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Tcp10 Promoter-directed Expression of the *Nek2* Gene in Mouse Meiotic Spermatocytes

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Nek2 is a cell cycle regulator that is involved in diverse cell cycle events. The expression pattern and biochemical properties of Nek2 in mammalian male germ cells suggested its involvement on regulation of the meiotic cell cycle. To further investigate specific roles of Nek2 in meiosis, we generated transgenic mice in which the Nek2 transgenes were expressed specifically in spermatocytes using the *Tcp10* promoter. The *Nek2* transgenic mice did not reveal any significant defect in gross testicular anatomy as well as in fertility. However, we observed significant increases in defective spermatogenic cells, such as apoptotic cells and giant degenerating cells, in the *Tcp10/Nek2* transgenic mice. These results revealed that even only slightly elevated production of the Nek2 protein disturbed the normal development of male germ cells, possibly in meiosis.

Keywords: Cell Cycle; Nek2; Spermatogenesis; *Tcp10* Promoter; Transgenic Mouse.

Introduction

The cell cycle consists of a complicated series of cellular processes for duplication. However, control of cell cycle progression occurs only at a limited number of events, called checkpoints. Cells at checkpoints make sure completion of the previous cell cycle events, and then proceed to the next step. The Cdk activity plays a pivotal role in driving these checkpoints. These controlling mechanisms are conserved among all eukaryotic cell cycles (Eilers, 1999).

It is clear that the meiotic cell cycle is controlled fundamentally in the same way as in the mitotic cell cycle (reviewed in Wolgemuth *et al.*, 1995). In fact, the presence of M-phase promoting factor, the complex of Cdc2 and the B-type cyclin, was initially identified as the maturation-promoting factor for the frog oocyte (Masui and Markert, 1971). However, meiosis also contains unique cell cycle events and checkpoints that cannot be observed in mitosis, such as homologous chromosome pairing and recombination, reduction division, and a specific meiotic arrest during oogenesis (reviewed in Wolgemuth *et al.*, 1995). Such unique aspects of the control of meiosis might be programmed genetically. Therefore, there might exist a group of genes whose functions are specific for meiosis-specific cell cycle events.

While investigating the cell cycle regulation in mouse male germ cells, we observed that expression patterns of many cell cycle-related genes were consistent with their predicted functions (reviewed in Wolgemuth *et al.*, 1998). For example, *Cdc2* was expressed most abundantly at meiotic spermatocytes in germ cell lineage and reduced below detection level once meiosis occurred, suggesting that Cdc2 play critical roles in meiosis of male germ cells (Rhee and Wolgemuth, 1995). In addition, we observed a few candidate genes whose functions were specifically related to the meiotic cell cycle. For example, *Cyclin A1* expression was limited to male germ cells, specifically at spermatocytes, and not in other tissues (Sweeny *et al.*, 1996). *Cyclin A1*-deficient male mice were sterile due to a block of spermatogenesis before the first meiotic division, whereas female mice were normal (Liu *et al.*, 1998). Cyclin A1 is therefore essential for spermatocyte passage

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Abbreviations: Nek, NIMA-related kinase; NIMA, never in mitosis A; TUNEL, terminal deoxynucleotide transferase-mediated dUTP nick-end labeling.

into the first meiotic division in male mice. Another example may be Pftaire1, a novel member of the Cdk family. In male germ cell lineage, *Pftaire1* was expressed specifically in spermatocytes right before meiosis, suggesting its involvement in the meiotic processes (Besset *et al.*, 1998).

Nek2 is another candidate cell cycle regulator that may play specific roles in the meiotic cell division. Nek2 is a mammalian protein kinase that shares a strong similarity with *Aspergillus* NIMA biochemically as well as structurally (Fry *et al.*, 1995; Schultz *et al.*, 1994). Loss-of-function mutations in the *Aspergillus nimA* gene arrested the cell cycle at G2 phase, while overexpression of *nimA* induced a premature mitosis like chromosome condensation and bipolar spindle formation (Osmani *et al.*, 1988; 1991). Based on these observations, it was proposed that NIMA plays a critical role in progression of the M phase by controlling chromosome condensation/decondensation (reviewed in Osmani and Ye, 1996). Recently, it was reported that NIMA might help promote chromosome condensation through histone H3 phosphorylation (De Souza *et al.*, 2000). Therefore, we hypothesized that, like NIMA, Nek2 functions as a cell cycle regulator, possibly controlling chromosome condensation/decondensation (Kim *et al*., 2002; Rhee and Wolgemuth, 1997).

