Suppression of *Nek2A* in mouse early embryos confirms its requirement for chromosome segregation

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Summary

Nek2, a mammalian structural homologue of *Aspergillus* protein kinase NIMA, is predominantly known as a centrosomal kinase that controls centriole-centriole linkage during the cell cycle. However, its dynamic subcellular localization during mitosis suggested that Nek2 might be involved in diverse cell cycle events in addition to the centrosomal cycle. In order to determine the importance of Nek2 during mammalian development, we investigated the expression and function of *Nek2* in mouse early embryos. Our results show that both Nek2A and Nek2B were expressed throughout early embryogenesis. Unlike cultured human cells, however, embryonic Nek2A appeared not to be destroyed upon entry into mitosis, suggesting that the Nek2A protein level is controlled in a unique manner during mouse early embryogenesis. Suppression of Nek2 expression by RNAi resulted in developmental defects at the second mitosis. Many of the blastomeres in *Nek2*-suppressed embryos showed abnormality in nuclear morphology, including dumbbell-like nuclei, nuclear bridges and micronuclei. These results indicate the importance of Nek2 for proper chromosome segregation in embryonic mitoses.

Key words: Nek2, RNAi, Mitosis, Spindle pole, Centrosome, Early embryogenesis

Introduction

Mitotic kinases have been named for their importance in the execution and regulation of mitotic events. The Cdc2-cyclin B complex plays a major role in both entry of and exit into mitosis. Other mitotic kinases take part in execution of specific mitotic events in close association with the Cdk activity. It is interesting that a single mitotic kinase is often involved in multiple cellular processes during mitosis (Ke et al., 2003). For example, aurora B is a kinase for histone H3 whose phosphorylation is critical for chromosome condensation and/or segregation (Hsu et al., 2000; Giet and Glover, 2001). Aurora B can also phosphorylate Cenp-E for targeting checkpoint proteins into the kinetochore (Ditchfield et al., 2003) and vimentin at the cleavage furrow during cytokinesis (Goto et al., 2003). Another example is Plk1, which can phosphorylate a number of cell cycle proteins, such as Cdc25, Wee1, Myt1, topoisomerase IIα and Incenp (Elia et al., 2003). In fact, Plk1 is involved in diverse cell cycle events, including activation of Cdc2 kinase at onset of the G2/M transition (Kumagai and Dunphy, 1996; Liu and Erikson, 2002), centrosome maturation (Lane and Nigg, 1996; Liu and Erikson, 2002), Golgi inheritance (Sutterlin et al., 2001) and activation of the anaphase-promoting complex (Descombes and Nigg, 1998). These mitotic kinases are usually located at specific sites within a cell and undertake their designated roles.

Nek2 was initially described as a mammalian structural homologue of *Aspergillus* NIMA whose function was linked to chromosome condensation in mitosis (Schultz et al., 1994; De Souza et al., 2000). However, the importance of Nek2 in centrosomal function has been studied extensively (Fry, 2002). The Nek2 protein was found in association with the centrosome, preferentially at the proximal ends of centrioles (Fry et al., 1998a). C-Nap1, a centrosomal protein, was identified as a specific substrate of Nek2 (Fry et al., 1998a). Premature splitting of centrosomes was induced by overexpression of Nek2 as well as by microinjection of the C-Nap1 antibody (Fry et al., 1998b; Mayor et al., 2000). Based on these results, it was proposed that Nek2 controls centriole-centriole linkage of the cells entering mitosis by phosphorylating C-Nap1 (Fry, 2002).

It is worthy of note that Nek2 is not exclusively centrosomal. In fact, we observed that subcellular distribution of Nek2 changed dynamically during mitosis (Kim and Rhee, 2001; Kim et al., 2002). Association of Nek2 with chromosomes became evident once cells entered mitosis and was maintained until the end of metaphase. Chromosomal association of Nek2 is also observed in meiotic cells (Rhee and Wolgemuth, 1997). Once cells enter anaphase, Nek2 is dissociated from the condensed chromosomes and redistributed throughout the cytoplasm. Distinct localization of Nek2 on the mid body of the telophase cells was evident (Kim et al., 2002). Such dynamic behavior of Nek2 allowed us to propose that Nek2 might be involved in diverse cell cycle events, in addition to the centrosomal cycle.

A specific role of Nek2 on chromosome behavior has been proposed by recent studies on spindle checkpoint. Hec1, a kinetochore protein, is required for a proper spindle checkpoint by recruiting Mps1 kinase and Mad1/Mad2 complexes to

kinetochores (Martin-Lluesma et al., 2002). Depletion of Hec1 impairs chromosome congression and causes persistent activation of the spindle checkpoint (Martin-Lluesma et al., 2002). Interestingly, it was reported that Hec1 is a substrate of Nek2, suggesting possible involvement of Nek2 in regulation of spindle checkpoint (Chen et al., 2002). Recently, it was also reported that Nek2A interacts physically with Mad1 and is required for proper response to spindle damage (Lou et al., 2004).

Two C-terminal splicing variants of Nek2, named Nek2A and Nek2B, were identified in *Xenopus* embryos (Uto et al., 1999; Fry et al., 2000) and in cultured human cells (Hames et al., 2001). As Nek2B lacks a destruction box, it is resistant to mitotic destruction mediated by the anaphase-promoting complex and withstands destruction until early G1 phase (Hames et al., 2001; Hames and Fry, 2002). The Nek2B protein is present exclusively in early *Xenopus* embryos and the Nek2A protein appears only after the neurula stage (Uto et al., 1999). It was recently suggested that Nek2B acts to promote assembly of a functional zygotic centrosome in fertilized *Xenopus* eggs (Twomey et al., 2004).

In the current study, we suppressed *Nek2* expression in mouse early embryos by RNAi. Our results revealed that Nek2 is critical for proper segregation of chromosomes during embryonic mitosis.

Materials and Methods

Embryo collection, culture and micromanipulation

Super-ovulated Fvb female mice were mated with Fvb males and checked for vaginal plugs the next morning. One-cell zygotes were collected and cultured in CZB medium containing 0.5% BSA (Chatot et al., 1989). 24 hours later, embryos were transferred to CZB medium without EDTA but supplemented with glucose (1 mg/ml) and cultured for up to three more days.

Micromanipulation was performed following a standard procedure (Nagy et al., 2002). In brief, one-cell embryos were placed in HEPES-buffered CZB medium containing 20 mM HEPES and 5 mM sodium bicarbonate under mineral oil (Sigma) for 10 minutes prior to micromanipulation. 10 pl dsRNA was microinjected into the cytoplasm of a zygote using a constant flow system (Transjector, Eppendorf). After microinjection, the embryos were cultured in CZB medium supplemented with 5 mg/ml BSA in a 5% CO_2 atmosphere at 37°C.

