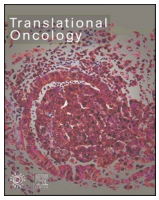
Translational Oncology 51 (2025) 102186

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon



NEK2 promotes colorectal cancer progression by activating the TGF-β/Smad2 signaling pathway

Hai Qin [a,1,*], Manqin Yuan [b,1], Yaqin Yuan [c], Fengqiong Xia [a], Yonghong Yang [a,*]

[a] Department of Clinical Laboratory, Beijing Jishuitan Hospital Guizhou Hospital, No. 206, Sixian Street, Baiyun District, Guiyang City, Guizhou Province, China
[b] Department of Clinical Laboratory Medicine, Guizhou Medical University, No. 9, Beijing Road, Yunyan District, Guiyang City, Guizhou Province, China
[c] Microbiological Laboratory, Guizhou Center for Medical Device Testing, No.247, South Section of Xintian Avenue, Yunyan District, Guiyang City, 550001, Guizhou Province, China

ARTICLE INFO

Keywords:
NEK2
Colorectal Cancer
TGF-β/Smad2
Stemness
Ubiquitination

ABSTRACT

Colorectal cancer (CRC) is a prevalent malignancy with poor patient survival, and NIMA-associated kinase 2 (NEK2) has been implicated in the pathogenesis and progression of various cancers, including CRC. This study aimed to investigate the impact of NEK2 on CRC cell functionality and its interaction with the TGF-β/Smad signaling pathway. NEK2 expression in CRC tissues and cell lines was assessed, and its association with patient survival was analyzed. Functional assays, including NEK2 knockdown via lentiviral infection, RT-qPCR, Western blotting, CCK-8 assay, Transwell migration, invasion assays, and goblet cell formation assays, were employed to evaluate NEK2′s effects on CRC cell proliferation, migration, invasion, and stemness. Mechanistic studies explored the TGF-β/Smad2 signaling pathway, utilizing co-immunoprecipitation (Co-IP) and protein interaction analyses. *In vivo* experiments further evaluated NEK2′s role in tumor initiation, metastasis, and chemoresistance. NEK2 was found to be upregulated in CRC tissues and correlated with poor survival. NEK2 knockdown inhibited CRC cell behaviors, while NEK2 activated the TGF-β/Smad2 signaling pathway through Smad2/3 phosphorylation. Overexpression of Smad2/3 reversed NEK2 knockdown effects, confirming the importance of this pathway in CRC. *In vivo*, NEK2 promoted tumor initiation, metastasis, and chemoresistance, effects partially reversed by Smad2/3 overexpression. These findings reveal the critical role of NEK2 in CRC progression and underscore its potential as a therapeutic target, offering new insights into the molecular mechanisms driving CRC and informing targeted therapy development.

Introduction

Colorectal cancer (CRC) is a prevalent and formidable global health challenge, representing a major cause of morbidity and mortality [1]. Despite advancements in diagnosis and treatment, the molecular mechanisms driving CRC progression remain complex and not fully elucidated. The exploration of novel molecular targets is crucial for advancing our understanding of CRC pathogenesis and developing targeted therapeutic strategies.

Tumor heterogeneity, a hallmark of cancer, encompasses diverse cell populations with distinct molecular profiles and functional capabilities within a single tumor [2]. Notably, tumor heterogeneity includes the presence of cancer stem cells (CSCs) or tumor-initiating cells (TICs), which contribute to therapy resistance, recurrence, and metastasis [3,4]. Understanding the role of tumor heterogeneity, particularly the integration of CSCs, is imperative for developing targeted therapies that can effectively eradicate all subpopulations within a tumor.

The NEK family of kinases has garnered attention in the context of various cancers due to their involvement in cell cycle regulation, mitosis, and cellular proliferation [5]. Among the NEK family of kinases, NEK2 has been shown to promote aerobic glycolysis in multiple myeloma through regulating splicing of pyruvate kinase (PKM) activity by binding to the intronic sequence flanking exon 9 of PKM pre-mRNA [6]. Additionally, NEK2 can directly bind to other proteins, such as NEK2 can directly bind to and phosphorylate PD-L1 to maintain its stability, causing PD-L1-targeted pancreatic cancer immunotherapy to have poor efficacy [7]. Furthermore, NEK2 can promote the progression of Esophageal Squamous Cell Carcinoma (ESCC) via binding to and

* Corresponding authors.
E-mail addresses: 18786665889@163.com (H. Qin), 2301444608@qq.com (Y. Yang).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.tranon.2024.102186
Received 23 September 2024; Received in revised form 28 October 2024; Accepted 30 October 2024

phosphorylating at Thr143 of YAP, subsequently enhancing YAP stability [8]. Similarly, NEK2 acts at an early upstream step to promote dephosphorylation of MST2 and inactivate the Hippo signaling cascade in cervical cancer [9]. Notably, the upregulation of NEK2 has been shown to be associated with poor prognosis in colorectal cancer and shorter cancer-specific survival [10,11]. However, the specific role of NEK2 in CRC and its association with crucial signaling pathways remain areas of active investigation.

In this study, we aimed to bridge the existing knowledge gaps by investigating the role of NEK2 in CRC progression. NEK2′s involvement in cell cycle regulation and potential contributions to cancer initiation and metastasis prompted us to explore its specific impact on CRC cell behavior. Moreover, based on online dataset analysis, our focus on the TGF-β/Smad signaling pathway stems from its recognized importance in cancer progression and the intriguing connection suggested by our preliminary analyses [12,13]. Our research aimed to provide a detailed characterization of NEK2′s influence on CRC cell proliferation, migration, invasion, stemness, and chemotherapeutic sensitivity. By utilizing a combination of *in vitro* and *in vivo* experiments, we sought to unravel the functional consequences of NEK2 dysregulation in CRC. Importantly, we investigated the underlying molecular mechanisms, particularly its interaction with the TGF-β/Smad2 signaling pathway.

