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Nek2A Contributes to Tumorigenic Growth and Possibly Functions as Potential Therapeutic Target for Human Breast Cancer

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ABSTRACT

Nek2A (NIMA-related kinases 2A) has been known as an important centrosome regulatory factor. The aim of this study was to investigate the expression of Nek2A and the role it played in different stages of breast cancer. We detected the expression of Nek2A in both mRNA and protein levels in MCF10 cell lines including MCF-10A, MCF-10DCIS.com, MCF-10CA1a and in human breast samples which contained normal breast tissue (NBT), breast ductal carcinoma in situ (DCIS), and invasive ductal carcinoma (IDC). Our study revealed that the mRNA and protein expression of Nek2A were significantly up-regulated in MCF-10DCIS.com and MCF-10CA1a cell lines as well as in human primary breast cancer tissue (DCIS and IDC). Our study also presented a correlation between Nek2A mRNA expression and some clinic pathological factors. We found that Nek2A mRNA expression was associated with molecular subtypes, ER, PR and Ki-67 immunoreactivity ($P < 0.05$) in DCIS and associated with histological grade, lymph node metastasis, molecular subtypes, c-erbB-2, and Ki-67 expression ($P < 0.05$) in IDC. In addition, we observed that ectopic expression of Nek2A in "normal" immortalized MCF-10A breast epithelial cell resulted in increased Nek2A which lead to abnormal centrosomes. Furthermore, knockdown of Nek2A in MCF-10DCIS.com could remarkably inhibit cell proliferation and induce cell cycle arrest in MCF-10DCIS.com cell line. These data suggested that Nek2A might bear a close relationship with development and progression of breast carcinoma, and highlighted its role as a novel potential biomarker for diagnosis and a possible therapeutic target for human breast cancer especially for DCIS. J. Cell. Biochem. 113: 1904–1914, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: Nek2A; BREAST CANCER; DCIS; IDC; MCF10 CELL LINES

NIMA-related kinase2A (Nek2A) is a serine/threonine kinase located at centrosome and regulates its separation. Nek2A is one of the main splice variants of Nek2 [Wu et al., 2007]. It regulates centrosome cohesion and separation through phosphorylation of structural components of the centrosomes [Bahmanyar et al., 2008]. Previous studies provided confirmed evidence that Nek2A had an important function in maintaining centrosome structure, and together with other protein kinases such as Cdk1, Plk1, and Aurora-A, Nek2A also greatly involved in mitotic progression. As a result, the altered activity of Nek2A leads to aneuploid defects which can be observed in kinds of malignancies.

Abbreviations: Nek2A, NIMA-related kinases 2A; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma in situ; NBT, normal breast tissue; siRNA, small interfere RNA.

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It has been established that abnormal centrosome was a distinct feature of most cancer cells. The vast majority (80–100%) of breast tumors displays abnormal centrosome [Guo et al., 2007]. When compared to normal breast tissue (NBT), breast carcinoma cells have a much higher frequency of centrosome defects [Lingle et al., 1998; Guo et al., 2007], including amplification of number, increased volume, and supernumerary centrioles. Furthermore, the result of our former study [Niu et al., 2009] has demonstrated that centrosomal aberrations might play key roles in the early stage of breast tumorigenesis. During interphase, centrosomes are joined together by the linker proteins C-Nap1 and rootletin [Yang et al., 2006]. While at the onset of mitosis, these linker proteins are phosphorylated and displaced from centrosomes by the Nek2A, and then centrosomes were separated exactly. Though the hypothesis that dysfunction of Nek2A may lead to centrosome abnormalities (CA) which may contribute to the development of breast cancer has been proposed in some literatures, it is still far from demonstrating a causal relationship between CA and mammalian tumorigenesis.

A comprehensive and consistent picture of the genetic changes that underlie breast cancer initiation, development, and progression remains unresolved. The MCF10 model includes series of cell lines which featured as "normal" immortalized breast cell (MCF-10A), carcinoma in situ (MCF-10DCIS.com) and invasive carcinoma (MCF-10CA1a), and the three cell lines can resemble the initiation, development, and progression steps of breast carcinoma, respectively. Their cytogenetic and molecular variation may help to reveal genetic changes relevant to breast cancer [Worsham et al., 2006]. MCF-10A (a non-transformed, near diploid, spontaneously immortalized human mammary epithelial cell line) has been used as "normal" immortalized breast epithelium controls [Vazquez-Martin et al., 2008] for studies of human breast cell lines.

Most of previous researches about Nek2A conducted in the cell lines of breast invasive ductal carcinoma (IDC), while few studies conducted in the cell lines of ductal carcinoma in situ (DCIS) or in human breast samples. In our study, we detected the mRNA and protein expression of Nek2A not only in IDC cell line (MCF-10CA1a), but also in DCIS cell line (MCF-10DCIS.com) and "normal" breast cell line (MCF-10A), as well as NBT, DCIS, and IDC tissues. In addition, by induced ectopic expression of Nek2A in "normal" immortalized MCF-10A breast epithelial cells, we found that increased Nek2A contribute to abnormal centrosomes. Furthermore, knockdown of Nek2A expression in MCF-10DCIS.com could remarkably inhibits cell proliferation and induce cell cycle arrest. This research can give some information of Nek2A expression in patients' tissue and suggest that it may bear a close relationship with development and progression of breast carcinoma. And our study provided the clue to explore Nek2A as a novel potential biomarker for diagnosis and a new target for therapeutic intervention for human breast cancer especially for DCIS.

MATERIALS AND METHODS

TISSUE SPECIMENS

All cases of breast surgical specimens, formalin-fixed and paraffin-embedded, were anonymized after collection from the archival file of the Breast Pathology Department, Tianjin Tumor Hospital. In situ hybridization (ISH) of Nek2A and immunohistochemistry (IHC) of Nek2, oestrogen receptor (ER), progesterone receptor (PR), c-erbB-2, Ki-67, and p53 were performed in 66 cases of DCIS, 66 cases of IDC and 66 cases of NBT which were randomly selected from patients who were admitted during January 2008 to December 2009. And tissues of 66 NBT cases were obtained from the quadrant far away from the original foci in the surgical specimens of 66 cases of IDC as control. The pathologic diagnosis was confirmed by two senior pathologists (Y.N. and N.L.) according to the 2003 WHO histological classification of tumors of the breast (Tavassoli and Devilee, 2003). Tissue collection and analysis in this study were approved by the Ethical Committee of Tianjin Medical University Tumor Hospital, China.