Double-stranded RNA (dsRNA) preparation

Double-stranded RNA was prepared by annealing two complementary RNAs transcribed by T7 or SP6 RNA polymerase in vitro. The cDNA fragments for dsRNA were initially subcloned into the *pGEM-T* vector. For *Nek2*, we prepared four independent dsRNAs: *Nek2* Fragment 1 (501 bp in size at the kinase domain common to both *Nek2A* and *Nek2B* using primers 5′-CGA ACC AAC ACA ACC CTG TA-3′ and 5′-GCC ATC AGA GTA GCG GTA GG-3′); *Nek2* Fragment 2 (501 bp in size at the regulatory domain common to both *Nek2A* and *Nek2B* with 5′-GCA GAC ATG GTT GCA GAA GA-3′ and 5′-CTG CCT GCT CTT TAG CTG GT-3′); *Nek2* Fragment 3 (412 bp in size at the 3′ untranslated region specific to *Nek2A* with 5′-GCA TTG CTT GTG GTC TGC AA-3′ and 5′-GAC CTT CCT GGA ATG GGA AT-3′); and *Nek2* Fragment 4 (480 bp in size corresponding to a part of coding region and the 3′ untranslated sequences specific to *Nek2B* using 5′-TCC ACC TCA CAT GAG GAT-3′ and 5′-AGC TGA GAG TTC TCC ATC TT-3′). The *E-Cadherin* dsRNA was a 330 bp-fragment prepared using primers 5′-CTG CTG CTC CTA CTG TTT CT-3′ and 5′-GAA CAC CAA CAG AGA GTC GT-3′. The *MmGFP* dsRNA was a 443 bp-fragment prepared with 5′-CAC ATG AAG CAG CAC GAC TT-3′ and 5′-ACG AAC TCC AGC AGG ACC AT-3′.

After RNAs were synthesized using the T7 and SP6 RNA polymerase (Roche), DNA templates were removed with the DNase I treatment. The RNA products were extracted with phenol:chloroform and ethanol precipitated. To anneal sense and antisense RNAs, equimolar quantities of both sense and antisense RNAs were mixed in the annealing buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) to a final concentration of 2 μM each, heated for 1.5 minutes at 94°C, and incubated at room temperature for several hours. To remove leftover single-stranded RNA, the mixture was treated with 2 μg/ml RNaseT1 (Calbiochem) and 1 μg/ml RNaseA (Sigma) for 30 minutes at 37°C. The dsRNA was treated with 140 μg/ml proteinase K (Sigma), phenol:chloroform extracted, ethanol precipitated, washed in 75% ethanol and dissolved in water. The quality of dsRNA was confirmed by running on an agarose gel. The dsRNA samples were diluted to a final concentration of 2-4 mg/ml and stored at –70°C before use.

Quantification of mRNA in the embryos

Total RNAs were isolated by the guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Ten embryos were added to a tube containing 300 μl of solution D (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-lauryl sarcosyl and 0.1 M 2-mercaptoethanol) on ice and immediately plunged into liquid nitrogen for storage until use. After thawing, 0.1 volume of 2 M sodium acetate (pH 4.0), one volume of water-saturated phenol and 0.2 volume of chloroform:isoamyl alcohol (49:1) were added. After vortex mixing, the mixture was incubated on ice for 10 minutes. Total RNA was then fractionated by centrifugation at 10,786 ***g*** for 15 minutes at 4°C and precipitated from supernatant in the presence of one volume of isopropanol. The pellet was washed with 75% ethanol and dissolved in water.

For reverse transcription, the RNA sample was heated in the presence of 100 pmol random hexanucleotides in a final volume of 8 μl at 65°C for 5 minutes (Shim et al., 1997). After brief centrifugation at 4°C, 12 μl master mix [200 U RNaseH-MMLV reverse transcriptase, 4 μl dNTP mix (2.5 mM each), 1 μl RNasin (26 U/μl), 2 μl 0.1 M DTT, and 4 μl of 5×RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM dithiothreitol)] was added, and the reaction mixture was incubated at 37°C for 1 hour. The reaction was terminated by incubating the sample at 75°C for 15 minutes.

The amounts of specific mRNA were determined with reverse transcription-PCR based methods. To quantify the *Nek2* mRNA, we used a pair of the following primers that correspond to a part of the kinase domain and that generate a 167 bp-fragment: 5′-CGA ACC AAC ACA ACC CTG TA-3′ and 5′-AGC GAG GAA GAC ACT GTG AG-3′ or the primer set for Fragment 1 as described above. For *E-Cadherin* mRNA, we used a primer set which resulted in 385 bp fragment: 5′-GCT GGA CCG AGA GAG TTA-3′ and 5′-TCG TTC TCC ACT CTC ACA T-3′.

The PCR amplification was carried out with 2 μl RT reaction mixture in 20 μl PCR reaction solution containing 2 μl 10×PCR buffer, 1.6 μl dNTP mix (2.5 mM each), 10 pmol each of PCR primers and 1 U Ex-Taq polymerase (Takara). The sample was subjected to a 35-cycle amplification on a GeneAmp PCR System 2400 (Perkin Elmer). 6 μl PCR product were analyzed by 1% agarose gel electrophoresis.

Competitive RT-PCR

To determine relative amounts of specific mRNAs, we carried out competitive RT-PCR in which a specific DNA fragment with an insertion sequence was added into the PCR reaction mixture along

with reverse transcribed cDNAs. The competitive PCR amplification was carried out by adding 0.5-8 μl reverse transcribed cDNA and a fixed amount of the mutant DNA into 40 μl PCR reaction mixture. As a result, two specific PCR fragments were amplified: one from the fragment of the insertional mutant and the other from reverse-transcribed cDNA. The Nek2 primers generated a 167 bp fragment from *Nek2* mRNA and a 294 bp fragment from the insertional mutant DNA. The *E-Cadherin* primers generated a 385 bp-fragment from *E-Cadherin* mRNA and a 524 bp-fragment from the insertional mutant DNA. The *Nek2* and *E-Cadherin* cDNA was PCR-amplified for 35 cycles. The PCR products were electrophoresed on 1% agarose gel, stained with ethidium bromide and the intensity of a specific band was determined by a densitometry.

Immunocytochemical staining of the embryos

Embryos were fixed in 3.7% formaldehyde in PBS for 10 minutes at room temperature, neutralized with 50 mM NH_4Cl in PBS for 10 minutes and post-permeabilized with 0.25% Triton X-100 in PBS for 10 minutes. Immunocytochemical staining was performed by incubating the fixed samples with primary antibodies for 60 minutes, followed by secondary antibodies conjugated with TRITC or FITC for 30 minutes. Polyclonal antibodies against Nek2 (Rhee and Wolgemuth, 1997) and γ-tubulin (Santa Cruz Biotechnology) were diluted 1:100 and monoclonal antibodies against E-Cadherin (Transduction Laboratories), β-tubulin (Sigma) and γ-tubulin (Sigma) were diluted 1:200. The secondary antibodies conjugated to TRITC or FITC (Jackson ImmunoResearch Laboratories) were diluted 1:200. The slides were observed under a fluorescence or confocal microscope.

