5 mRNAs were significantly down-regulated in the testis of Nm23-M5 shRNA transgenic mice (Fig. 4A and B). Real-time RT-PCR analyses revealed that GPX-5 expression decreased by 20% and 60% in Nm23-M5 shRNA heterozygous and homozygous transgenic testes, respectively. Also, testis

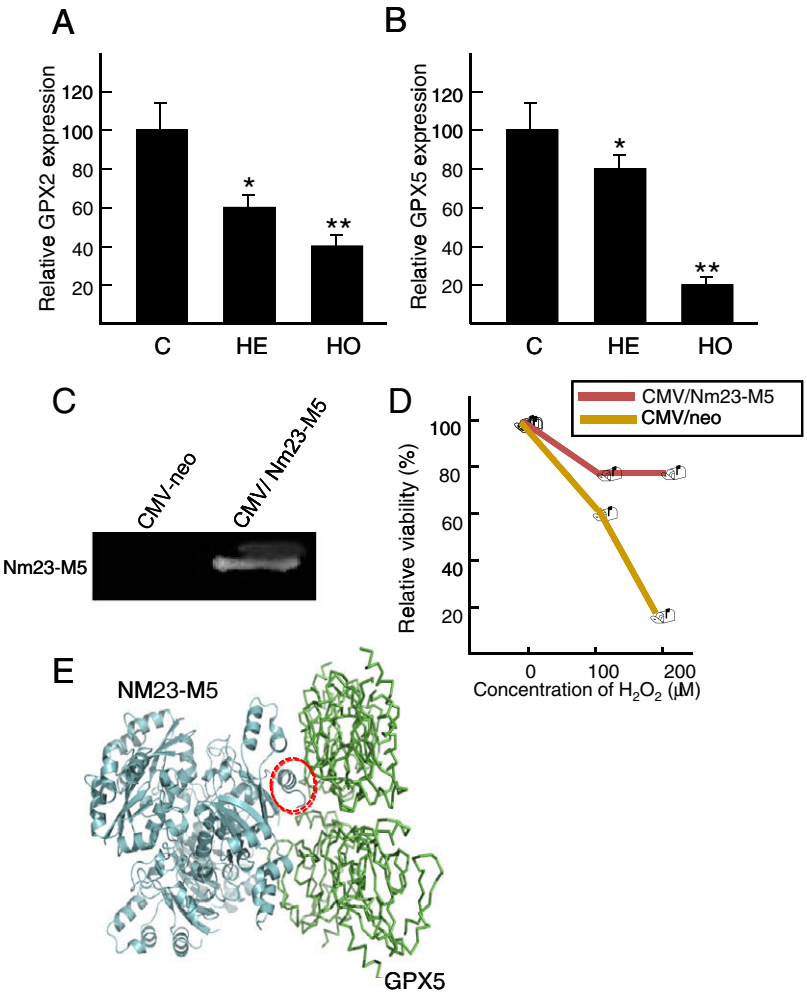

Fig. 4. Glutathione peroxidase 5 regulation by Nm*23*-M5. (A and B) RT-PCR was performed with testis-derived RNAs expressed in the wild type (C), Nm23-M5 shRNA heterozygous (HE) and homozygous (HO) transgenic mice. (C) Establishment of a CHO cell line stably expressing Nm23-M5. (D) Apoptosis comparison by different H_2O_2 treatments in a CHO cell line stably expressing Nm23-M5. (E) Possible docking mode between Nm23-M5 and mGPX-5 modeled using the HEX 4.5 program. A ribbon diagram shows the hexameric Nm23-M5 homology model structure. An alpha-carbon back bone diagram shows the tetrameric mGPX-5 homology model structure.

cells were severely depleted in homozygous transgenic mice, especially round and elongated spermatids, compared to those of heterozygous transgenic and control mice.

To better understand the structural basis of the interaction between Nm23-M5 and mGPX-5, we generated homology model structures of NM23-M5 and mGPX-5. A possible interaction between Nm23-M5 and mGPX-5 was predicted using a docking simulation program. This result suggests that the C-terminal extension region may mediate the specific interaction between Nm23-M5 and GPX-5 (Fig. 4E). Considering that antioxidants protect cells against oxidative DNA damage, our results suggest that Nm23-M5 plays an important role in spermiogenesis by providing protection via GPX-5 against damage induced by ROS.