Materials and methods

Online dataset analysis

The Kaplan Meier plotter was used to analyze the correlation between the overall survival of CRC patients and gene expression [14]. ROC Plotter - Online ROC analysis was used to determine the potential for predicting chemotherapy [15]. TNMplot: differential gene expression analysis in Tumor, Normal, and Metastatic tissues was utilized to evaluate the expression of genes in online clinical samples, including CRC and normal adjacent tissues, and the correlation between gene expressions [16].

Cell culture and reagents

CRC cell lines HCT-116, SW620, SW480, HT-29, CT-26, SW1116, and normal colon epithelial cell line NCM460, and HEK293T cells were bought from Chinese Academy of Sciences Cell Resource Center (Shanghai, China). To establish a cisplatin-resistant SW620 (SW620-cis-R) cell line, SW620 cells were seeded at 30–50% confluency and exposed to an initial cisplatin concentration of 0.5 μM. The cisplatin concentration was then progressively increased by 1 μM increments following each adaptation phase, with final exposure levels reaching 5–10 μM. Once the cells demonstrated stable proliferation under the final cisplatin concentration, resistance was confirmed through IC50 assays and functional testing. All cells were maintained in DMEM (Thermo Fisher Scientific, Inc., Waltham, MA) with the existence of 10% heated inactivated fetal bovine serum (HyClone, Beijing, China), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were kept at 37 °C in a humidified incubator with 5% CO_2.

Lentivirus package and infection

HEK293T cells were cultured to 70 - 80% confluency and transfected with a lentiviral vector plasmid containing NEK2 shRNA, Smad2 coding sequences or Smad3 coding sequences along with packaging plasmids (psPAX2 and pMD2.G) using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA). Lentivirus-containing supernatant was collected 72 h post-transfection, processed to remove debris, and stored at −80 °C. For stable cell line selection, target cells were plated, and lentivirus containing the gene of interest was added with Polybrene. Following a 24-hour incubation, cells were replenished with fresh growth medium and allowed to recover for 72 h. Selection was initiated with puromycin, and resistant colonies were expanded and characterized for the expression of the gene of interest.

Real time fluorescence quantitative PCR (RT-qPCR)

Total RNA was extracted by lysing the tissues or cells with TRIzol reagent (Invitrogen, USA). The concentration and purity of RNA were measured by Nano Drop UV spectrophotometer. cDNA was synthesized by reverse transcription with PrimeScript TM RT reagent kit (TaKaRa, China). cDNA was used as template and amplified according to the instructions of PCR kit (Invitrogen, USA). The internal reference was β-actin, and relative gene expression was measured using the $2^{-\Delta\Delta Ct}$ method.

Western blot

The total protein was extracted by adding tissue or cell lysis solution and lysed on ice for 30 min. The protein concentration was determined by BCA. 30 μg of protein was taken from each sample for SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes and incubated with 5% skim milk for 2 h. The primary antibody was added and incubated overnight at 4 °C in a shaker. After TBST was washed, horseradish peroxidase-labeled secondary antibody was added and incubated at room temperature for 2 h, and ECL (Thermo Fisher Scientific) was added to develop and exposed to take pictures.

CCK-8

Cells of logarithmic growth stage were digested, centrifuged to remove supernatant, and resuspended in culture medium to prepare cell suspensions for counting. 4000 cells were inoculated in each well of the 96-well plates. After the cells were plastered, the culture medium was aspirated and discarded at different time points. Then, 100 μL containing 10% CCK-8 solution was added and incubated for 2 h at 37 °C in an incubator. The absorbance values at 450 nm were measured using an enzyme marker. The data were analyzed and the activity curves of each group of cells were plotted.

Transwell migration and invasion assay

For cell invasion detection, transwell chambers with matrigel were removed from the −20 °C refrigerator and rewarmed at room temperature. After rewarming, 200 μL PBS was added to the transwell chambers and incubated at 37 °C with 5% CO_2 for 1 hour. Then, DMEM medium containing 10% FBS was added to the lower chamber. 30,000 cells were inoculated into the lower chamber, and serum-free medium was used in the upper chamber. After inoculation, cells were incubated for 48 hour. The original medium was poured off, and the lower chamber was fixed in 4% paraformaldehyde for 30 min. After fixation was completed, the PBS was rinsed 2 - 3 times. After the washing was completed, the lower chamber was placed in 0.1% crystal violet staining solution for 30 min. Then the crystalline violet staining solution was aspirated from the wells and rinsed 5 times repeatedly with water. The cells remaining in the chamber were wiped off with cotton swabs and then dried in a ventilated place. The pictures were observed and taken under the microscope. The migration experiment was performed as the invasion experiment except that matrigel was not applied.

Sphere-formation assay

The detailed procedure was referred to our previous study [17].

Immunohistochemistry

Tissue sections were adhered to anti-release slides and stored at room temperature after baking the sections at 60 °C for 2 hour. Paraffin

sections were dewaxed and hydrated with xylene and ethanol. The sections were then placed in boiling citric acid antigen repair solution for 10 min for antigen repair. The tissue sections were removed and cooled at room temperature for 30 min. The sections were washed 3 times with PBS for 5 min each time. Later, the sections were placed in a blocking solution for 1 hour at room temperature. The primary antibody solution was increased dropwise to tissue and incubated overnight at 4 °C in a wet box. The sections were washed twice with PBS and dropwise with biotinylated secondary antibody solution was incubated for 40 min. The slices were washed twice with PBS and DAB solution was incubated for 5 min. Hematoxylin re-staining was applied for 30 s after full rinsing with tap water, and the slices were dehydrated again with gradient alcohol and xylene after rinsing with tap water. Finally, the slices were sealed with neutral resin, dried at room temperature, observed under a microscope and images were collected.

Co-immunoprecipitation (Co-IP)

The collected cell precipitate was lysed on ice for 10 min by adding protein lysate and then centrifuged for 5 min. The supernatant was transferred to a new l.5 mLEP tube, 20 μL agarose beads (Beyotime, Beijing, China) was added for pre-purification of the extracts by incubation at 4 °C in a shaker for 2 hour. The supernatant was removed after centrifugation and the bead-containing precipitate was collected. After that, agarose beads were washed 3 - 5 times by adding PBST wash into the precipitate, and the washed lysates were incubated with the magnetic bead mixture uniformly overnight at 4 °C. After washing the lysates 3 times with binding buffer, they were separated by elution with SDS-PAGE sample buffer. Finally, the bound proteins were blended with the loading buffer and subjected to western blot analysis.