Immunoblot analysis

Protein samples from embryos, testes or cultured cells were solubilized in Laemmli sample buffer, resolved by 8% SDS-PAGE, and blotted onto a nitrocellulose membrane. The membrane was blocked by soaking in Blotto (Tris-buffered saline with 0.3% Triton X-100 and 5% non-fat dried milk) for 1.5 hours and incubated overnight with the primary antibody in blocking solution. The membrane was then washed three times with TBST (Tris-buffered saline with 0.3% Triton X-100), incubated with a secondary antibody conjugated with horseradish peroxidase for 45 minutes and washed five times with TBST. The signal was detected with the ECL western blotting detection reagents (Amersham) following the manufacturer's recommendations. The affinity-purified Nek2 antibody was diluted 1:100 and secondary antibody was diluted 1:5000.

Results

Nek2 expression in mouse early embryos

As an initial step in the investigation of the role of Nek2 during mouse development, we decided to confirm *Nek2* expression in the early embryos. For detection of *Nek2* at the RNA level, we carried out RT-PCR analysis using three different sets of *Nek2* primers: for detection of *Nek2A* only (see Fragment 3 in Fig. 4A), for detection of *Nek2B* only (Fragment 4 in Fig. 4A) and for simultaneous detection of both *Nek2A* and *Nek2B* (Fragment 1 in Fig. 4A). The results showed that both *Nek2A* and *Nek2B* mRNAs were present in oocytes as well as in embryos of all stages examined (Fig. 1A).

Immunoblot analyses were carried out to detect Nek2 proteins in mouse early embryos. First, we wished to ensure that the polyclonal antibody that we had raised was able to detect both the Nek2A and Nek2B proteins. The results showed that the antibody detected the Nek2A protein from the

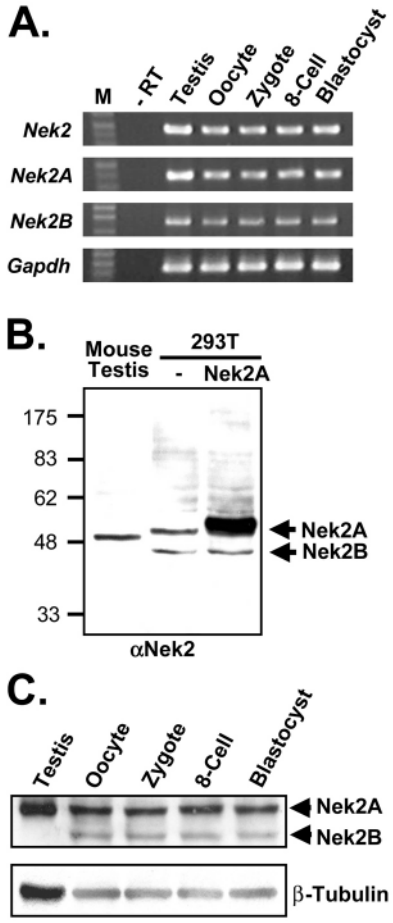


Fig. 1. *Nek2* expression in mouse early embryos. (A) RT-PCR analysis. Total RNA isolated from mouse testis, oocytes and embryos at indicated stages was reverse-transcribed followed by PCR amplification with primers specific to a common region of *Nek2A* and *Nek2B* (*Nek2*), to *Nek2A* or to *Nek2B*. *GAPDH* was detected as a control. (B) Detection of the Nek2A and Nek2B proteins. Immunoblot analysis was carried out with lysates from the mouse testis, the human 293T cell line and 293T cells transfected with *pNek2RHA1*, which encodes the HA-tagged Nek2A protein. Mouse Nek2A protein routinely appeared smaller than the human Nek2A protein. (C) Immunoblot analysis of Nek2 in mouse early embryos. Protein samples were prepared from the mouse testis, oocytes and embryos at indicated developmental stages and were subjected to immunoblot analysis with a polyclonal antibody specific to Nek2. β-Tubulin was detected as a control.

endogenous human *Nek2* as well as exogenous mouse *Nek2* genes (Fig. 1B). In addition, the human Nek2B protein was detected specifically with estimated molecular weight of 45 kDa in size (Hames and Fry, 2002). Identity of the Nek2B band was also confirmed by its disappearance in the embryos injected with the *Nek2B*-specific dsRNA (see Fig. 4C). In the testis, we detected the mouse Nek2A protein but not Nek2B protein, suggesting that the *Nek2B* mRNA is not translated efficiently in the testis or the Nek2B protein is present below detection levels. In the early embryos and oocytes, we detected both the Nek2A and Nek2B proteins (Fig. 1C). The Nek2A protein appeared more abundant than Nek2B in all experimental groups. These results are in contrast to those

reported with *Xenopus* embryos in which the Nek2B protein was present predominantly in oocytes and early embryos whereas the Nek2A protein started to appear only after the neurula stage (Uto et al., 1999).

In order to examine the Nek2 protein levels during the first mitosis of mouse embryos, we collected mitotic embryos at specific stages based on their microscopic morphology (Fig. 2A) and carried out immunoblot analysis. The results showed that levels of both the Nek2A and Nek2B proteins remained constant throughout the mitosis (Fig. 2B). These results are in contrast to a previous report where, in cultured human cells, Nek2A was destroyed upon entry into mitosis by APC/Cdc20 ubiquitin ligase whereas Nek2B was stable until late mitosis and early G1 phase (Hames et al., 2001).

Subcellular localization of the Nek2 proteins in mouse early embryos was determined immunohistochemically. The mouse zygotes that were about undergo mitosis were immunostained with the Nek2 antibody. The results showed that the Nek2 antibody stained not only the spindle poles, which were co-stained with the antibody specific to γ-tubulin but also the metaphase chromosomes (Fig. 2C). The subcellular distribution of Nek2 changed dynamically during mitosis, so that Nek2 was associated with mitotic chromosomes at prophase and metaphase and then dissociated from them thereafter (Fig. 2D). At telophase, the Nek2 protein was detected at the mid body. Nek2 appeared in association with spindle poles throughout mitosis. Such dynamic distribution of the Nek2 proteins in the first mitotic embryos is consistent with the previous observation in cultured cells (Kim et al., 2002).