IN SITU HYBRIDIZATION (ISH)

To detect the mRNA expression of Nek2A, specific dignoxigenin (DIG)-labeled oligonucleotide probe was generated. The sequence of Nek2A probe was 5′-TCTTAATTACTGAGGATGGAAGATTAA-GAAGTTCTGGATTACTTGCCA-3′. ISH analysis was preformed according to the manufacturer's instruction. Briefly, 4-μm paraffin sections were dewaxed and rehydrated, dipped in 0.2 M hydrochloric acid for 10 min, pretreated with proteinase K compound digesting solution for 5 min at 37°C. Then the sections were incubated at 37°C for 2 h with pre-hybridization working solution provided by the ISH kits (HanYang Biotech Co.), followed by pipetting the hybridization solution containing Nek2A probe on the sections. Hybridization was performed in a humidity chamber at 37°C and incubated overnight. After hybridization, the sections were rinsed in 2 × SSC for 5 min, three times, 0.2 × SSC for 5 min, three times, 0.1 M Tris-buffered saline (TBS) for 5 min, three to five times. Thereafter the sections were incubated with biotinylated anti-Digoxin (DIG) antibody, and subsequently high sensitivity avidin-biotin peroxidase complex at 37°C for 30 min. The hybridization signal was developed in the dark with DAB. Positive controls (tumor known to be positive for Nek2A expression) and negative controls (using known positivity sample digested with RNase or using hybridization solution without a probe) were included in every test. The sections were observed by light microscopy. Five high-power fields (×400) were chosen per case and 150–200 cells were counted for each power field. Nek2A was expressed in the cytoplasm. Five areas of every slide were chosen randomly, and we counted 150–200 carcinoma cells per area. The scoring system was modified and used according to evaluation standards [Tanaka et al., 2000]. The percentage of the staining cells (P) was scored as follows: 0 (staining of $<5\%$ of cells), 1 (5–25% of cells), 2 (25–50%), 3 (50–75%), and 4 (75–100%). Immunoreactivity intensity (I) was graded as follows: 0 (no reactivity), 1 (weak reactivity), 2 (moderate reactivity), 3 (intense reactivity). Samples in each power field were evaluated for both factors, that is, P multiplied by I. The scoring of each case was a mean value of that of five fields. Eventually the sections were graded as follows: (−, negative) scoring 0–1, (+) scoring 2–5, (++) scoring 6–8, (+++) scoring over 8 and +, ++, +++ were defined as positive.

IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry was performed using the labeled streptavidin–biotin technique with antibodies against Nek2, Ki-67, ER, PR, c-erbB-2, and p53 in all cases. Briefly, tissue sections were deparaffinized and rehydrated with xylene and a series of grades of alcohol. Antigen retrieval was carried out in 5 mM citrate buffer (pH 6.0) or EDTA (pH 9.0) for 2 min in an autoclave, followed by cooling at room temperature for 45 min. After inactivation of endogenous peroxidase with 3% H_2O_2, sections were blocked with 10% normal goat serum for 30 min and then incubated with either anti-Nek2 antibody (1:100 dilution; Abcam, UK) or anti Ki-67 antibody (1:75; Zymed) at 4°C overnight or with either anti-ER antibody (1:150; Zymed) or anti-PR antibody (1:150; Zymed) or anti-c-erbB-2 antibody (1:600; Newmarker) anti-p53 antibody (1:100; Zymed) at 37°C for 2 h. Normal mouse serum served as a negative control. After incubation with biotin-conjugated secondary antibody for 20 min at 37°C, and streptavidin–horseradish peroxidase (Zymed) for 20 min at 37°C, colour was developed by incubation with 3, 3′-diaminobenzidine tetrahydrochloride (DAB). The sections were counterstained with haematoxylin. All steps were preceded by rinsing with phosphate-buffered saline (PBS; pH 7.6).

Nek2 was expressed in the cytoplasm and/or nuclear. The evaluation standards of Nek2 cytoplasm expression was the same as that described in ISH part and the nuclear reactivity was valuated by the percentage of tumor cells. Ki-67 immunoreactivity was valuated by the percentage of tumor cells with nuclear reactivity counted across five representative fields (−, <10% of cells reactive; +, 10–25% of cells reactive; ++, 26–49% of cells reactive; +++, >50% of cells reactive). According to guidelines [Wolff et al., 2007; Hammond et al., 2010] published by American Society of Clinical Oncology, ER/PR negativity by immunohistochemical (IHC) analysis was defined as <1% nuclear staining and c-erbB-2 positive was defined as strong membrane staining in >30% of the tumor cells. Breast carcinoma were classified into five molecular subtypes [Rouzier et al., 2005] including: luminal A (ER+ and/or PR+, c-erbB-2−), luminal B (ER+ and/or PR+, c-erbB-2+), triple-negative (ER−, PR−, c-erbB-2−) and HER2/neu(ER−, PR−, c-erbB-2+).

CELL CULTURE

MCF-10A ("normal" immortalized breast epithelial cell line) and MCF-10CA1a (malignant breast epithelial) cell lines were purchased from Barbara Ann Karmanos Cancer Institute Wayne State University. MCF-10DCIS.com (carcinoma in situ) was purchased from Asterand business development representative. MCF-10A cell was cultured in DMEM: Ham's F-12 supplemented with 5% horse serum, 10 mm HEPES buffer, 10 ng/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 0.5 mg/ml hydrocortisone. MCF-10DCIS.com and MCF-10CA1a cell lines were cultured in DMEM: Ham's F-12 with 5% horse serum. Cells were fed twice a week and were passaged on a weekly basis. They were maintained at 37°C in a humidified atmosphere of 5% CO_2. All the cell lines were passaged for <6 months in this study.

PLASMID CONSTRUCTION, PREPARATION OF siRNA, AND TRANSFECTION

Nek2A expression plasmid (pcDNA-mycNek2A) was generated by cloning its coding region into the pcDNA3.0-myc vector (Invitrogen, Beijing, China). And the pcDNA3.0-myc vector was used as control group. The sense and antisense strands of Nek2A small interfering RNA (siRNA) obtained from Invitrogen were shown in Table I (3#:HSS107083, 4#:HSS107084, 7#:HSS181487). Negative control siRNA (Invitrogen, cat number 12935–300) was used as control. Seventy-two hours after transfections using the Lipotectamin PLUS transfection reagent (Invitrogen), cells were harvested and used for further experiments.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION, REAL-TIME REVERSE TRANSCRIPTASE PCR

Total RNA was extracted with the TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. The first-strand complementary DNA was synthesized with oligo (dT) primer by using the Reverse Transcription System (Promega Biotech, Beijing, China). Five nanogram of complementary DNA were used in real-time polymerase chain reaction (PCR) analysis with the SYBR Premix Ex Taq Kit (TaKaRa Biotechnology, Dalian, China) on the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Beijing,