It has been shown that *Nek2* was expressed in many tissues tested, but most abundantly in the testis (Rhee and Wolgemuth, 1997; Tanaka *et al.*, 1997). Within the testis, *Nek2* was expressed predominantly in spermatocytes and its expression was reduced below detection level in haploid cells after meiosis (Rhee and Wolgemuth, 1997). Nek2 appeared to be associated with meiotic chromosomes in spermatocytes. Furthermore, in an *in vitro* experimental setting in which meiotic chromosome condensation was induced with okadaic acid, a concomitant induction of Nek2 kinase activity was observed (Rhee and Wolgemuth, 1997). These observations suggested that Nek2 was involved in events of meiosis, including but not limited to chromosome condensation (Rhee and Wolgemuth, 1997).

In order to further investigate specific roles of Nek2 during the meiotic cell cycle, we generated transgenic mice to overexpress the *Nek2* transgenes specifically in the spermatocytes using the *Tcp10* promoter. We observed that the resulting slightly elevated Nek2 levels disturbed normal development of the male germ cells, yielding a small but demonstrable increase of apoptotic cells and giant degenerating cells. These results supported the importance of regulatory functions of Nek2 in male germ cell development, possibly in meiosis.

Materials and Methods

Construction of the vectors The *Tcp10* eukaryotic expression vector consisted of the *Tcp10*^{t} promoter of 1.6 kb in size (*Eco*RI/*Nhe*I fragment; Ewulonu *et al.*, 1993) and the SV40 polyadenylation signal sequence of 450 bp in size. The *Nek2* open reading frame (ORF) (1-443 a.a.) and a truncated *Nek2* mutant ORF (1-343 a.a.) were subcloned into the *Tcp10* expression vector to produce the *Tcp10/Nek2* and *Tcp10/Nek2DC* transgenic constructs, respectively.

Generation of transgenic mice Transgenic mice were generated by microinjection of the linearized *Tcp10/Nek2* constructs into fertilized eggs according to standard techniques (Hogan *et al.*, 1986). DNA was purified, linearized, and suspended at a concentration of 5 ng/μl in sterile water before microinjection. Verification of transgenic animals was performed by Southern blot hybridization analysis of tail DNA with the sequence of the SV40 polyadenylation signal as a probe. All transgenic families were generated and maintained in C57BL/6 × DBA/2 F1 mixed genetic background.

Northern blot hybridization analysis Northern blot hybridization analysis was carried out as described previously (Rhee and Wolgemuth, 1997). In brief, total RNA from the cell lines was prepared by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). Total RNA was electrophoresed in a denaturing 0.85% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, and hybridized with the *Nek2* riboprobe. Ethidium bromide staining of the 18S and 28S RNAs was used to determine equal loading for each sample.

Immunoblot analysis The immunoblot analysis was carried out as described previously (Rhee and Wolgemuth, 1997). In brief, cell lysates were separated by 10% SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked with Blotto (5% skim milk in TBST) at room temperature for 1 h. After blocking, the membrane was incubated with a primary antibody at room temperature for 3 h, washed with TBST 3 times for 10 min each, incubated with a secondary antibody (HRP-conjugated IgG, 1:5,000) for 30 min, and washed again with TBST 3 times for 10 min each. Specific signals were detected with ECL reaction. The affinity-purified anti-Nek2 (Rhee and Wolgemuth, 1997) was diluted to 1:50.

Histological analysis Histological analyses were carried out as described previously (Liu *et al.*, 1998). In brief, testes were dissected, fixed in 4% paraformaldehyde and embedded in paraffin. Sections (5 μm thick) were prepared and stained with haematoxylin and eosin. *In situ* labeling of apoptotic cells was performed on tissue sections using the ApoTag Plus peroxidase kit (Oncor, USA), according to the manufacturer's instructions. The testicular morphology and stages of the seminiferous tubules were assessed as described by Russell *et al.* (1990).

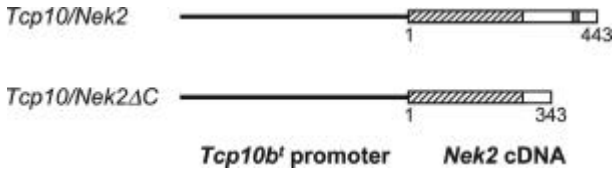

Fig. 1. Construction of the *Tcp10/Nek2* chimeric genes. The *Tcp10*^{t} promoter of 1.6 kb in size was linked to the mouse *Nek2* ORF sequences. The Nek2ΔC lacks 100 amino acids at the C-terminal end where the KEN box (marked as a gray box) is localized. The kinase domain of Nek2 is marked as a shaded box.