Suppression of *Nek2* expression with RNAi

We chose the RNAi approach to investigate Nek2 function during mouse early embryogenesis. dsRNA specific to *Nek2* was injected into one-cell mouse embryos and *Nek2* mRNA levels were determined using RT-PCR. Double-stranded RNAs specific to *E-Cadherin* or *GFP* were also injected as controls. The results showed that the *Nek2* mRNA levels were reduced significantly within 12 hours of injection of the *Nek2*-specific dsRNA but not of the other dsRNAs specific to *E-Cadherin* or *GFP* (Fig. 3A). Such suppression lasted for at least 72 hours after injection. In accordance, *E-Cadherin* mRNA levels were reduced significantly in embryos injected with the specific dsRNA, but not with the other dsRNA specific to *GFP* or *Nek2* (Fig. 3A). The *GAPDH* mRNA level was not reduced by dsRNA injection. These results indicated that the RNAi method is an effective way to suppress specific gene expression in mouse early embryos.

Competitive PCR was carried out to determine the amount of suppression with RNAi. A fixed amount of the mutant DNA template from which a gene-specific primer set could amplify a larger PCR fragment than that from the wild-type template was added into the PCR reaction mixture along with variable amounts of reverse-transcribed cDNA from embryonic RNA samples. As a result, two specific PCR bands corresponding to the competitive DNA and the reverse-transcribed cDNA were detected (Fig. 3B). Their relative intensities reflect relative amounts of reverse-transcribed cDNA in comparison to a fixed amount of the reference mutant DNA. The results showed that for *Nek2*, addition of 2 μl reverse-transcribed cDNA from uninjected embryos produced a PCR band with intensity comparable to that of the reference mutant DNA. In contrast,

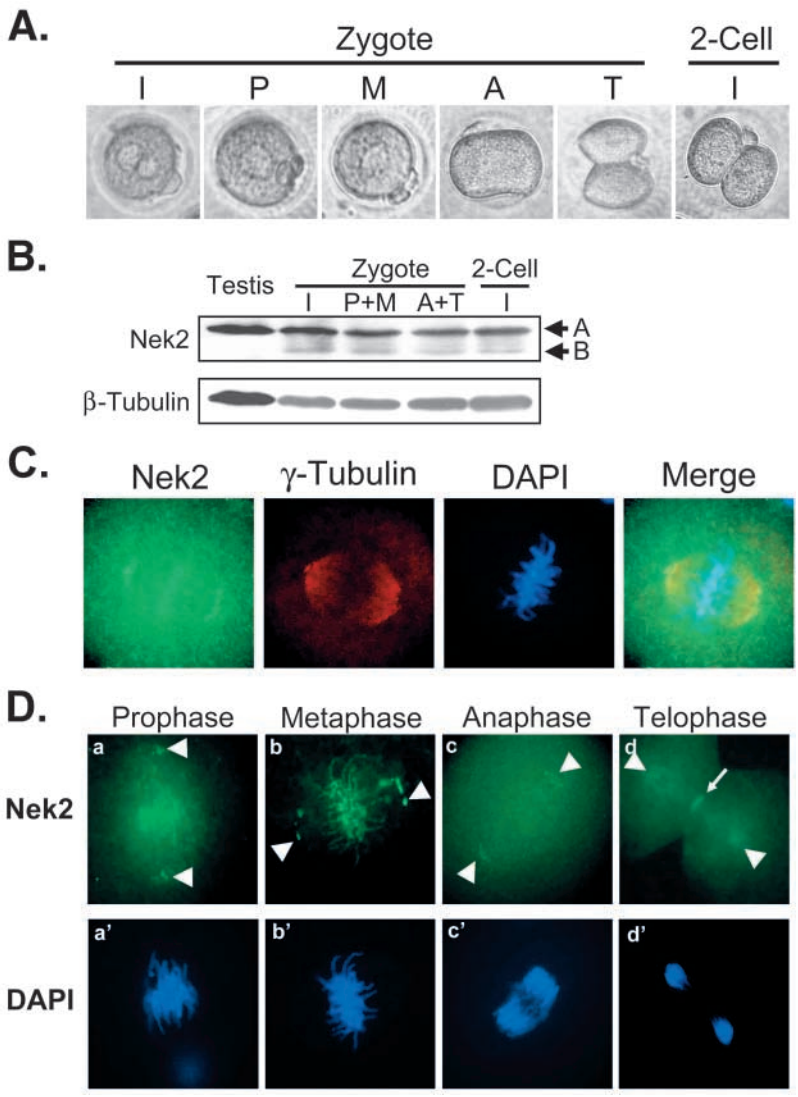


Fig. 2. Expression and localization of the Nek2 proteins during the first mitosis of the mouse embryo. (A) Mitotic stages of mouse embryos that underwent the first mitosis were determined based on their morphology under a dissecting microscope. I, interphase; P, prophase; M, metaphase; A, anaphase; T, telophase. (B) Mouse embryos were collected based on their mitotic stages and were subjected to immunoblot analysis with the Nek2 antibody. The Nek2A- and Nek2B-specific bands are indicated on the right side of the figure. β-Tubulin was used as a loading control. (C) A zygote at metaphase was co-immunostained with antibodies specific to Nek2 and γ-tubulin. DNA was stained with DAPI.
(D) Immunostaining of the Nek2 protein during the first mitosis of the mouse embryos. Zygotes that were about to undergo the first mitotic division were immunostained with the Nek2 antibody. DNA was stained with DAPI. Arrowheads indicate spindle poles and arrow indicates the mid body.