TABLE I. List of Sequences of PCR Primers and siRNA Used

PCR primer	Forward (5′–3′)	Reverse (5′–3′)
Nek2A(RT-PCR)	TCTTAATCTTCCATCCTCAG	CTATACAGAAAGGCATGGCT
Nek2A (real-time RT-PCR)	AAACTTAAAGGATTACCATCGACC	GCCAGTTTGTCCTCTGCTAGTCT
Ki-67	GTGGGCACCTAAGACCTGAAC	ACTCTTGTTTTCCTGATGGTTGA
TGFB2	TGTGGAAATGGATACACGAACC	CGCAGCAAGGAGAAGCAGAT
IL8	GCATACTCCAAACCTTTCCACC	GCAACCCTACAACAGACCCAC
OPA1	TCTAAACCATTGTAACCTTTGTCG	TCTTCTTCTCACCATCTTCAGCA
DLGAP5	AAGAATTGCAGCGAGAAATCG	TTCTGGTGATGTAGTTTGTTGTGG
PTP4A1	TGTTGTATTGCTGTTCATTGCG	ACAGTTGTTTCTATGACCGTTGG
GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTTGCTGTAGCCAAATTCGTTGT
siRNA	**Sense (5′–3′)**	**Antisense (5′–3′)**
HSS107083 (3#)	AUAAUCCGAUCAUAGUAACGAACGA	UCGUUCGUUACUAUGAUCGGAUUAU
HSS107084 (4#)	UUUGUAAUUACACUAGCCAGAUCCC	GGGAUCUGGCUAGUGUAAUUACAAA
HSS181487 (7#)	UGCACUUGGACUUAGAUGUGAGCUG	CAGCACAUCUAAGUCCAAGUGCA

siRNA, small interfere RNA.

China). Each reaction was carried out in duplicate. The expression of GAPDH was used as the internal control. Gene-specific primers were listed in Table I. The reaction products were subjected to computer-assisted densitometry after electrophoresis on a 2% agarose gel and staining with ethidium bromide.

WESTERN BLOTTING

Seventy-two hours after transfections with plasmid or siRNA, cells were harvested and washed with ice-cold phosphate buffer. Cells were lysed in the RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris–HCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, and 1 μmol/L phenylmethylsulfonyl fluoride). Protein concentrations were determined by BCA method (Thermo, pierce Biotechnology). Proteins were resolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transferred onto polyvinylidene fluoride membranes (Millipore, New Bedford, MA). The membranes were incubated at 4°C overnight with primary antibodies for Nek2 (1:500, Sigma), γ-tubulin (1:1,000, Sigma), myc (1:2,000, Cell Signaling Technology), and β-actin (1:2,000, Santa Cruz). After being incubated with the respective second antibody, immune complexes were detected by ECL (Thermo, Pierce Biotechnology) Western blotting reagents. The expression of β-actin was used as the internal control.

INDIRECT IMMUNOFLUORESCENCE MICROSCOPY

Cells grown on glass coverslips were fixed with cold methanol for 5 min. Cells were then blocked by 2% bovine serum albumin (BSA) in PBS for 30 min. Cells were then fixed and double-stained with anti-myc (1:2,000; Cell Signaling Technologies) or anti-γ-tubulin (1:1,000, Sigma) or anti-centrin2 antibodies (1:50, Santa Cruz). Then cells were incubated with Alexa Fluor 594 goat anti-mouse or Alexa Fluor 488 goat anti-rabbit (1 g/ml; Invitrogen) as the secondary antibodies, followed by staining with 4′, 6-diamidino-2-phenylindole (DAPI; Biosource) for 5 min. Coverslips were then mounted with antifading mount media (Beyotime, China) and examined by Leika fluorescence microscopy.

SULFORHODAMINE B STAINING (SRB)

Cells were seeded at 2×10^4 cells per well in 24-well plates. After various days, cells were fixed with 50% trichloroacetic acid and stained with 0.4% SRB dissolved in 1% acetic acid. The cells were then washed with 1% acetic acid to remove unbound dye. The protein-bound dye was extracted with 10 mM Tric–HCl base to determine the optical density at 490 nm wavelength.

IMMUNOCYTOCHEMISTRY (ICC)

Cells were fixed with ice acetone for 20 min followed by being washed three times with PBS. The preparations were incubated in a 3% H_2O_2 for 10 min in order to quench endogenous peroxidase activity. Cells were blocked with 5% goat serum for 20 min at room temperature then incubated overnight at 4°C with the anti-Ki-67 antibody (1:75; Zymed) or anti-Nek2 antibody (1:100, abcam, UK) while the PBS was served as negative controls. After incubation with biotin-conjugated secondary antibody for 20 min at 37°C and then streptavidin–horseradish peroxidase (Zymed) for 20 min at 37°C, color was developed by incubation with DAB and counterstained with hematoxylin. All steps were preceded by rinsing with PBS. Five areas of every slide were chosen randomly and counted 150–200 carcinoma cells per area to obtain a mean value of positive immunostaining cells.

FLOW CYTOMETRY

Cell-cycle distributions were confirmed by flow cytometry. Cells were harvested 72 h after transfections with plasmid or siRNA and fixed in 70% ethanol and stored at −20°C. They were then suspended with 5 ml cold PBS and then centrifuged, followed by staining of the DNA with propidium iodide (50 mg/ml) at 4°C for 30 min in the dark. Then, each sample was analyzed using a BD FACS caliber and the proportion (percentage) of cells within the G1, S, and G2/M phases of the cell cycle was determined.

GENETIC EXPRESSION PROFILING DETECTION

RNA of Nek2A siRNA (3# and 4#) and control siRNA transfection MCF-10DCIS.com cell lines were extracted with TRIZOL reagent (Invitrogen, Gaithersburg, MD) and purified with the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA quality was assessed by formaldehyde agarose gel electrophoresis and quantified spectrophotometrically. Human Genome U133 plus 2.0 Array Gene Chips was hybridized and analyzed by CapitalBio Corporation (Beijing, China). Methods for RNA extraction, probe labeling reactions, and microarray hybridization were as described previously [Feng et al., 2010]. A gene was considered to be significantly differentially expressed, if the ratio of the expression level in the Nek2A siRNA transfection groups to the control group was more than twofold (or lower than 0.5).

STATISTICAL ANALYSIS

The SPSS 15.0 software package was used for statistical analysis. Mann–Whitney *U*-test, Kruskal–Wallis test were performed for group comparisons, One-way analysis of variance (ANOVA) and Student's *t*-test were performed for continuous variable and correlations between two variables were evaluated by Spearman's rank correlation. All data were presented as means of three independent experiments. The results were considered significant when $P < 0.05$.