Ubiquitination assay

For *in vivo* ubiquitination assays, cells were treated with MG132 (10 μM) for 6 h before lysis. For the exogenous interaction study, Flag-NEK2 and HA-Smad2/3 plasmids were used. Commercial antibodies against Flag and HA were used for immunoprecipitation and detection. HEK293T cells were cultured and transfected with these plasmids using an appropriate transfection method. Co-IP was performed using protein A or G beads coupled with the specific antibodies. The immunoprecipitated proteins were subjected to SDS-PAGE, transferred to a membrane, and analyzed by Western blot using anti-Flag or anti-HA antibodies. The presence of the target proteins in the immunoprecipitates indicated their interactions.

In vivo experiments

For detecting the effects of NEK2 on CRC cell proliferation ability *in vivo*, male nude mice between the ages of 4 - 6 weeks were stochastically separated into 2 groups, each with 6 mice, housed in SPF environment. Stably transfected cell lines in good condition were selected, digested and counted. The cells were inoculated in a certain number (1×10^7) in the right axilla of mice. After that, nude mice were continued to be reared for 2 - 3 weeks to observe the growth of subcutaneous tumors. The size of tumors was recorded periodically. For chemotherapy analysis, cisplatin (2.5 mg/kg) was used for this experiment. After 4 weeks of rearing, the nude mice were executed to remove the tumors. The size of the tumor was measured and the weight of the tumor was weighed. The volume of the tumor was calculated (volume = long diameter × short diameter^2/2) and the growth curve of the tumor was plotted. The tumor tissues were embedded with paraffin and then subjected to IHC assay to examine the level of relevant protein indicators in tumor tissues.

For determining the effects of NEK2 on the metastatic ability of CRC cells, cells with or without NEK2 knockdown were injected into (i.v) male nude mice between the ages of 4 - 6 weeks. 28 days later, the lung tissues were separated and subjected to HE staining to observe the metastatic nodes.

For evaluating the effects of NEK2 on the tumor-initiating ability of CRC cells, Extreme Limiting Dilution Analysis (ELDA) (https://bioinf.wehi.edu.au/software/elda/) was performed using CRC cells with or without NEK2 stably knockdown [18]. 1×10^6, 1×10^5, 1×10^4 cells/mice were conducted to inoculate in the right axilla of mice. 14 days later, the tumor-initiating cell ratio was calculated following the protocols described in ELDA.

Statistical analysis

GraphPad Prism statistical software (San Diego, California, USA) was applied to analyze the data. Data are presented as mean ± standard deviation (SD). Statistical significance was determined using Student's *t*-test or one-way ANOVA followed by Tukey's post hoc test. P-values < 0.05 were considered statistically significant. Analyses were performed using GraphPad Prism 8.0 software. $p < 0.05$ means the difference was statistically significant.

Results

NEK2 is highly expressed in CRC patients and cell lines

To investigate the effects of NEKs family on CRC progression, we initially investigated the expression levels of NEKs family genes in CRC and normal adjacent tissues through online datasets. As shown in Fig. 1**A–D**, the mRNA levels of NEK1, NEK7, NEK9, and NEK11 were downregulated in CRC tissues. On the contrary, NEK2, NEK3, and NEK4 exhibited a higher level in CRC tissues compared to that in normal adjacent issues, specifically, NEK2 displayed the most significant difference with a 2.75-fold change (Fig. 1**E–**G). Thus, we focused on NEK2 by subsequently evaluating its expression correlation between the overall survival of CRC patients. It was found that NEK2 expression exhibited a negative correlation with the overall survival of CRC patients, especially the Stage 1 and 4 (Fig. 1**H** and **I, Supplementary Figure S1**). These results suggest that NEK2 might promote the occurrence and progression of CRC. Consistently, NEK2 expression was examined in CRC cell lines and found to be increased in CRC cells, especially SW620 and HT-29 (Fig. 1**J** and **K**), which were chosen for the further experiments.

NEK2 promotes the proliferation, migration, and invasion ability of CRC cells

We then constructed CRC cell lines with stable-knockdown of NEK2 using lentivirus infection and the knockdown efficiency was confirmed through RT-qPCR and western blot analysis (Fig. 2**A** and 2**B**). CCK8 assay was performed to detect the effects of NEK2 knockdown on CRC cell proliferation ability and it was found that NEK2 knockdown significantly suppressed the proliferation ability of CRC cells (Fig. 2**C** and 2**D**). Additionally, transwell migration analysis revealed that the migration ability of CRC cells was attenuated by NEK2 knockdown (Fig. 2**E** and 2**F**). Consistently, NEK2 knockdown inhibited the invasion ability of CRC cells through transwell invasion assay (Fig. 2**G** and 2**H**).

NEK2 enhances the stemness of CRC cells and negatively correlated with drug sensitivity

Subsequently, we analyzed the correlation between NEK2 expression and chemotherapy response through online datasets. It was shown that NEK2 expression was higher in chemoresistant groups than that of chemosensitive groups, and exhibited a negative correlation with chemotherapy (Fig. 3**A** and 3**B**). To confirm this result, we examined the expression of NEK2 in SW620-cis-R and the parental SW620 cells, and found that NEK2 indeed was highly expressed in SW620-cis-R cells (Fig. 3**C**). As CSCs have been regarded to be one of the roots of