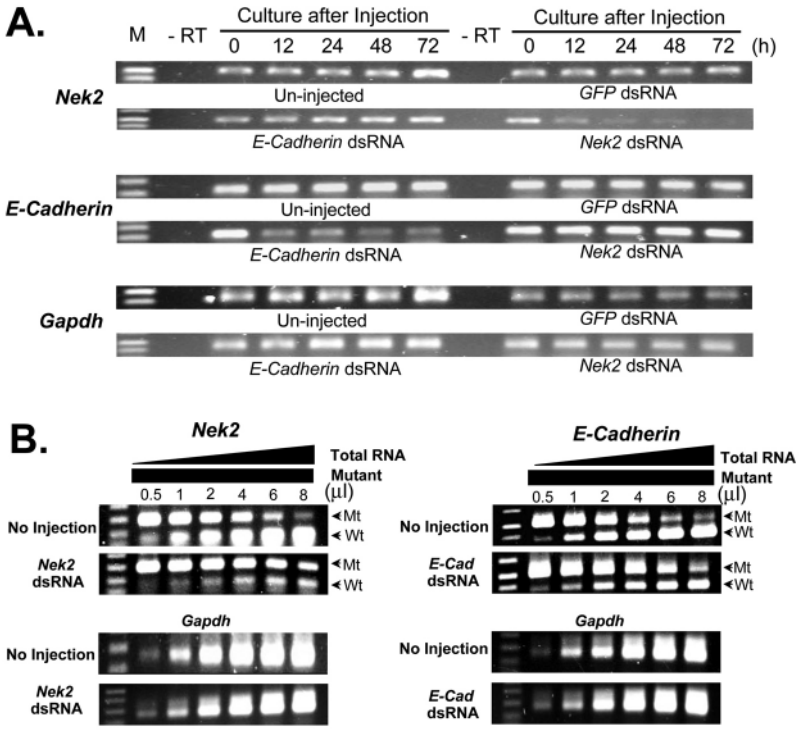


Fig. 3. Suppression of *Nek2* expression with RNAi in mouse early embryos. (A) Specific suppression of *Nek2* and *E-Cadherin* mRNA with RNAi. Double-stranded RNAs specific to *Nek2* or *E-Cadherin* were injected into zygote embryos. The *GFP* dsRNA was also injected as a non-specific control. After culture for the indicated times, the embryos were subjected to RT-PCR for detection of levels of *Nek2* and *E-Cadherin* mRNA. *GAPDH* mRNA was detected as a control for RT-PCR. (B) Suppression of *Nek2* and *E-Cadherin* expression with RNAi. Competitive PCR was used to determine suppression levels. A fixed amount of the insertional mutant DNA fragments (Mt) specific to *Nek2* or *E-Cadherin* was added to the PCR reaction mixture along with a variable amount of the reverse-transcribed cDNA (Wt). *GAPDH* mRNA was detected as a control for the RT-PCR reaction.

addition of 8 µl reverse-transcribed cDNA from *Nek2* dsRNA-injected embryos produced a band comparable to the reference band. These results indicate that the *Nek2* mRNA level was reduced about fourfold with RNAi (Fig. 3B). Similarly, 2 µl of cDNA from uninjected embryos and 6 µl of cDNA from *E-Cadherin* dsRNA injected embryos produced comparable amounts of PCR-amplified DNA in comparison to the competitive DNA, indicating that *E-Cadherin* mRNA was reduced about threefold (Fig. 3B). Such a suppression rate was somewhat less but comparable to published results (Svoboda et al., 2000) where dsRNA injections into mouse eggs suppressed specific mRNA levels by 80-90% based on their non-quantitative RT-PCR results.

In order to achieve the isoform-specific suppression of *Nek2* expression, we prepared four different *Nek2* dsRNAs that corresponded to parts of the kinase domain (Fig. 4A, fragment 1), the regulatory domain (Fragment 2), the 3′ untranslated sequences specific to the *Nek2A* mRNA (Fragment 3), and a part of coding region and the 3′ untranslated sequences specific to *Nek2B* (Fragment 4). When *Nek2* dsRNA corresponding to the domain common to the Nek2 variants (Fragment 2) was injected, both the *Nek2A* and *Nek2B* transcript levels appeared significantly reduced (Fig. 4B). When *Nek2A*-specific dsRNA (Fragment 3) was injected, only the *Nek2A* transcript level was reduced without any effect on the *Nek2B* transcript level (Fig. 4B). At the same time, the *Nek2B*-specific dsRNA (Fragment 4) solely suppressed the *Nek2B* specifically (Fig. 4B). Interestingly, the total *Nek2* transcript level, which was detected with the common domain primers (Fragment 1), was reduced with *Nek2A*, but not with *Nek2B* dsRNAs, indicating that *Nek2A* is the predominant species of the *Nek2* mRNA in mouse early embryos (Fig. 4B). We also carried out an immunoblot analysis and observed that *Nek2* dsRNAs suppressed the Nek2 isoform proteins in a sequence-specific manner (Fig. 4C).

Reduction of protein expression with the RNAi method was confirmed immunohistochemically (Fig. 4D). The E-Cadherin antibody specifically stained the cytoplasmic membrane of the blastomeres. Such a staining pattern largely disappeared in embryos injected with *E-Cadherin* dsRNA, but not in those with *GFP* or *Nek2* dsRNA. When control embryos were immunostained with antibody specific to Nek2, nuclei of the blastomeres undergoing mitosis stained strongly. Such Nek2-specific staining disappeared in embryos injected with *Nek2* dsRNA (Fragment 2) but not with dsRNAs specific to *GFP* or *E-Cadherin* (Fig. 4D). These results indicate that RNAi could suppress protein levels sufficiently to remove most of the specific staining in immunohistochemical analyses.

Mitotic defects in *Nek2*-suppressed embryos

Next, we investigated effects of *Nek2* suppression on mouse early embryogenesis. We routinely observed that over 80% of the fertilized eggs reached the blastocyst stage in a four-day culture period. Comparable rates of embryonic development were also observed in vehicle-injected or non-specific (*GFP*) dsRNA-injected eggs (Fig. 5A). On the other hand, only 33% of the fertilized eggs with *E-Cadherin* dsRNA reached the blastocyst stage, which is consistent with a previous report (Wianny and Zernicka-Goetz, 2000). In such conditions, we injected a *Nek2* dsRNA (Fragment 2) into the one-cell embryos and observed that only 25% of the eggs reached the blastocyst stage (Fig. 5A). These results indicate that Nek2 is necessary for development of the mouse embryos in the pre-implantation stage.

In order to confirm specificity of *Nek2* suppression in the embryos, we injected the four different dsRNAs used earlier. Embryonic development was blocked significantly in eggs injected with Fragments 1 or 2, which could suppress both *Nek2A* and *Nek2B* expression (Fig. 5B). Injection of the *Nek2A*-specific dsRNA (Fragment 3) also blocked embryonic development as efficiently as common *Nek2* dsRNAs (Fragments 1 and 2). However, injection of the *Nek2B*-specific dsRNA (Fragment 4) did not affect development of cultured