RESULTS

Nek2A mRNA AND Nek2 PROTEIN EXPRESSION WERE ELEVATED IN MCF-10DCIS.COM AND MCF-10CA1a CELL LINES COMPARED WITH MCF-10A CELL LINE

We examined the mRNA expression of Nek2A in the cell lines of MCF-10A, MCF-10DCIS.com, and MCF-10CA1a by RT-PCR. The increased expression of Nek2A was observed in breast carcinoma cell lines (MCF-10DCIS.com and MCF-10CA1a), whereas it was hardly detectable in "normal" immortalized breast epithelial cell line MCF-10A (Fig. 1A). Which was confirmed by our following finding in Western blotting that was carried out to detect the protein expression of Nek2 in breast carcinoma cell lines (Fig. 1B). As shown in Figure 1A,B, the above results indicated that expression level of Nek2A and Nek2 were raised, at least in part, at the transcriptional level in a tumor-specific manner.

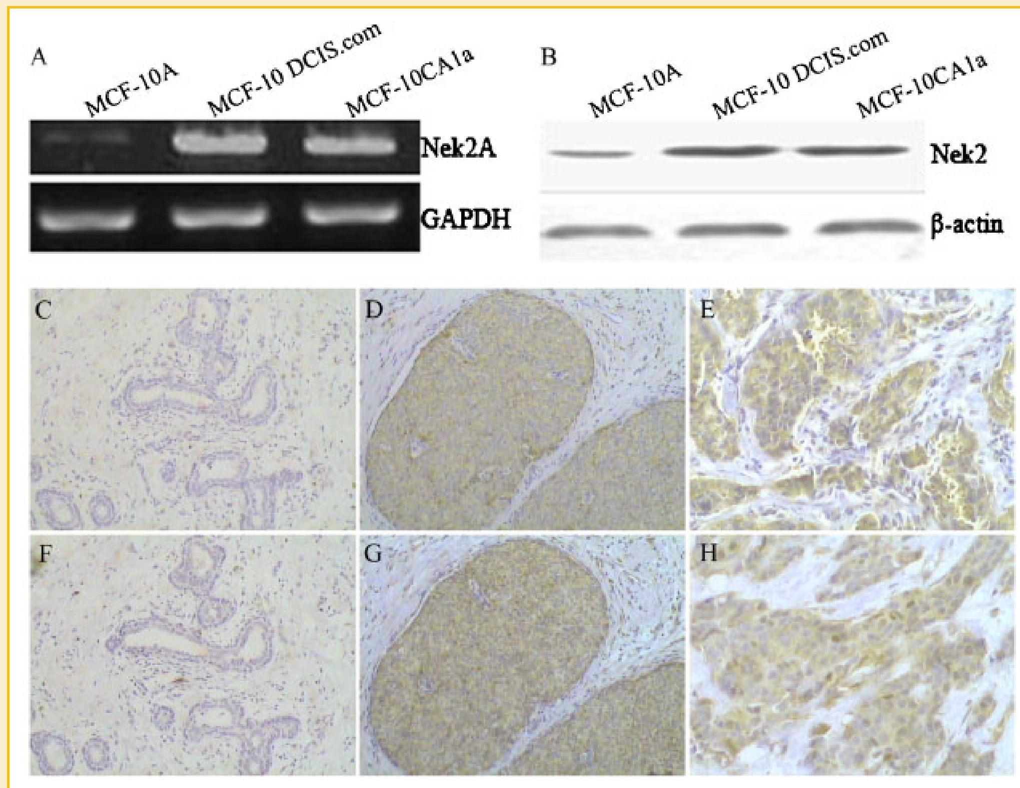

Fig. 1. The expression analysis of Nek2 and Nek2A in MCF10 cell lines and human breast samples. A: Compared with MCF-10A, the expression of Nek2A mRNA (340 bp) was increased in MCF-10DCIS.com and MCF-10 Ca1 detected by RT-PCR, and GAPDH (127 bp) serves as an internal control. B: Nek2 protein expression was higher in MCF-10DCIS.com and MCF-10 Ca1 than MCF-10A cell line by Western blotting. C–E: Nek2A mRNA was expressed in the cytoplasm of tumor cells by in situ hybridization (ISH). And Nek2A mRNA expression was higher in DCIS (D) and IDC (E) than NBT (C). F–H, Nek2 protein expressed in the cytoplasm and/or nucleus of tumor cells by IHC (immunohistochemical), Compared with NBT (F), the expression of Nek2 was increased in DCIS (G), and IDC (H). C–H, original magnification ×200. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Nek2A mRNA AND Nek2 PROTEIN WERE OVER-EXPRESSED IN HUMAN BREAST CANCER TISSUES AND THE EXPRESSION CORRELATES WITH CLINICOPATHOLOGICAL PARAMETERS

Compared with NBT (Fig. 1C), mRNA expression of Nek2A was increased in DCIS (Fig. 1D), and IDC (Fig. 1E, $P < 0.001$ and $P = 0.004$ separately, Table II). In DCIS, the expression level of Nek2A mRNA was observed different among cases with different Ki-67 expression ($P < 0.01$, Table III), so is it in ER ($P < 0.01$, Table III) group and PR group ($P < 0.01$, Table III) and groups of molecular subtypes. In IDC, there was different expression level of Nek2A mRNA in different groups of histological grades ($P < 0.01$, Table IV), lymph node metastasis ($P < 0.01$, Table IV), Ki-67 ($P < 0.01$, Table IV), c-erbB-2 ($P < 0.01$, Table IV) as well as molecular subtypes ($P < 0.05$, Table IV). The interesting founding was that Nek2A mRNA expression was positively correlated with Ki-67 ($r = 0.369$ $P = 0.002$) in DCIS and the similar relationship can also be seen in IDC ($r = 0.392$, $P = 0.001$).