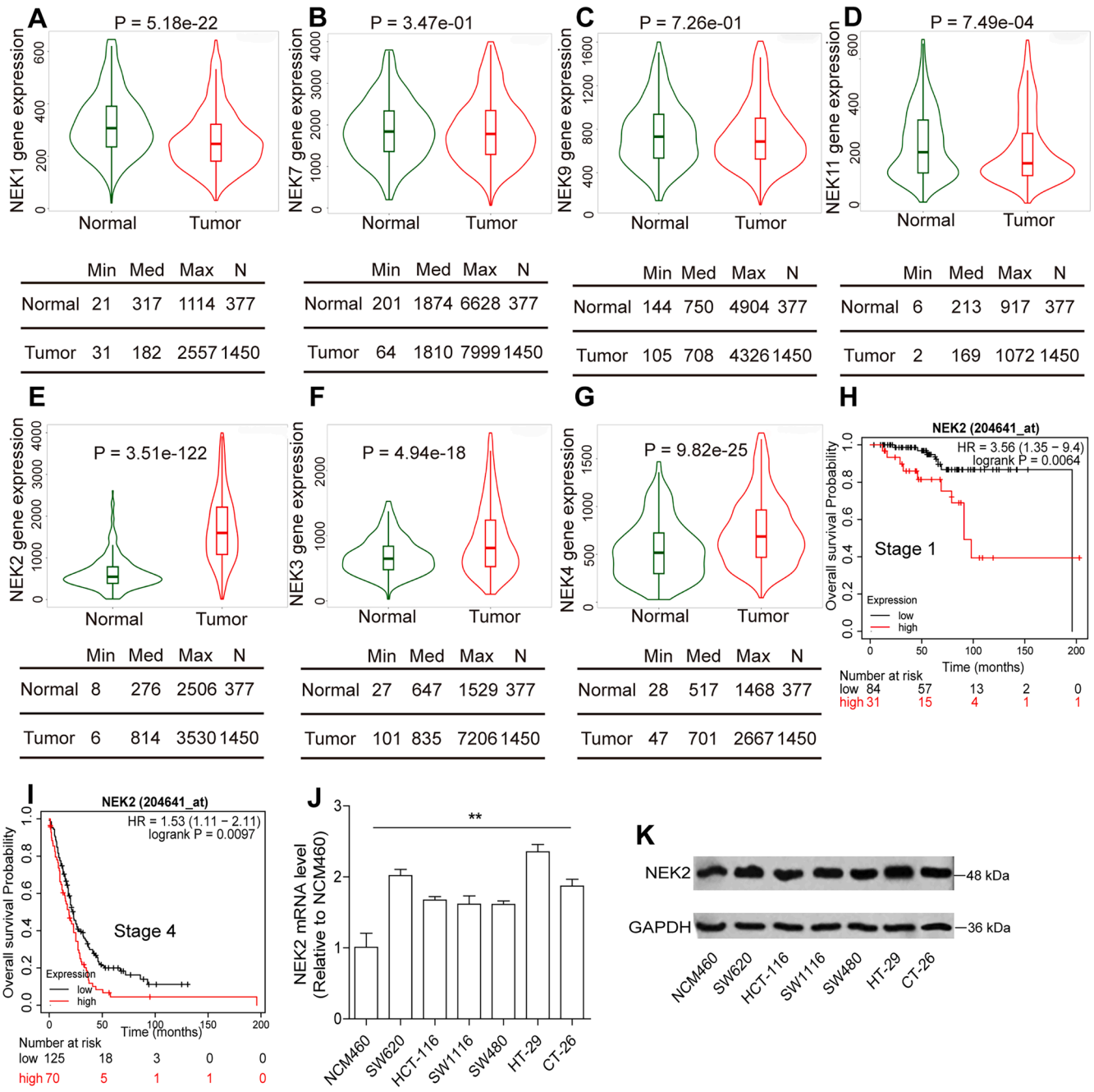


Fig. 1. NEK2 is highly expressed in CRC patients and cell lines. (A - G) The expression levels of NEK family genes were examined through online dataset analysis. (H and I) The correlation between NEK2 expression and the overall survivals (Stage 1 and 4) of CRC patients was evaluated based on online dataset analysis. (J and K) The expression levels of NEK2 were determined in different types of CRC cell lines and normal colon epithelial cell line. $^{**}P < 0.01$ vs NCM460.

chemoresistance and tumor initiation, we further examined the effects of NEK2 on the stemness of CRC cells. As shown in Fig. 3**D**–**F**, NEK2 knockdown reduced the expression of stemness markers (Oct4, Sox2, Nanog). Additionally, the sphere-formation ability, which has been validated to be positively correlated with CSC stemness, was significantly attenuated by NEK2 knockdown, as evident by the decrease of sphere size and number (Fig. 3**G** and 3**H**). There results suggest that NEK2 can promote the progression and occurrence of CRC cells.

NEK2 facilitates the tumor-initiation and metastatic ability of CRC cells

To validate the *in vitro* results, we further constructed the *in vivo* experiments. Firstly, the tumor-initiation ability, which has been confirmed to be positively correlated with cell stemness, was measured through ELDA. As show in Fig. 4**A** and 4**B**, we identified that the tumor-formation ratio of CRC cells with NEK2 knockdown was significantly decreased. In addition, through ELDA, the ratio of CSC was reduced by NEK2 knockdown (Fig. 4**C** and 4**D**). Consistently, IHC analysis indicated that the expression of stemness markers (Oct4 and Sox2) was decreased in tumors derived from CRC cells with NEK2 knockdown (Fig. 4**E** and 4**F**). Furthermore, the metastatic ability of CRC cells with or without NEK2 knockdown was determined through *i.v* model. Through HE analysis, it was found that the lung metastatic nudes exhibited a decrease of size and number from CRC cells with NEK2 knockdown (Fig. 4**G** and 4**H**).