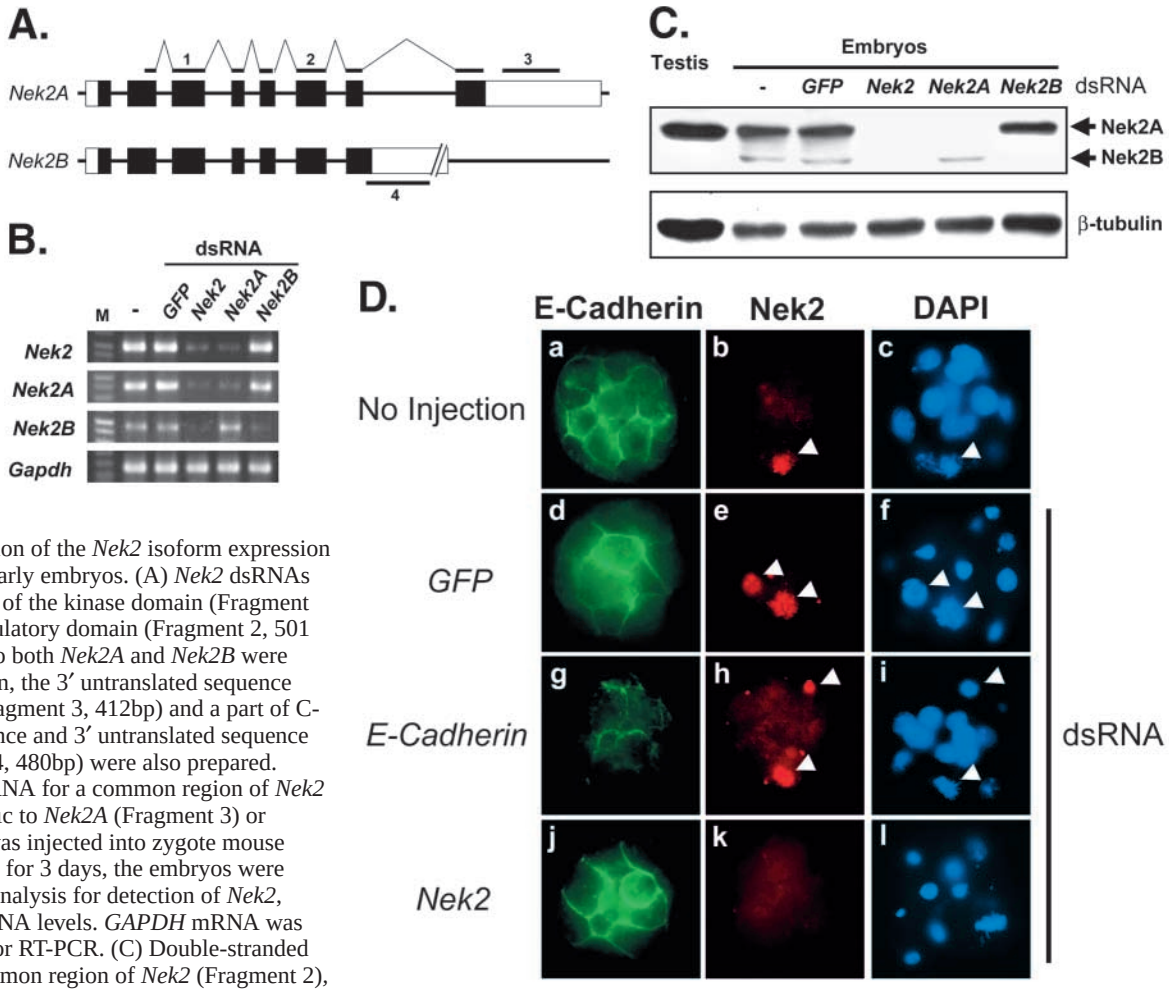


Fig. 4. Specific reduction of the *Nek2* isoform expression with RNAi in mouse early embryos. (A) *Nek2* dsRNAs corresponding to parts of the kinase domain (Fragment 1, 501 bp) and the regulatory domain (Fragment 2, 501 bp) that are common to both *Nek2A* and *Nek2B* were constructed. In addition, the 3′ untranslated sequence specific for *Nek2A* (Fragment 3, 412bp) and a part of C-terminal coding sequence and 3′ untranslated sequence for *Nek2B* (Fragment 4, 480bp) were also prepared. (B) Double-stranded RNA for a common region of *Nek2* (Fragment 2) or specific to *Nek2A* (Fragment 3) or *Nek2B* (Fragment 4) was injected into zygote mouse embryos. After culture for 3 days, the embryos were subjected to RT-PCR analysis for detection of *Nek2*, *Nek2A* and *Nek2B* mRNA levels. *GAPDH* mRNA was detected as a control for RT-PCR. (C) Double-stranded RNA specific to a common region of *Nek2* (Fragment 2), to *Nek2A* (Fragment 3) or *Nek2B* (Fragment 4) was injected into mouse zygote embryos. The *GFP* dsRNA was also injected as a non-specific control. After culture for 3 days, the embryos were subjected to immunoblot analyses for detection of the Nek2 proteins. The testis lysate was used as a control and β-tubulin was detected as a loading control. (D) One-cell embryos injected with dsRNA specific to *GFP* (d-f), *E-Cadherin* (g-i) or *Nek2* (j-l) were cultured for 72 hours and immunostained with antibodies specific to E-Cadherin and Nek2. Nuclei were stained with DAPI and mitotic nuclei were marked with arrowheads. Uninjected embryos were used as a control (a-c). The E-Cadherin antibody stained the cytoplasmic membrane of the embryonic cells, whereas the Nek2 antibody stained nuclei of the mitotic cells as marked with arrowheads.

embryos at all (Fig. 5B). These results indicate that Nek2A plays a critical role in mouse early embryogenesis.

Typical morphologies of *Nek2*- or *E-Cadherin*-suppressed eggs are shown in Fig. 6. The *E-Cadherin* dsRNA-injected embryos developed normally up to the eight-cell stage but could not undergo compaction, and, as a result, cavitation did not occur. Such phenotypes have been observed previously in *E-Cadherin*-null embryos (Larue et al., 1994) and in *E-Cadherin*-suppressed embryos with RNAi (Wianny and Zernicka-Goetz, 2000). In the case of the *Nek2* dsRNA-injected eggs, developmental abnormality was seen as early as the four-cell stage (Fig. 6). The size of the blastomeres appeared uneven and there were usually fewer than four blastomeres in a single embryo. These results indicated that *Nek2*-suppressed embryos have defects beginning at the second mitotic division.

To have a better understanding of morphological characteristics of the *Nek2*-suppressed embryos, we visualized cellular microtubules and MTOC with antibodies specific to β-tubulin and γ-tubulin, respectively (Fig. 7A). Distinct MTOCs near nuclei were observed in blastomeres of the control embryos (Fig. 7A) (Houliston et al., 1987; Meng et al., 2004). Spindle poles in the mitotic blastomere looked focused with typical spindle arrays (Fig. 7A). However, we could not detect any discrete MTOC structure in the *Nek2*-suppressed embryos. Furthermore, abnormal spindle structures, such as multipolar and disorganized spindles, were observed in the mitotic blastomeres (Fig. 7A). In fact, there was a significant increase in number of mitotic blastomeres in *Nek2*-suppressed embryos (Fig. 7B). These results suggest that abnormal spindle structures in the *Nek2*-suppressed embryos cause a delay in the progression of the M phase of the cell cycle.