IHC was performed to evaluate the protein expression of Nek2 in DCIS tissues, IDC tissues and NBTs, and the representative immunohistochemistry staining patterns and intensities of Nek2 were illustrated in Fig. 1F,G). The Nek2 cytoplasm staining was observed in 50 cases in DCIS (75.76%), 51 in IDC (77.27%), while only 2 in NBT (7.58%), and statistical analysis showed that compared with NBT, Nek2 cytoplasm expression was significantly increased in DCIS and IDC ($\chi^2 = 92.078$, $P < 0.001$, Table II). Nek2 nuclear expression was also observed in DCIS (13/66, 19.70%) and

TABLE II. Positive Expression of Nek2A and Nek2 in IDC, DCIS, and Normal Tissue Detected by IHC and ISH

		Normal		DCIS		IDC			
		–	Positive (+, ++, +++)	–	Positive (+, ++, +++)	–	Positive (+, ++, +++)	χ^2	*P*-value
ISH	Nek2A cytoplasm, n (%)	63 (95.5)	3 (4.5)	20 (30.30)	46 (69.70)	16 (24.24)	50 (75.76)	81.887	<0.001
IHC	Nek2 cytoplasm, n (%)	61 (92.42)	2 (7.58)	16 (24.24)	50 (75.76)	15 (22.73)	51 (77.27)	92.078	<0.001
	Nek2 nuclear, n (%)	65 (98.48)	1 (1.52)	53 (80.30)	13 (19.70)	58 (87.88)	8 (12.12)	11.091	0.004

IDC, breast invasive ductal carcinoma; DCIS, breast ductal carcinoma in situ; IHC, Immunohistochemistry; ISH, in situ hybridization.
P-values were calculated by Kruskal–Wallis test.

TABLE III. Comparison and Association of Nek2A mRNA Expression and Some Clinicopathological Characteristics in 66 DCIS

	Nek2A				χ^2/Z	*P*-value
	−	+	++	+++		
Ki-67					10.336	0.016*
−	4	1	1	0		
+	7	2	2	0		
++	7	19	6	7		
+++	2	3	1	4		
ER					−3.083	0.002**
Negative	4	9	6	8		
Positive	16	16	4	3		
PR					−2.763	0.006**
Negative	6	12	6	9		
Positive	14	13	4	2		
Molecular subtype					7.979	0.046*
Luminal A	12	12	4	2		
Luminal B	5	6	2	2		
Triple-negative	2	4	3	3		
HER-2/neu	1	3	1	4		

DCIS, breast ductal carcinoma in situ.
*P-values were calculated by Kruskal–Wallis test.
**P-values were calculated by Mann-Whitney U-test.

IDC (8/66, 12.12%), while the nuclear staining was detected in only one case of 66 NBT cases (1.52%, $P < 0.05$, Table II).

ELEVATED EXPRESSION OF NEK2A INDUCES SUPERNUMERARY CENTROSOMES

After 72 h of transfection with myc-Nek2A and control vector in MCF-10A, the expression of Nek2A and Nek2 in mycNek2A-transfected group was detected higher than that in control group by RT-PCR and Western blotting. To determine whether altered Nek2A expression affect centrosome in MCF-10A, we conducted immunofluorescence (IF) to investigate the ectopic expression of Nek2A in normal breast epithelia cell line MCF-10A. As shown in Figure 2B–E, staining by antibodies against myc (Fig. 2F) and centrin2 (Fig. 2G), γ-tubulin (data not shown) revealed that Nek2A transfection group has more cells with abnormal centrosome (7.17 ± 0.55%) than the control group (1.93 ± 0.3%). Ectopically expressed Nek2A colocalized with centrin2 and γ-tubulin at the centrosome in MCF-10A cells as expected. Over-expression of Nek2A did not appear to cause a significant block to progression towards mitosis, as flow cytometric analyses of DNA profiles revealed no changes in the cell cycle distribution of Nek2A-transfected cells.

TABLE IV. Comparison and Association of Nek2A mRNA Expression and Some Clinicopathological Characteristics in 66 IDC

	Nek2A				χ^2/Z	*P*-value
	−	+	++	+++		
Histological					9.422	0.009*
I	9	8	5	0		
II	6	11	3	2		
III	1	10	7	4		
Ki-67					11.723	0.008*
−	2	3	0	0		
+	9	1	4	0		
++	5	25	11	5		
+++	0	0	0	1		
LN					−2.944	0.003**
Negative	13	11	7	0		
Positive	3	18	8	6		
c-erbB-2					−2.727	0.006**
Negative	15	21	8	3		
Positive	1	8	7	3		
Molecular subtype					7.897	0.048*
Luminal A	13	17	7	2		
Luminal B	0	5	4	0		
Triple-negative	2	4	1	1		
HER-2/neu	1	3	3	3		

IDC, breast invasive ductal carcinoma; LN, lymph node metastasis.
*P-values were calculated by Kruskal–Wallis test.
**P-values were calculated by Mann–Whitney *U*-test.

Nek2A siRNA INHIBITED THE PROLIFERATION OF MCF-10 DCIS.COM

In this study, we inhibited Nek2A expression in MCF -10DCIS.com by using three different siRNAs. In siRNA-treated cells, the expression of Nek2A was confirmed to be decreased compared with those of control siRNA-treated cells by RT-PCR (Fig. 3A) and by Western blot analysis (Fig. 3B). We subsequently examined the effect of decreased Nek2A expression on the growth of breast carcinoma cells. The results of SRB staining showed that growth of MCF-10 DCIS.com cells was substantially suppressed in siRNA treated cells (Fig. 3C). Consistent with the above finding, the percentage of Ki-67 positive cells in Nek2A-siRNA-treated cells (56%; Fig. 3D,b) were detected lower than control-siRNA treated cells (98%) by ICC (Fig. 3D,a). All together, these data indicated that knockdown of Nek2A gene can significantly inhibited the proliferation of MCF-10 DCIS.com breast cancer cells.

EFFECT OF Nek2A siRNA ON CELL CYCLE DISTRIBUTION IN MCF-10 DCIS.COM CELL

To further evaluate the effect of Nek2A siRNA on cell cycle profiles, we performed flow cytometry. Compared with control siRNA group, Nek2A siRNA-treated group had a higher proportion of cells in G0/G1 and a decreased one in S phase (Fig. 4A–E). These results showed that Nek2A siRNA could block MCF-10 DCIS.com cell cycle by inhibiting G0/G1-S phase transition and arresting cells in G0/G1.