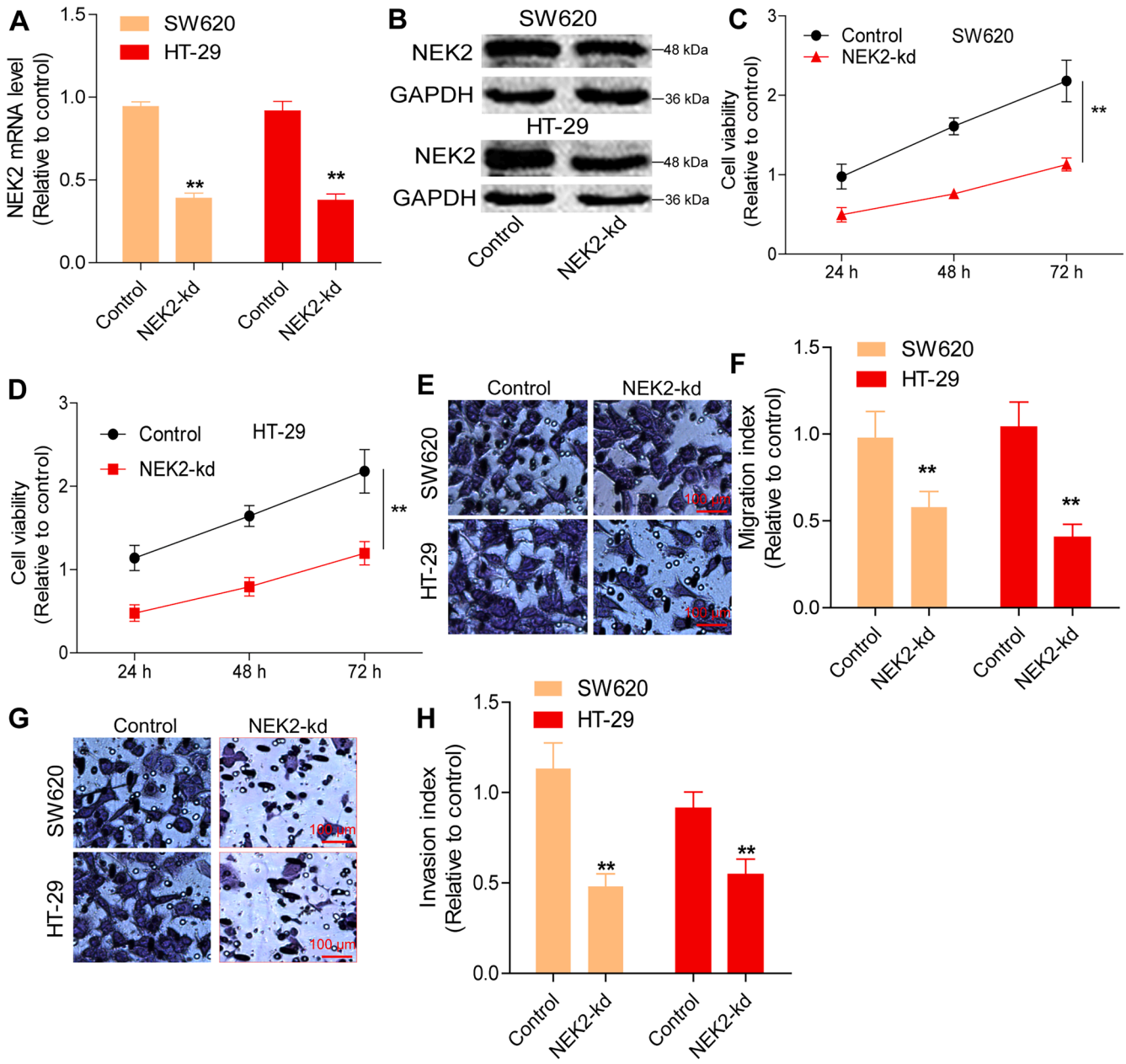


Fig. 2. NEK2 promotes the proliferation, migration, and invasion ability of CRC cells. (A) NEK2 mRNA level was determined in CRC cells with or without NEK2 knockdown. (B) NEK2 protein level was measured in CRC cells with or without NEK2 knockdown. (C and D) Cell viability of CRC cells with or without NEK2 knockdown was detected through CCK-8 analysis. (E and F) The migration ability of CRC cells with or without NEK2 knockdown was evaluated through transwell-migration assay. (G and H) The invasion ability of CRC cells with or without NEK2 knockdown was examined through transwell-invasion analysis. $^{**}P < 0.01$ vs control group.

NEK2 increases the tumor progression ability and decreases chemotherapeutic effects

The roles of NEK2 in CRC cell proliferation and chemotherapeutic sensitivity were further explored. CRC cells with or without NEK2 knockdown were inoculated in the right axilla of mice for 21 days, tumor volume was measured every three days and tumor weight was finally detected at the ending of this experiment. As show in Fig. 5**A** and 5**B**, it was found that NEK2 knockdown significantly suppressed the tumor progression, as evident by the tumor size and volume. Consistently, the tumor weight was also reduced by NEK2 knockdown (Fig. 5**C**). Furthermore, when combined with cisplatin treatment, NEK2 knockdown could enhance the sensitivity of cisplatin *in vivo*, as evident by the decrease of tumor progression (Fig. 5**D–F**). Taken together, these findings suggest that NEK2 acts a tumor-promoter for CRC progression.

NEK2 activates the TGF-β/Smad2 signaling pathway in CRC cells by phosphorylating Smad2

To explore the underlying mechanisms by which NEK2 promotes the progression of CRC cells, we screened out the genes exhibiting a positive correlation with NEK2 expression in CRC tissues through online datasets analysis (**Supplementary Table S1**), which were subjected to KEGG analysis combined with Gene Set Enrichment Analysis (GSEA). We found that the TGF-β signaling pathway was mostly enriched (Fig. 6**A**). In consistently, the phosphorylation level of Smad2/Smad3, but not Smad4, was significantly decreased following NEK2 knockdown in CRC cells (Fig. 6**B**). As NEK2 belongs to NIMA-related kinases, we wondered