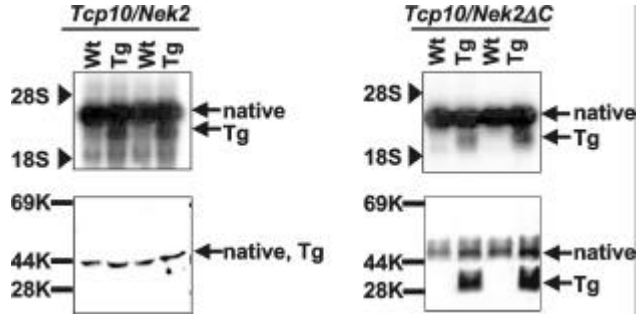

Fig. 2. Expression of the *Tcp10/Nek2* transgenes in the testis. Northern blot hybridization (upper panels) and immunoblot (lower panels) analyses were carried out to determine expression of the *Tcp10/Nek2* and *Tcp10/Nek2DC* transgenes in the testis. Samples were obtained from testes of the two-independent lines of transgenic mice (Tg) and their littermates (Wt). Specific signals were indicated at the right sides of the figures as native (endogenous Nek2) or Tg (transgene products). Reference markers are indicated on the left sides of the figures.

Results

Construction of the *Tcp10/Nek2* chimeric genes We chose to use the promoter from *Tcp10b*^{t}, one of the *Tcp10* haplotype genes, for specific expression of the transgenes in spermatocytes (Ewulonu and Schimenti, 1997; Vidal *et al.*, 1998). The *Tcp10* genes are located in the mouse *t* complex on chromosome 17 and were thought to play a role in segregation distortion associated with mouse *t* haplotypes (Ewulonu *et al.*, 1996; Schimenti *et al.*, 1988; Silver, 1985). It was reported that the *Tcp10b*^{t} gene was expressed exclusively in male germ cells from the spermatocyte stage onward (Schimenti *et al.*, 1988). The 1.6 kb fragment of the *Tcp10b*^{t} promoter was sufficient to confer spermatocyte-specific expression of the transgenes (Ewulonu *et al.*, 1993; Vidal *et al.*, 1998).

The *Tcp10* promoter was linked to the ORF of the mouse *Nek2* gene (*Tcp10/Nek2*) (Fig. 1). In addition, we generated an additional construct in which the 100 amino acid-long of the C-terminal end of the *Nek2* ORF was truncated (*Tcp10/Nek2DC*) (Fig. 1).

Expression of the *Tcp10/Nek2* transgenes in the testis More than three independent lines of transgenic mice with the *Tcp10/Nek2* and *Tcp10/Nek2DC* constructs were generated, respectively. We carried out northern blot hybridization analysis to determine expression of the *Nek2* transgenes in the testis (Fig. 2 upper panels). Since the transgene constructs lacked the 3′ untranslated sequence of the *Nek2* cDNA, their mRNA products appeared smaller than the endogenous *Nek2* mRNA. The results showed that the endogenous *Nek2* mRNA was detected both in the transgenic mice and in their littermates, whereas the transgene mRNA products were detected only in the transgenic mice (Fig. 2 upper panels). The levels of the endogenous *Nek2* mRNA were higher than those of the transgenic mRNA, suggesting that the promoter activity of the endogenous *Nek2* gene was stronger than that of the exogenous *Tcp10* sequence in male germ cells.

The protein products of the *Nek2* transgenes were detected with the immunoblot analysis (Fig. 2 lower panels). The endogenous Nek2 protein of 46 kDa in size was detected both in the transgenic mice and in their littermates. Since the protein product from the *Tcp10/Nek2* construct was identical to the endogenous Nek2 protein, we were not able to distinguish them in the immunoblot analysis (Fig. 2 lower left panel). On the other hand, the Nek2ΔC protein of 30 kDa in size could be detected as distinct from the endogenous Nek2 protein (Fig. 2 lower right panel). Thus, even if the mRNA level of the *Nek2DC* transgene was lower than that of the endogenous *Nek2* gene, the protein level of Nek2ΔC was about comparable to the endogenous protein (Fig. 2). In fact, the Nek2ΔC protein lacks the KEN box that is important for anaphase-promoting complex-mediated proteolysis (Pfleger and Kirschner, 2000), and, therefore, it appeared more stable in a cell than the endogenous protein did. Even if we could not distinguish the transgene product of *Tcp10/Nek2* from the endogenous protein that had migrated with an identical velocity in the immunoblot data, we believe that the amount of the *Nek2* transgene product was much less than the endogenous one. First, in the immunoblot analysis, we were not able to observe any significant enhancement of the Nek2-specific bands in the transgene lysates, compared to that in the littermate samples. Second, the levels of the *Nek2* transgene product should have been less than the endogenous level, if the *Nek2DC* transgene product, which is more stable than the wild type, were present at about equal levels to the endogenous protein.