GENE EXPRESSION PROFILING AND VALIDATION OF SOME DIFFERENTIALLY EXPRESSED GENES USING REAL-TIME RT-PCR

Compared with control siRNA transfection groups, there were 46 expressed common genes of Nek2A 3# siRNA (HSS107083) and 4# siRNA (HSS107084) including 20 down-regulated genes and 26 up-regulated. The gene expression profiling of Nek2A siRNA and control transfection were shown in supplementary data (Supplementary Fig. S1 and Table S1). To confirm the results obtained by microarray, Nek2A, Ki-67, transforming growth factor beta 2 (TGFB2), interleukin 8 (IL8), optic atrophy 1 (OPA1), and protein tyrosine phosphatase type IVA, member 1 (PTP4A1) which were related to cell proliferation, cell apoptosis, and tumorigenesis, were selected from the differentially expressed common genes and detected by real-time RT-PCR, and GAPDH as the control gene. Consistent with the microarray data, all six of these genes were differentially expressed when compared with the control group. Figure 4F shows that the mRNA expression levels of TGFB2, IL8, and PTP4A1 were presented at higher levels in Nek2A-siRNA (3#), while

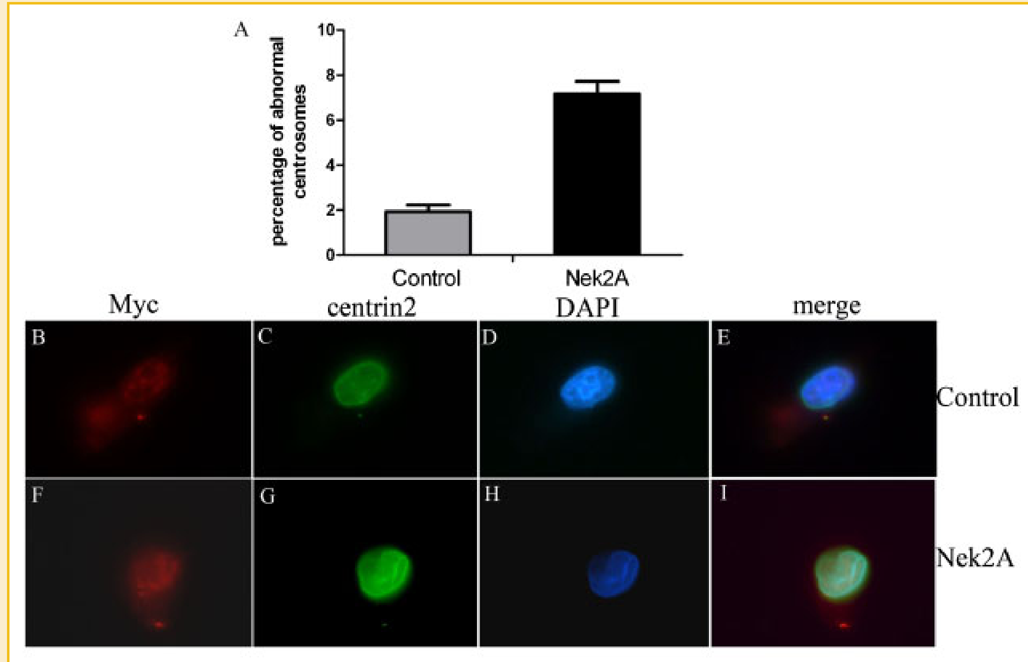

Fig. 2. Elevated expression of Nek2A induces abnormal centrosomes in MCF-10A cells. A, histogram showing percentage of cells with abnormal centrosomes after 72-h transfection of either myc-control or myc-Nek2A; 100–200 transfected cells were counted in three independent experiments, t = 18.375, $P < 0.001$ (Student's *t*-test). B–E: Images display a normal pattern of centrosomes in the control group. F–I: Images of abnormal centrosomes in MCF-10A over-expressing myc-Nek2A stained with antibodies against the myc-tag (red) and centrin2 (green). DNA was stained with DAPI (blue). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

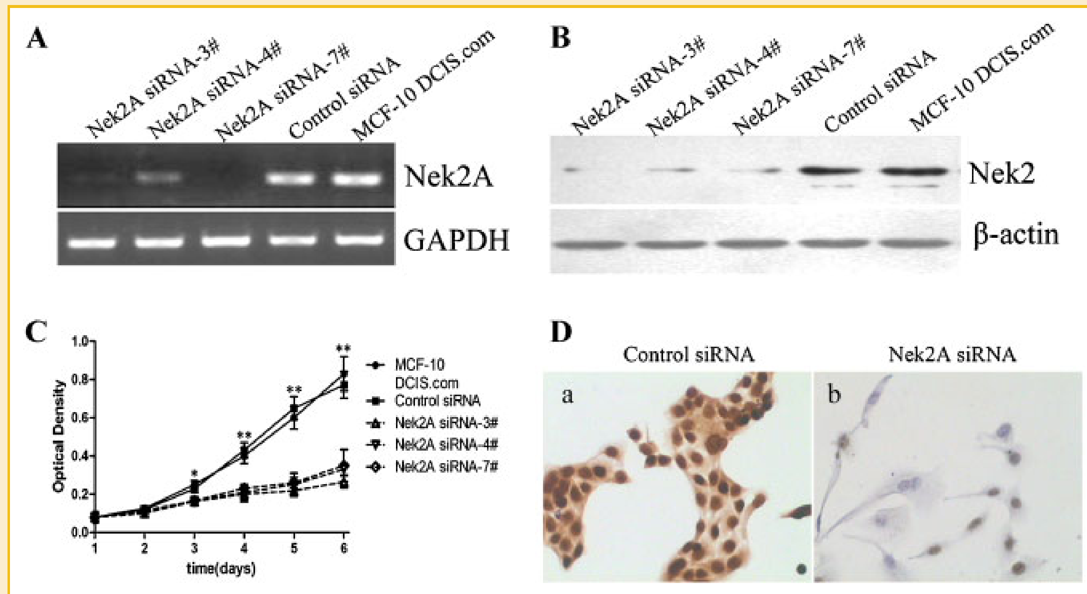

Fig. 3. Nek2A short-interfering RNA (siRNA) treatment inhibits cell growth in MCF-10DCIS.com breast cancer cell line. Confirmation of Nek2A decreased expression by RT-PCR (A) and Western blotting (B) in MCF-10DCIS.com cells transfected for 72 h. Degrade expression of Nek2A decreases the proliferation in cells of MCF-10DCIS.com by SRB assay (C, * and ** *P*-values was lower than 0.05 and 0.01, control a siRNA vs. Nek2A siRNA group, respectively) and by ICC (D), D: The expression of Ki-67 (proliferation index) was lower in Nek2A-siRNA (b) group than in the control-siRNA (a) group, 100–200 transfected cells were counted in three independent experiments, One-way analysis of variance (ANOVA) with multiple comparison(LSD) showed statistic difference ($P < 0.05$, Nek2A siRNA vs. control siRNA). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