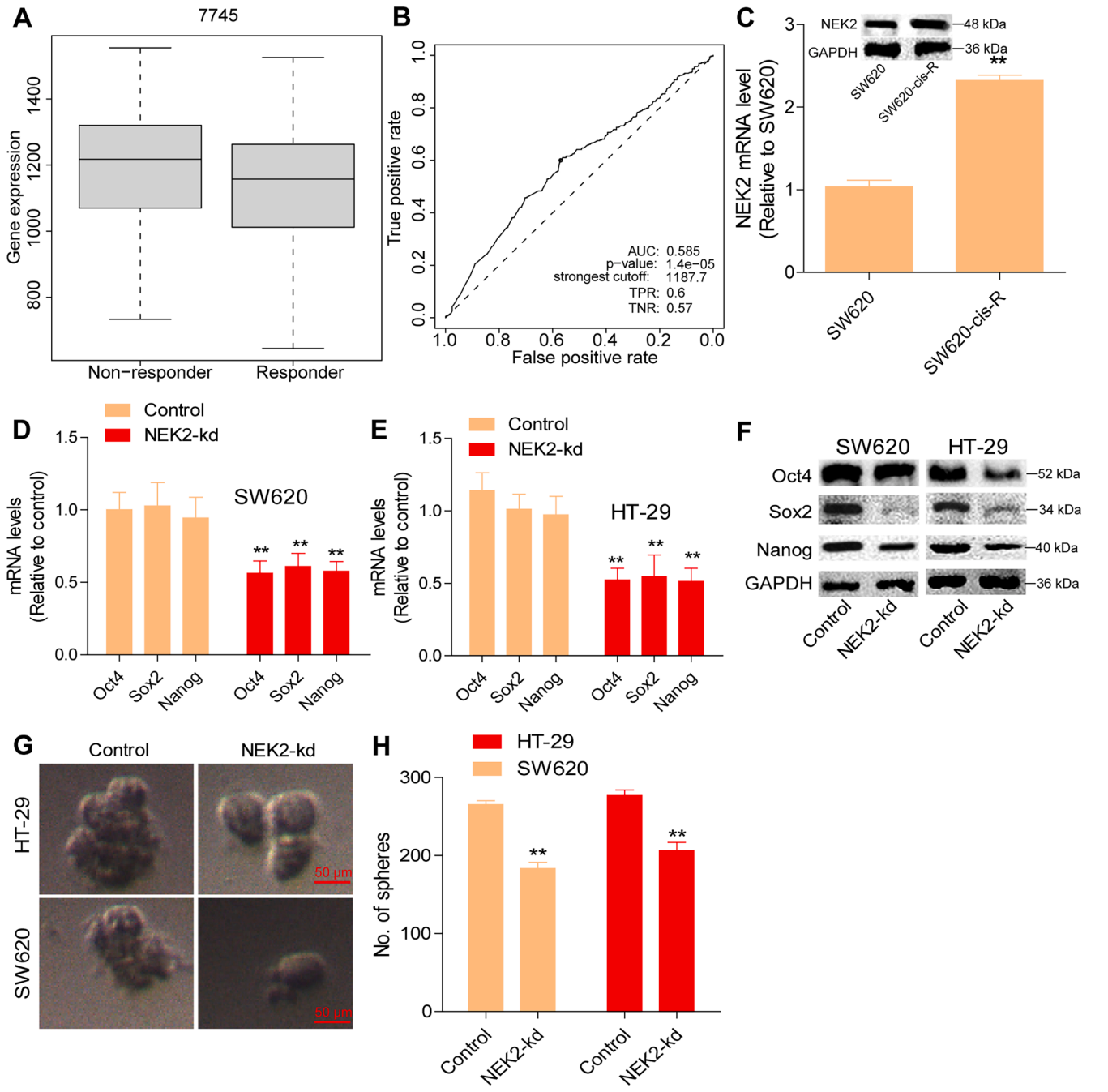


Fig. 3. NEK2 enhances the stemness of CRC cells and negatively correlated with drug sensitivity. (A and B) The expression of NEK2 was detected in the responders and non-responders of chemotherapy through online dataset analysis. (C) NEK2 expression level was detected in cisplatin-sensitive and -resistant CRC cells. (D - F) The expression levels of stemness markers were determined in CRC cell lines with or without NEK2 knockdown. (G and H) The sphere-formation ability of CRC cells was measured in CRC cells with or without NEK2 knockdown. $^{**}P < 0.01$ vs control group.

whether NEK2 could interact with Smad2 or/and Smad3 to phosphorylate them. As expected, co-IP experiments revealed that NEK2 could interact with Smad2 and Smad3, but not Smad4 in CRC cells (Fig. 6**C**). In addition, the ubiquitination level of Smad2/3 was increased in CRC cells with NEK2 knockdown (Fig. 6**D**). In consistent, the expression of Smad2/3 downstream effectors (PAI-1, CTGF, ALDH1A2, Snail) was reduced in CRC cells with NEK2 knockdown (Fig. 6**E** and 6**F**). There results demonstrate that NEK2 can activate the TGF-β/Smad2/3 signaling pathway through interacting and phosphorylating Smad2/3.

NEK2 promotes the progression of CRC cells dependent on the TGF-β/Smad2 signaling pathway

Finally, we investigated whether NEK2 exerted its promoting effects on CRC progression through the TGF-β/Smad2/3 signaling pathway. Then Smad2/3 was overexpressed in CRC cells with NEK2 knockdown, respectively. The overexpression efficiency was confirmed by western blot analysis (Fig. 7**A**). Cell proliferation, migration, and invasion analysis indicated that Smad2/3 overexpression rescued the inhibitory effects of NEK2 knockdown on the proliferation, migration, and invasion ability of CRC cells (Fig. 7**B**–**F**). Consistently, the decreased stemness of CRC cells led by NEK2 knockdown were partially reversed by Smad2/3