Histological analysis of the *Tcp10/Nek2* transgenic mice We did not observe any reduction in fertility in the *Tcp10/Nek2* transgenic lines, compared to their littermates. At the same time, we did not observe any gross anatomical defect in the testis of the transgenic mice, ei-ther. However, we were able to detect significant morpho-

Table 1. Increase in the rate of apoptosis in the male germ cells of the *Tcp10/Nek2* transgenic mice.

Experiments*	Number of testicular tubules with 5 or more TUNEL-positive cells	
	Littermates	Tcp10/Nek2
Slide 1	0	4
Slide 2	0	3
Slide 3	0	2
Average	0	3

* The slides for testis sections were prepared from the *Tcp10/Nek2* transgenic mice and their littermates of different founders.

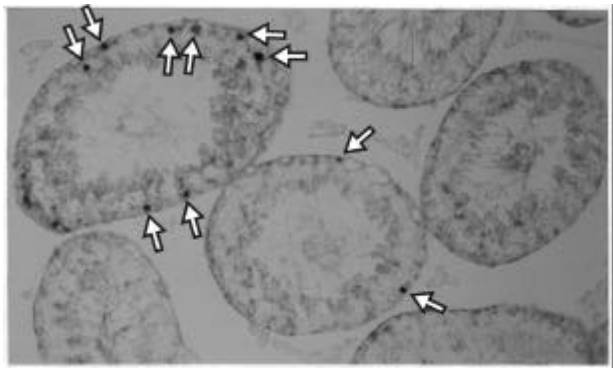

Fig. 3. Detection of apoptotic cells in testis of *Tcp10/Nek2* transgenic mouse. Apoptotic cells in testicular sections were detected with the TUNEL assay. Arrows indicate TUNEL-positive cells in this representative picture.

Table 2. Increase in the rate of giant degenerating cells in the male germ cells of *Tcp10/Nek2* transgenic mice.

Stages of seminiferous tubules	Littermates		*Tcp10/Nek2* Tg	
	Total number of tubules examined	Number of tubules with giant cells (%)	Total number of tubules examined	Number of tubules with giant cells (%)
II-VIII	104	0 (0)	116	12 (10)
IX-I	44	2 (5)	73	48 (66)

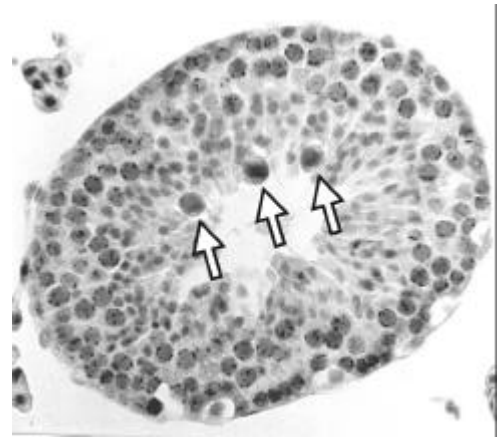

Fig. 4. Detection of giant degenerating cells in testis of *Tcp10/Nek2* transgenic mouse. Giant degenerating cells in the testicular section were detected with microscopic observations. Arrows indicate giant degenerating cells in this representative picture.

logical defects at the microscopic levels. First, apoptotic cells were detected more frequently in the *Tcp10/Nek2* transgenic mice (Fig. 3). We observed the presence of seminiferous tubules with > 5 apoptotic cells in testis sections from the transgenic mice, while none in those from their littermates (Table 1).