To determine chromosomal defects in the *Nek2*-suppressed embryos, we stained the embryos with DAPI (Fig. 7C). Normal embryos contained blastomeres with identically sized nuclei and one or two polar bodies (Fig. 7C panel a). On the other

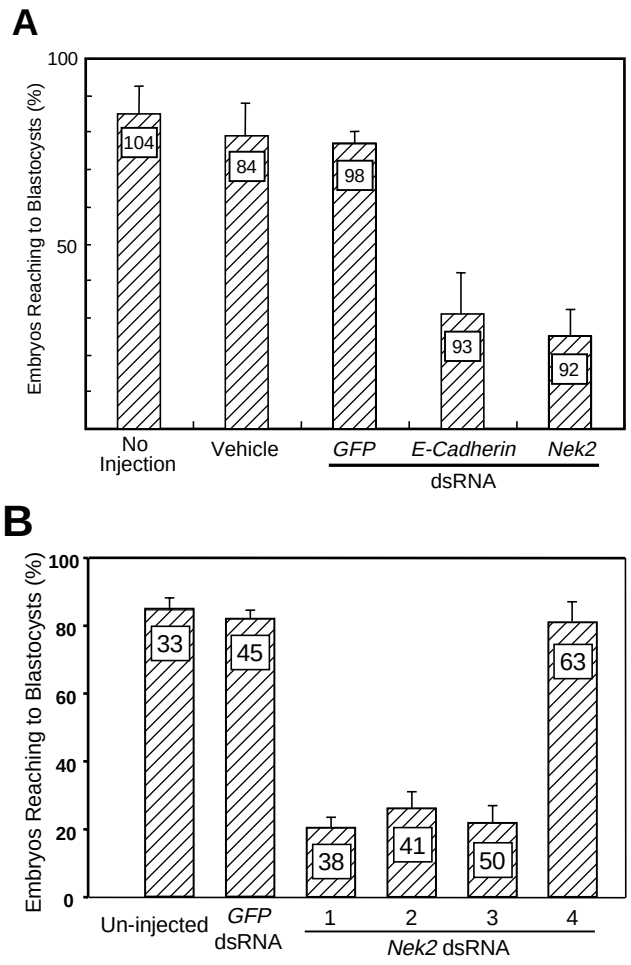


Fig. 5. Effects of dsRNA specific to *Nek2* and *E-Cadherin* on mouse early embryo development in culture. (A) One-cell embryos injected with dsRNA specific to *GFP*, *E-Cadherin* or *Nek2* were cultured for 4 days and the number of embryos reaching the blastocyst stage was counted. Uninjected and vehicle-injected groups were included as controls. Experiments were repeated five times. Data are presented as mean±s.e.m. The number of embryos used for each experimental group is indicated within the bar. (B) Four kinds of *Nek2* dsRNAs (see Fig. 4A) were injected into mouse one-cell embryos. Four days later, the number of embryos reaching the blastocyst stage was counted. *GFP* dsRNA was used as a control. Experiments were repeated five times and data are presented as mean±s.e.m. The number of embryos used for each experimental group is indicated within the bar.

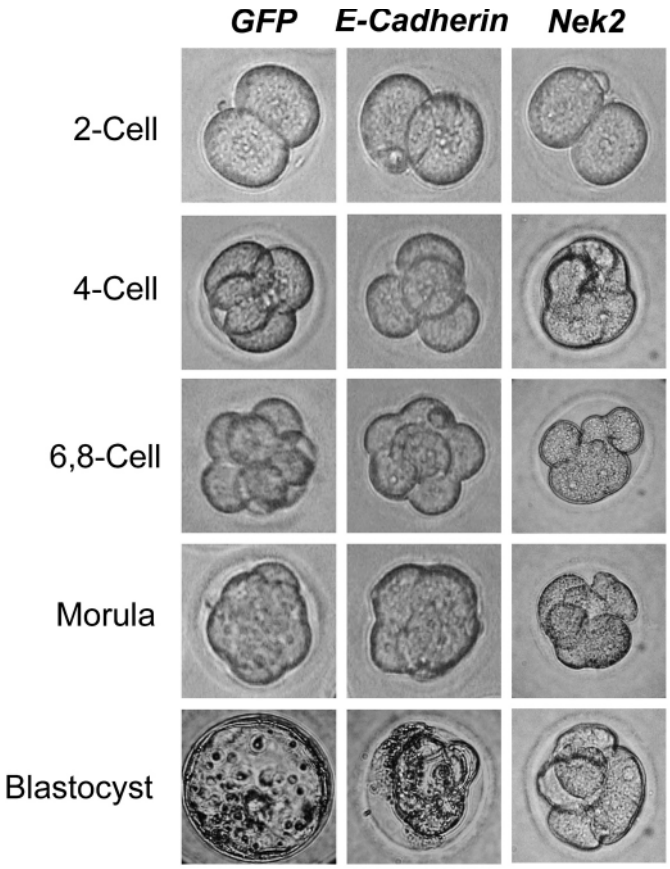


Fig. 6. Morphological phenotypes of the *Nek2*-suppressed embryos. One-cell embryos that had been injected with dsRNA specific to *GFP*, *E-Cadherin* or *Nek2* were cultured for up to 4 days, and embryos with typical morphologies were photographed at the indicated developmental stages.

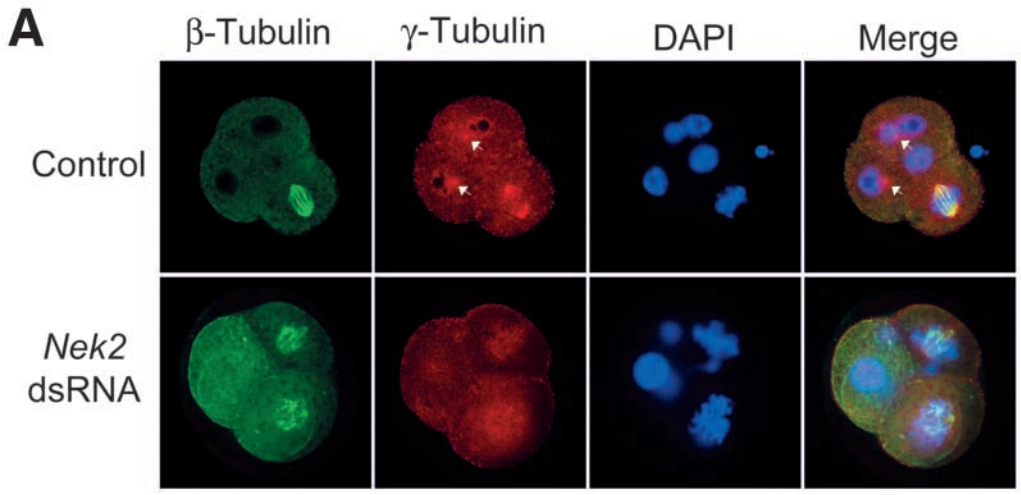
hand, many of the blastomeres in *Nek2*-suppressed embryos showed abnormal nuclear morphology, including dumbbell-like nuclei, nuclear bridges and micro-nuclei (Fig. 7C panels b,c). Such chromosomal defects might result from incomplete chromosomal segregation and chromosomal disjunction of the *Nek2*-suppressed embryos (Cutts et al., 1999; Liu and Erikson, 2002).