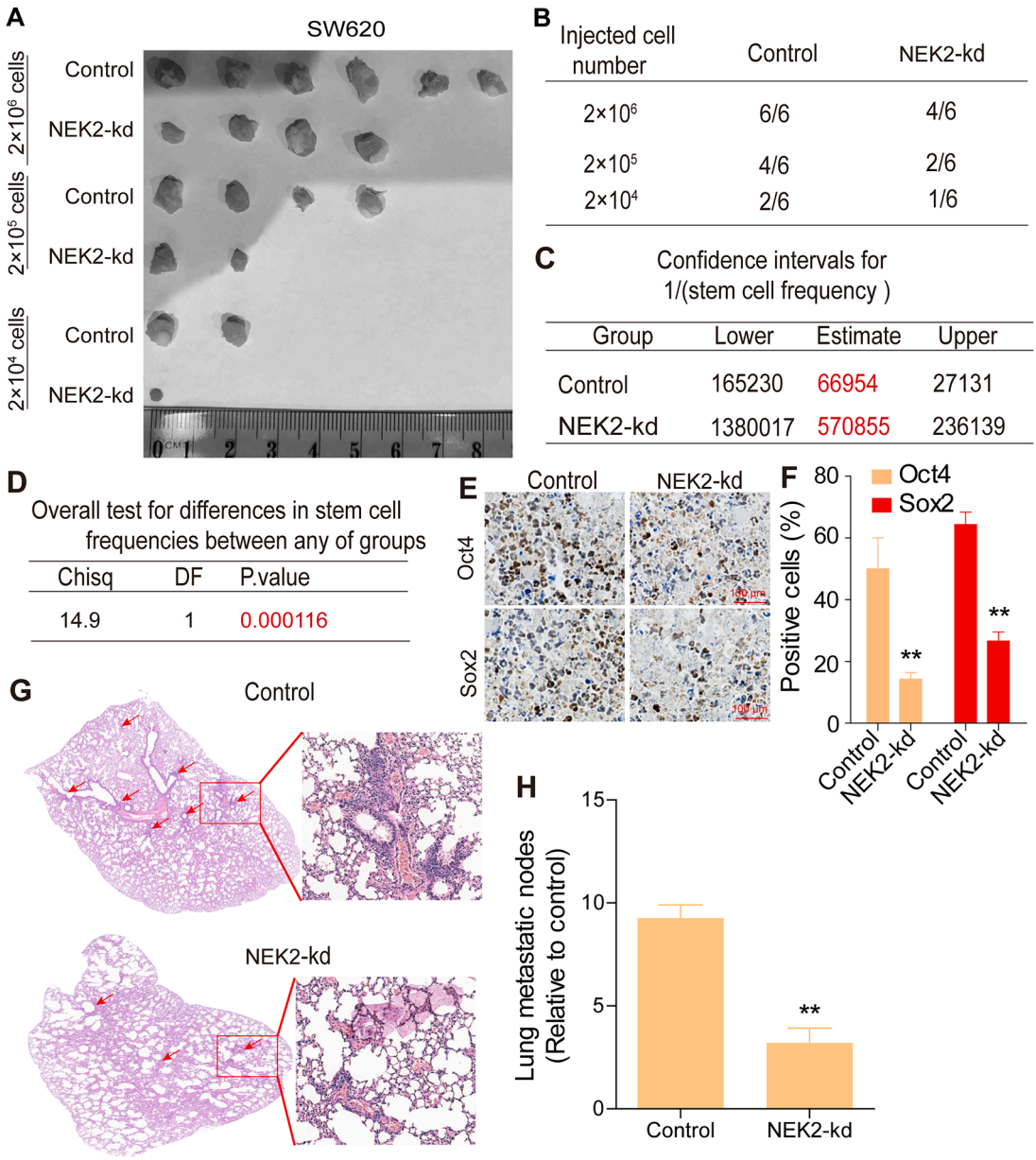


Fig. 4. NEK2 facilitates the tumor-initiation and metastatic ability of CRC cells. (A) Tumor images derived from CRC cells with or without NEK2 knockdown at different cell number as indicated. (B) Tumor-formation ratio was calculated as described in (A). (C) The stem cell frequency was obtained through ELDA based on the tumor-formation ratio in (B). (D) The differences in stem cell frequencies between CRC cells with or without NEK2 knockdown was calculated through ELDA. (E and F) The expression of Oct4 and Sox2 was detected in tumors derived from CRC cells with or without NEK2 knockdown. (G and H) The metastatic ability of CRC cells with or without NEK2 knockdown was determined through *i.v* model. $^{**}P < 0.01$ vs control group.

overexpression (Fig. 7**G**–K). Furthermore, we constructed CRC cells with NEK2 knockdown and Smad2/3 overexpression via lentivirus infection, which were subjected to tumor-initiating ability detection. As shown in Fig. 8, the suppressive effects of NEK2 knockdown on the progression ability of CRC cells were partially abrogated by Smad2/3 overexpression, as evident by the recovery of tumor volume and tumor weight. Overall, our findings indicate that NEK2 can promote the progression of CRC cells through the TGF-β/Smad2 signaling pathway.

Discussion

Our study significantly contributes to the understanding of CRC by highlighting the distinctive role of NEK2 in CRC progression. The unique aspect of our research lies in the comprehensive investigation of NEK2′s influence on multiple facets of CRC cell behavior, ranging from proliferation and migration to stemness and chemotherapeutic sensitivity.

The analysis of NEK family gene expression in CRC tissues revealed a notable upregulation of NEK2, setting it apart from other family members. This differential expression pattern suggests that NEK2 may play a specific and crucial role in CRC pathogenesis, making it a promising target for further exploration. The connection between NEK2 and the TGF-β/Smad signaling pathway adds a layer of complexity to our findings. The activation of this pathway by NEK2, as demonstrated through experimental evidence, underscores the potential crosstalk between NEK2 and a well-established regulatory network in cancer progression. This interaction has not been extensively explored in the context of CRC. However, in addition to the TGF-β/Smad2 pathway, NEK2 is known to interact with several other signaling cascades that may significantly contribute to CRC progression. Notably, NEK2 has been shown to