Second, giant degenerating cells were detected more frequently in the transgenic mice (Fig. 4). The giant degenerating cells are multinucleated germ cells that can be observed in testis with defects in spermatogenesis (Rockett *et al.*, 2001; Rotter *et al.*, 1993). These giant degenerating cells could be distinguished easily by microscopic observations of the testis sections (Fig. 4). We scored more giant degenerating cells in testis sections from the *Tcp10/Nek2* transgenic mice than from their non-transgenic littermates (Table 2). Furthermore, the giant degenerating cells were detected more frequently in the tubular stage IX-I than in the tubular stage II-VIII (Table 2). Since male germ cells develop in a temporally defined progression along the tubule, there exists a characteristic association of germ cells in particular stages of spermatocytes. In the mouse, this progression of the cycle of the seminiferous tubule can be divided into 12 stages, and meiosis occurs at stage XII (Oakberg, 1956; Russell *et al.*, 1990). Therefore, the current results revealed a correlation between the appearance of giant degenerating cells and meiosis.

Discussion

In the present study, we generated transgenic mice in which *Nek2* was overexpressed specifically in meiotic spermatocytes. The *Nek2* transgenic mice did not reveal any significant defect in gross testicular anatomy as well as in fertility. However, we observed significant increases in defective spermatogenic cells, such as apoptotic cells and giant degenerating cells, in the *Nek2* transgenic mice. These results revealed that overproduction of the Nek2 protein disturbed the normal development of male germ cells. The mild phenotypes observed in the *Tcp10/Nek2* transgenic mice might be due to the fact that the levels of the exogenous Nek2 protein was not quite sufficient enough to override physiological functions of the endogenous protein.

It was proposed that Nek2 was involved in regulation of the meiotic cell division. The meiosis-specific expression pattern of *Nek2* supports the notion (Rhee and Wolgemuth, 1997). An additional piece of evidence was obtained in the current study in which more giant degenerating cells were observed in the *Nek2* transgenic mice than in their littermates. It was known that that the giant cells were resulted from the inability of primary 4N spermatocytes to undergo meiotic divisions to generate haploid cells (Rotter *et al.*, 1993). Furthermore, defects in meiosis might produce more the giant degenerating cells at around tubular stage XII, at which meiosis occurred. In consistent with this prediction, we observed that more giant degenerating cells appeared in tubular stage XI-II than in the tubular stage III-VIII.

Even if it is likely that overexpression of *Nek2* affected meiosis during male germ cell development, it was difficult to pinpoint specific meiotic events that had been affected in the *Tcp10/Nek2* transgenic mice. It is possible that processes in meiotic chromosome condensation/decondensation might be affected, since Nek2 was in association with the meiotic chromatin and its activity was induced in association with pharmacological induction of meiotic chromosome condensation (Rhee and Wolgemuth, 1997). Additional possibilities can be predicted from roles of Nek2 in mitotic cells. It was reported that Nek2 was in association with centrosome (Fry *et al.*, 1998). Overexpression of *Nek2* induced a splitting of centrosomes, whereas prolonged expression of *Nek2* led to dispersal of centrosomal material and loss of a focused microtubule-nucleating activity (Fry *et al.*, 1998). Centrosomal localization of Nek2 was also observed in *Xenopus* early embryos (Fry *et al.*, 2000; Uto and Sagata, 2000). These results indicated that Nek2 plays a critical role in centrosomal cycle.

Recently, we observed a dynamic expression and subcellular distribution of the Nek2 protein during the cell cycle, not limited to centrosome, but also in chromosomes and in midbody (Kim *et al.*, 2002). Moreover, Nek2 was in association with an ER/Golgi protein, suggesting that Nek2 may play a role in changes of the ER/Golgi during the cell cycle (J. C. Y., S. K. J., D. J. W., K. K., K. R., submitted). All these observations propose that Nek2 is a cell cycle regulator that is involved in multiple cellular processes. Therefore, overexpression of *Nek2* might have affected in multiple cellular processes, not limited to meiotic chromosome condensation/decondensation.

The present study revealed that the *Tcp10* promoter is a useful, although limited, tool for directing expression of the transgenes in spermatocytes. Several promoters have been introduced for specific expression of trangenes in male germ cells. The *protamine* promoter directed the expression in haploid cells after meiosis (Zambrowicz *et al.*, 1993), while the *phosphoglycerate kinase 2* (*PGK2*) promoter directed expression in meiotic and haploid male germ cells (Robinson *et al.*, 1989). With regard to specific expression of transgene within spermatocytes, the *Tcp10* promoter seems to be very useful. Even if the activity of the *Tcp10* promoter-driven transgene looked somewhat lower than that of the endogenous *Nek2* promoter, it certainly directed expression of the transgene enough for detection with northern blot hybridization analysis. However, if higher levels of transgene expression are needed, then one should turn to other, more robust promoters than the *Tcp10* promoter.

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