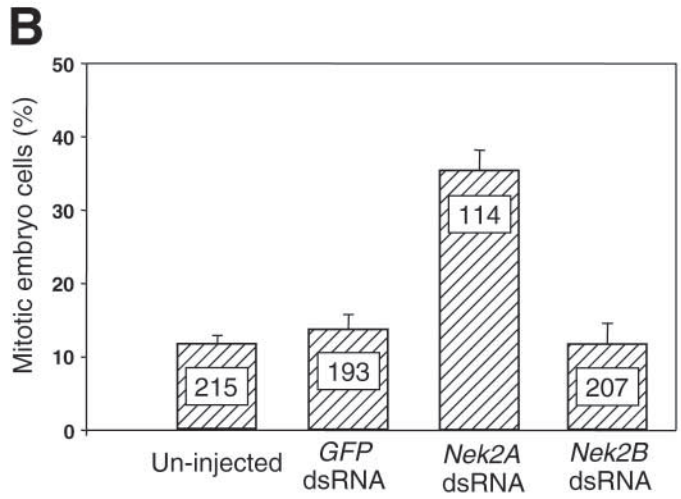
Discussion

In the present study, we report that suppression of *Nek2* expression by RNAi resulted in developmental defects at the second mitosis of the mouse early embryos. Many blastomeres in the *Nek2*-suppressed embryos were arrested at M phase with abnormal spindle structures. These results confirm that Nek2 is essential for embryonic mitosis, especially for proper segregation of chromosomes.

Phenotypes of *Nek2*-suppressed mouse embryos are comparable to those of the *Xenopus* embryos in which the Nek2 activity was reduced by microinjection of the mRNA for kinase-inactive form of *Nek2B* or of the Nek2 antibody (Uto and Sagata, 2000). Depletion of Nek2 resulted in fragmentation or dispersal of the centrosomes and eventually in interference of embryonic development of both species. In addition, our results are consistent with a previous report in which removal of the Nek2 proteins from *Xenopus* egg extracts did not disturb entry into mitosis and accompanying condensation of chromosomes (Fry et al., 2000). Rather, Nek2 appeared to be critical for segregation of the chromosomes.

Nek2 depletion caused abortive cleavage of both the mouse and *Xenopus* embryos with abnormal spindle formation, but we also identified features distinct to the mouse embryo. First, development of the *Nek2*-suppressed mouse embryos stopped at the second mitotic division, whereas development of the Nek2-depleted *Xenopus* embryos stopped at the 16- or 32-cell stage with irregularly sized blastomeres (Uto and Sagata,





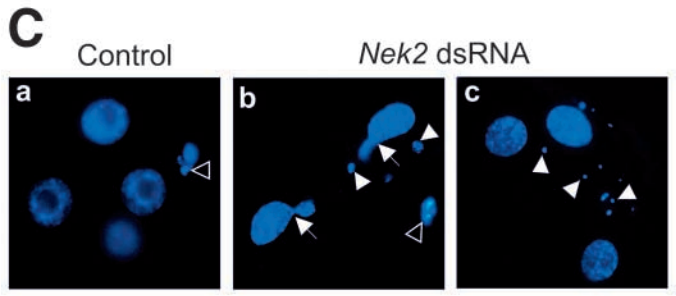


Fig. 7. Mitotic abnormalities in the *Nek2*-suppressed mouse embryos. (A) Control and *Nek2*-suppressed embryos were immunostained with antibodies specific to β-tubulin and γ-tubulin. Nuclei were stained with DAPI. Arrowheads indicate typical MTOCs observed in the control embryo. (B) The number of mitotic blastomeres was counted in uninjected embryos and in embryos injected with dsRNA specific to *GFP*, *Nek2A* or *Nek2B* and the proportions calculated. The experiments were repeated five times and data are presented as mean±s.e.m. The number of embryos used for each experimental group is indicated within the bar. (C) Nuclei of the *Nek2*-suppressed embryos (b,c) were stained with DAPI and compared with those of the uninjected control embryos (a). In the *Nek2*-suppressed embryos, abnormal segregations were observed such as dumbbell-like nuclei (arrows) and micronuclei (arrowheads). Nuclei of the polar body are indicated with open arrowheads.

2000). Such a difference might largely stem from different developmental processes of the mammalian and amphibian embryos. After fertilization, *Xenopus* embryos divide synchronously up to 12 cycles within several hours (Masui and Wang, 1998). Aborted cleavage of the Nek2-depleted *Xenopus* embryos, therefore, must be observed within a few hours after injection (Uto and Sagata, 2000). On the other hand, cleavage of mouse embryos proceeds much more slowly, so that each division occurs once in 12 hours. Therefore, it would appear to take longer for the *Nek2*-suppressed mouse embryos to reveal typical phenotypes of the second mitotic arrest than of the Nek2-depleted *Xenopus* embryos at the 16- or 32-cell stage.

Another difference was the presence of both the Nek2A and Nek2B proteins in mouse oocytes and throughout early embryos, whereas Nek2B was a sole Nek2 protein in *Xenopus* oocytes and early embryos (Uto et al., 1999). As Nek2A but not Nek2B was labile upon mitosis, it was proposed that maternal Nek2B might play a critical role in *Xenopus* early embryogenesis in which maternal gene products are needed for successive cell divisions until the mid-blastula transition (Uto et al., 1999). Mouse early embryos in which zygotic transcription is known to start from the two-cell stage may not need to reserve the maternal Nek2B proteins for subsequent mitosis. Furthermore, in contrast to the cultured human cells (Hames et al., 2001), embryonic Nek2A appeared intact upon entry into mitosis, suggesting that the Nek2A protein level is controlled in a unique manner during mouse early embryogenesis. Our results also indicated that Nek2B is not critical for cleavage of the mouse early embryos.

Spindle poles in mouse early embryos are known to have special features distinct from those in other cell types. Notably, centrioles are absent and normal looking centrioles become detectable later at the blastocyst stage (reviewed by Delattre and Gonczy, 2004). This led us to question the specific roles of Nek2 in spindle pole formation and chromosome segregation during mouse early embryogenesis. It is possible that Nek2 is involved in other regulatory functions, in addition to regulation of centriole-centriole linkage. Recently, Nek2 was proposed to cause recruitment of centrosomal proteins, including γ-tubulin and C-Nap1 (Faragher and Fry, 2003; Twomey et al., 2004). Indeed, there may be additional

substrates that are responsible for centrosomal functions of Nek2.

Ectopic expression of kinase-inactive Nek2A interfered with proper bipolar spindle formation and eventually resulted in chromosomal segregation defects such as unaligned chromosomes in metaphase, lagging chromosomes in anaphase and thin chromatin bridges in telophase (Faragher and Fry, 2003). Assuming that the kinase-inactive Nek2A functioned in a dominant-negative manner, the authors concluded that Nek2 is required for proper chromosome segregation. Consistent with this report, we observed chromosomal segregation defects in Nek2-depleted mouse embryos. Such segregation defects would result from problems in centrosome function (Faragher and Fry, 2003). However, we cannot rule out the possibility that they might be attributed to improper spindle checkpoint regulation caused by Nek2 depletion (Lou et al., 2004). It would be interesting to examine how chromosome segregation can be regulated by Nek2.

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