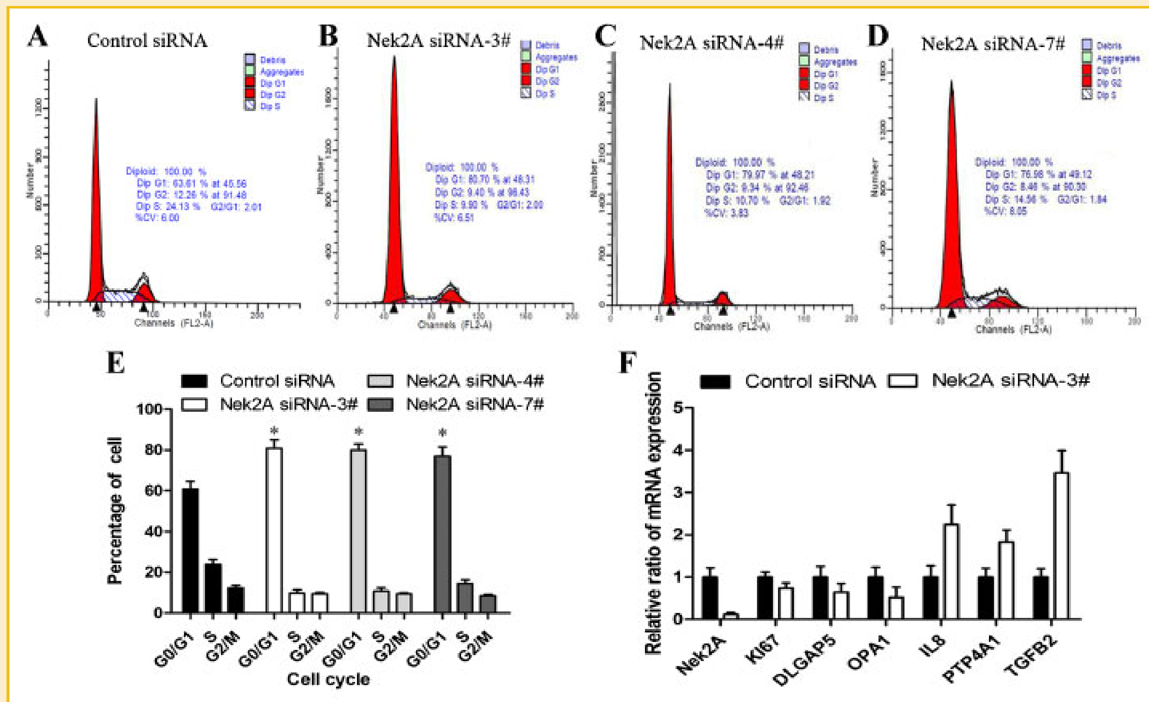

Fig. 4. A–E: Inhibition of Nek2A expression with its three siRNA (3#, 4#, and 7#) causes cell cycle arrest in MCF-10 DCIS.com cell line. Compared with control siRNA group (A), Nek2A siRNA treatment group have a higher proportion of cells in G0/G1 but decreased that in S phase (B–D). E: The graph showed that Nek2A siRNA could block cell cycle progression by inhibiting G0/G1-S phase transition and arresting cells in G0/G1. The percentages of cells in the G0/G1 phase were estimated to be 80.84 ± 4.15, 79.97 ± 3.01, 76.98 ± 4.45, and 60.83 ± 3.74 after treatment of Nek2A siRNA 3#, 4#, 7#, and control siRNA separately, and one-way analysis of variance (ANOVA) with multiple comparison (LSD) showed statistic difference ($P < 0.05$, Nek2A siRNA vs. control siRNA). F: Validation of some differentially expressed genes using real-time RT-PCR in Nek2A siRNA (3#) and control-siRNA treated MCF-10DCIS.com cell lines. Consistent with the microarray data, the mRNA expression levels of (Nek2A, Ki-67, DLGAP5, and OPA1) were present at lower or higher (TGFB2, IL8, and PTP4A1) levels in Nek2A-SiRNA (3#) and control siRNA. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

the expression of other genes (Nek2A, Ki-67, DLGAP5, and OPA1) was down-regulated.

DISCUSSION

CA contribute to carcinogenesis in many kinds of tumor especially in breast cancer. Recent research [Zeng et al., 2010] proposed that CA might precede mammary carcinogenesis. And centrosome aberrations [Ganem et al., 2009] were believed to be correlated with or even to precede the generation of aneuploidy and the acquisition of a chromosome instability phenotype in breast tumors. Importantly, centrosomal defects, aneuploidy, and chromosome instability have all been observed with high frequency in the early stage of tumors [Lingle et al., 2002]. The deregulation of centrosome function may be the major contributory factor to carcinogenesis. As the important centrosome regulatory factor, NIMA (never in mitosis A)-related kinase2 (Nek2) has three splice variants (Nek2A, Nek2B, and Nek2C), of which Nek2A is the most significant one [Wu et al., 2007]. And a study by Hames et al. [Hames and Fry, 2002] showed that Nek2A was the only splice variant which can induce abnormal centrosome splitting upon over-expression. Nek2 and Nek2A were thought to be of great importance in revealing molecular mechanisms for carcinogenesis and they might also be potential new targets for therapeutic intervention.

Nek2A is a serine/threonine protein kinase that locates at the centrosome and is essential for centrosome and exact chromosome segregation [Lou et al., 2004]. During interphase, centrosomes are held together by a proteinaceous linker that connects the proximal ends of the mother and daughter centriole. This linker is disassembled at the onset of mitosis in a process known as centrosome disjunction, thereby facilitating centrosome separation and bipolar spindle formation. Nek2A is implicated in disconnecting the centrosomes through disjoining the linker proteins C-Nap1 and rootletin [Mi et al., 2007]. Components of the Hippo pathway cooperate with Nek2A kinase to regulate centrosome disjunction in invasive cancer [Mardin et al., 2010].

And breast cancer initiation, development, and progression contain consistent genetic changes in the different stages. DCIS is an early step in the pathology of breast cancer in which a luminal epithelial cell lining the duct has undergone an early transformation event and proliferated, although the carcinoma is still bounded by the basement membrane surrounding the duct. Proliferation index [Stasik et al., 2011] and chromosomal instability (CIN) were the defining trait of early human DCIS and is believed to precipitate in breast carcinogenesis. DCIS accounts for 14–18% [Ernster et al., 2000, 2002] of newly diagnosed breast cancer, and some patients with DCIS may develop to IDC after years of diagnosis. However, the biological mechanisms involved in the progression of DCIS to

invasive cancer have not been fully understood and no effective prevention approaches have been reported to be able to interrupt the progression from DCIS to IDC. And most of previous studies about Nek2A were conducted in the cell lines of breast invasive ductal carcinoma (IDC), while few researches were carried out in the cell lines of DCIS, and the expression of Nek2A was rarely investigated in human breast tissue of DCIS and IDC either. In our study, we detected its protein and mRNA expression levels in MCF10 cell lines which MCF-10A, MCF-10DCIS.com, and MCF-10CA1a were included. To corroborate and extend the findings in cell lines, the expression and association of Nek2A with clinicopathological factors were explored in the human tissue. And NBT, DCIS, and IDC were obtained from human primary breast cancer tissue which can present different steps in breast cancer initiation, development, and progression.