4. Discussion

In this study, we investigated the mechanism by which Nm23-M5 protects CHO cells in vitro and male haploid germ cells in vivo against oxidative stress-induced apoptosis. Specifically, we focused on the level of expression and the C-terminal region of Nm23-M5 in protection against oxidative stress-induced apoptosis. We also explored the relationship between Nm23-M5 and GPX-5 during oxidative stress. Our results indicate that a high level of Nm23-M5 expression and Nm23-M5 protein in cells is critical to their protection against oxidative stress-induced apoptosis. When Nm23-M5 expression was reduced by a Nm23-M5 specific shRNA, round and elongated spermatids in testes of transgenic mice were significantly more sensitive to oxidative stress. In contrast, overexpression and maintenance of high levels of Nm23-M5 in CHO cells significantly increased resistance to oxidative stress.

The knock-down approach is useful for clarifying the physiological role of molecules in vivo [17]. To examine the effects of a loss-of function of Nm23-M5 in vivo, we used a Nm23-M5 short hairpin RNA (shRNA) to knock-down the expression in the testes of transgenic mice. The Nm23-M5 shRNA knocked-down the expression of Nm23-M5 and reduced the Nm23-M5 protein level in haploid spermatids derived from Nm23-M5 shRNA transgenic mice, as shown by RT-PCR and Western blot, respectively. More importantly, the reduction of Nm23-M5 expression resulted in a decrease in survival of round and elongated spermatids, as well as a decrease in expression of GPX-5. In the testis, GPX-5 has been used extensively as an indicator ROS scavenger activity in the testes of rodents and human. Our results suggest that after knock-down of Nm23-M5, the testes were severely damaged and haploid spermatids were rarely found.

The testis is sensitive to a variety of stressors, such as hyperthermia, inflammation, radiation, and oxidative stress [22–24]. Oxidative stress in the testis induces germ cell apoptosis and sev-

eral antioxidants (glutathione peroxidase, ascorbic acid, and vitamine E) have been shown to protect germ cells against oxidative DNA damage [25–28]. In our previous study, we reported that the overexpression of Nm23-M5 in fibroblasts enhanced cellular levels of several antioxidant enzymes, particularly glutathione peroxidase 5. Therefore, we hypothesized that Nm23-M5 controls the expression of glutathione peroxidase 5 in some way and that upregulation of antioxidant function serves to protect sperm membranes from oxidative damage [15].

Nm23-M5 mRNA expression was first detected at day 15 after birth, a time when primary spermatocytes in the early pachytene stage of meiosis are the most advanced germ cells present in the testis [15]. Therefore, we characterized the level of the Nm23-M5 protein more precisely to assess the differences in the expression of these two genes. A high level of Nm23-M5 protein is found only in round and elongated spermatids, depending on the stage of tubules, and is not observed outside of the testicular tubules; Nm23-M5 is not detected in spermatogonia or somatic cells. This expression pattern may reflect the cell-specific and/or stage-specific expression of Nm23-M5 in haploid cells. Thus, the murine Nm23-M5 mRNA appears to be somewhat differently expressed than its human counterpart.

In the testis of Nm23-M5 gene knock-down transgenic mice, expression of GPX-5 was profoundly down-regulated. GPX-5 is a 24-kDa secretory protein that acts independently of selenium and is expressed in the caput epididymis under the control of androgens [30,31]. The protein has been shown to bind to the acrosomic region of spermatozoa in the epididymis [32]. At that stage, GPX-5 behaves in vivo and in vitro as a glutathione peroxidase [33]. Thus, we hypothesize that Nm23-M5 controls the expression of GPX-5 and that this upregulation of antioxidant function serves to protect sperm membranes from oxidative damage.

In this study, we employed a shRNA knock-down system to analyze the functional role of the Nm23-M5 gene in transgenic mice. The Nm*23*-M5 shRNA heterozygous and homozygous transgenic mice exhibited an abnormal testis phenotype and significantly reduced cell numbers, particularly spermatids. Most likely, this cell death can be explained by the down-regulation in Nm23-M5 expression, in which Nm23-M5 is not present to activate glutathione peroxidase 5 expression, rendering the Nm23-M5 shRNA homozygous transgenic mice much more susceptible to oxidative DNA damage. At present, the mechanisms of spermatid cell death by Nm23-M5 knock-down are not clear, but previous experimental data on Nm23-M5 [15] allow us to hypothesize that increased oxidative stress by inactivation of glutathione peroxidase 5 leads to cell death. However, further study is needed to reveal the mechanism of glutathione peroxidase 5 activation by Nm23-M5 gene expression during spermiogenesis. These experiments are currently ongoing in our laboratory.

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