Our study showed Nek2A mRNA and protein expression were significantly up-regulated in DCIS and IDC of human cancer cell lines as well as human primary breast cancer tissues. So we infer that cells showing inappropriately high expression of Nek2A may affect the tumorigenesis. Nek2A in carcinoma cells were cytoplasm stained by ISH and Nek2 cytoplasm and/or nuclear stained of IDC and DCIS by IHC. By which we inferred that Nek2 protein has both nuclear and cytoplasm functions. This finding bears some resemblances with the results of Hayward et al. [2004]. Its up-regulation in DCIS tumors indicated that alteration of Nek2A and Nek2 expression levels occurred in breast tumors before invasion and metastasis.

From our data, we detected correlations between Nek2A and Ki-67, ER, PR expression and molecular subtypes in DCIS. And the different reactivity of Nek2A reactivity in different histological grades, lymph node metastasis, Ki-67, c-erbB-2, as well as molecular subtypes was observed in IDC. Importantly, Nek2A expression was positively related with Ki-67 both in DCIS and IDC. As we all know, breast carcinoma is now regarded as a heterogeneous disease classified into four molecular subtypes (luminal A, luminal B, triple-negative, and HER-2/neu). When comparing the prognosis of tumors within each of the different subtypes, it was shown [Carey et al., 2006] that triple-negative or HER-2/neu tumors showed a more aggressive clinical behavior, whereas luminal A tumors were associated with a better prognosis. Our study showed the expression of Nek2 was lower in luminal A, but higher in triple-negative or HER-2/neu. And it was known that the high proliferation index, high grade, high c-erbB-2 expression, and more lymph node metastasis are the poor prognostic indicators. So we inferred that the more up-regulation of Nek2 expression the poorer prognosis one patient may have. Possible mechanism [Rellos et al., 2007] of this may be that Nek2A regulates centrosome cohesion and separation through phosphorylation of structural components of the centrosome, and aberrant Nek2 can lead to aneuploid defects of cancer cells by regulating centrosome separation. Liu et al. [2010] believed that elevation of Nek2 might contribute to chromosome instability and promote aneuploidy through disrupting the control of the mitotic checkpoint. These findings highlighted Nek2A and Nek2 as novel potential biomarker for earlier period auxiliary diagnosis of the human breast cancer especial for DCIS.

In our study, over-expression of Nek2A in the "normal" immortalized MCF-10A cell line did induce the generation of abnormal centrosomes at a level higher than control transfection group. Previous studies have shown that Nek2A contributed to assembling and maintaining of centrosomes and to bipolar spindle formation [Faragher and Fry, 2003]. Therefore, it seems reasonable to predict that they have failed cytokinesis, perhaps as a result of some earlier defect in mitosis. And inappropriately high expression of Nek2A might interfere with centrosome segregation. As well as perturbing spindle formation, altered expression of Nek2A might interfere with other mitotic processes.

Nek2A has been reported to interact with the kinetochore proteins Hec1 and Mad1 suggesting a role in the spindle checkpoint [Chen et al., 2002; Lou et al., 2004]. Meanwhile, studies performed on Nek2A function proposed that it might regulates cohesion and separation of centrosomes through phosphorylation of centrosomal Nek2-associated protein 1 (C-Nap1) [Faragher and Fry, 2003], β-catenin [Bahmanyar et al., 2008] and Shugoshin1 (Sgo1) [Fu et al., 2007]. Previous investigation [Faragher and Fry, 2003] revealed that active Nek2A stimulated the sustained splitting of interphase centrioles indicative of loss of cohesion. Remarkably, Nek2A over-expression did not appear to cause a significant block to progression towards mitosis, as flow cytometric analyses of DNA profiles revealed no changes in the cell cycle distribution of Nek2A-transfected cells indicating that exogenous Nek2A was unable to impose a G2 arrest. Therefore, these data strongly suggested that Nek2-transfected cells entered mitosis in spite of having abnormal centrosomes. All together, our research supported the hypothesis that elevated Nek2A levels can generate errors in centrosomes segregation and/or mitotic progression that may contribute to breast carcinoma progression. Further studies will be needed to determine whether inappropriate expression of Nek2A can also cause cellular transformation or tumor formation in animal models.

RNA interference has emerged as a natural and highly efficient mechanism for gene silencing. Furthermore, our study showed that knockdown of Nek2A expression in MCF-10DCIS.com remarkably inhibited cell proliferation. These finding bears some similarities to the results of Tsunoda et al. [2009] and Westwood et al. [2009]. Nek2 siRNA could suppress anchorage independent growth, suggesting that breast carcinoma cells have a specific dependence on Nek2A for their carcinogenesis growth, which was proposed by Weinstein [Weinstein, 2002] as "oncogene addiction." This provided a strong rationale for further investigation of Nek2A as a new breast carcinoma therapy target. The mechanism of this may be partly explained by the increased cell population in the G1 phase and correspondingly decreased cell population in the G2/M phases [Zeng et al., 2010] which was also observed in our study.

By detecting the gene expression profiling of MCF-10DCIS.com cell lines between Nek2A siRNA interfering group and control, we found some differentially expressed genes were related to cell proliferation, cell apoptosis, and tumorigenesis. Interestingly, the down regulation of Nek2A expression was associated with a down regulation of the proliferative gene Ki-67, of DLGAP5 (predictors of poor outcome in adrenocortical tumors) [Fragoso et al., 2012] and of OPA1 (protect from apoptosis by preventing cytochrome c release independently from mitochondrial fusion) [Frezza et al., 2006; Piao et al., 2009]. Among these up-regulated transcripts, TGFB2 was an inhibitor in early stages breast tumorigenesis [Dave et al., 2011]. But

IL8, PTP4A1 were associated with a higher tumorigenicity, this maybe because of reverse feedback of down regulation of Nek2A. And the changes of Nek2A, Ki-67, DLGAP5, OPA1, IL8, PTP4A1, and TGFB2 were confirmed by real-time RT-PCR. It would be interesting to further investigate their roles to carcinogenesis.

In conclusion, our results demonstrated that Nek2 and Nek2A not only over-expressed in IDC but also in DCIS which represent a very early event in breast cancer development. Our study highlighted Nek2A as a novel potential adjunctive diagnosis biomarker for earlier period breast cancer. By knocking down the expression of Nek2A in MCF-10DCIS.com, remarkably inhibited cell proliferation and induced cell cycle arrest. It is plausible that Nek2A may be a new target for preventive drug for human breast cancer especial for DCIS in the future.

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