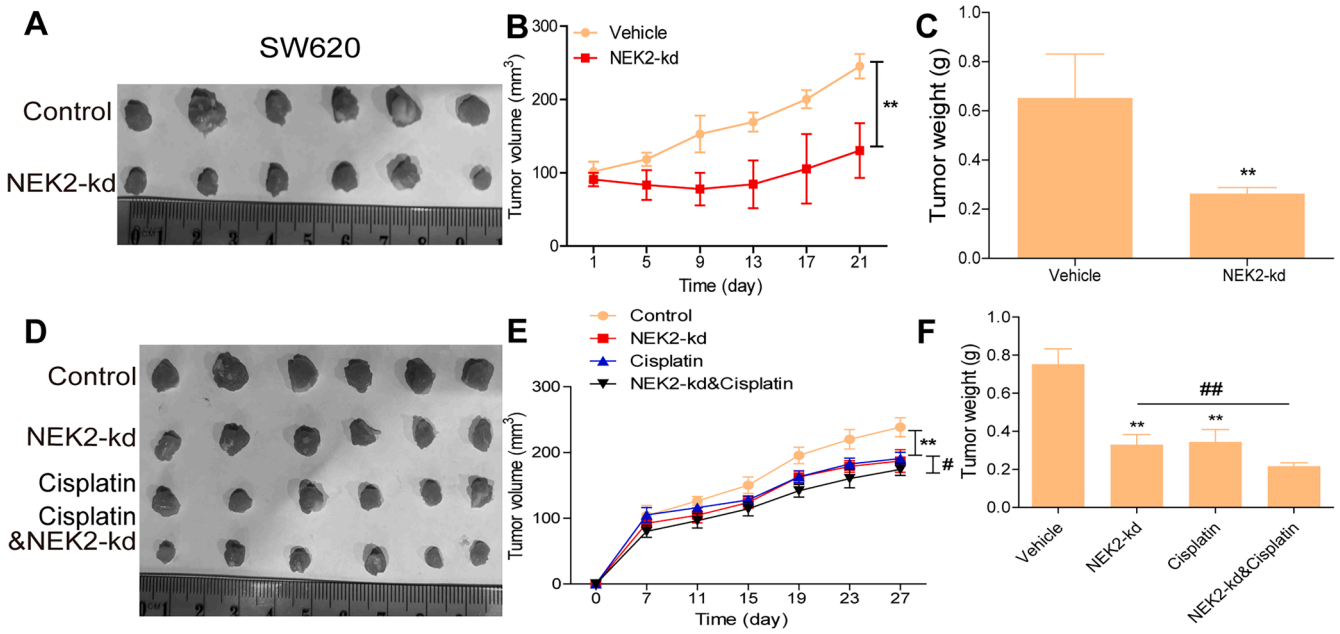


Fig. 5. NEK2 increases the tumor progression ability and decreases chemotherapeutic effects. (A) Tumor images derived from CRC cells with or without NEK2 knockdown. (B) Tumor volume was detected at different time-points as indicated in (A). (C) Tumor weight was measured for the tumors depicted in (A). (D) Tumor images derived from CRC cells with or without NKE2 knockdown plus cisplatin treatment or not. (E) Tumor volume was examined for tumors described in (D). (F) Tumor weight was determined for the tumors described in (A). $^{**}P < 0.01$ vs control group; $^{\#\#}P < 0.01$ vs NEK2-kd group.

enhance the nuclear translocation of β-catenin in the Wnt/β-catenin pathway, promoting tumor cell proliferation and migration [19,20]. Furthermore, NEK2 may activate the PI3K/Akt pathway, which is crucial for cell survival and often implicated in cancer growth and resistance to therapy [21,22]. Additionally, NEK2′s regulation of the MAPK pathway, including ERK and p38 MAPK, could influence the migratory and invasive capacities of cancer cells [23–25]. Therefore, further exploration of these interactions will provide a more comprehensive understanding of NEK2′s role in CRC, and we will incorporate this discussion in the revised manuscript.

Tumor heterogeneity, a critical aspect of CRC, encompasses diverse cell populations with distinct molecular profiles. Our study takes a step further by integrating the concept of tumor heterogeneity, specifically focusing on the influence of NEK2 on cell stemness. The observed effects of NEK2 on stemness markers and sphere-forming ability underscore its potential role in shaping the heterogeneous landscape of CRC. Consistently, the previous study has shown that RPL17 could promote the stemness of CRC cells through the NEK2/β-catenin signaling pathway [26], which also means that NEK2 might regulate CRC stemness through other signaling pathway as elucidated in our online dataset analysis (data not shown).

Understanding the molecular intricacies of NEK2 in CRC has significant clinical implications. The negative correlation between NEK2 expression and overall survival, especially in specific stages, highlights its potential as a prognostic marker. Moreover, our findings may pave the way for targeted therapies aimed at disrupting NEK2-mediated signaling, potentially enhancing the efficacy of existing treatment strategies (cisplatin treatment). It must be noted that targeting NEK2 has been shown to be used for the combination treatment for CRC with cisplatin [27]. While our research provides valuable insights, further studies are warranted to elucidate the precise mechanisms through which NEK2 activates the TGF-β/Smad signaling pathway. Some studies suggest that the TGF-β signaling pathway may be associated with the regulation of the cell cycle, particularly in the transition from G1 phase to S phase [28–31]. Due to the close correlation between NEK2 and cell cycle regulation, this may provide a potential link. Notably, it was found that Smad2/3 has no impact on the expression levels of NEK2 (Fig. 7**A**), which means Smad2/3 act as the downstream effectors of NEK2 in CRC cells. Additionally, exploring the interplay between NEK2 and other signaling cascades in CRC could uncover additional layers of complexity. Further researches could focus on the exploration of NEK2 inhibitors for the treatment of CRC progression or chemoresistance as there are numerous potential NEK2 inhibitors or antibody identified [32,33]. Notably, other NEKs, such as NEK3 and NEK4, also exhibited a higher expression in CRC tissues. And NEK3 has been implicated in the regulation of mitotic processes and is associated with cellular proliferation and survival. Studies have shown that NEK3 overexpression can enhance cell division and contribute to tumor growth in breast cancer [34]. Similarly, NEK4 plays a crucial role in cell cycle regulation and is involved in promoting cellular migration and invasion [35,36]. Its overexpression in CRC suggests that it may also facilitate tumor progression by enhancing these processes.

Conclusion

Our study sheds light on the pivotal role of NEK2 in colorectal cancer (CRC) progression. Through a comprehensive analysis, we have demonstrated that NEK2 is highly expressed in CRC patients and cell lines, promoting proliferation, migration, invasion, stemness, and chemoresistance of CRC cells. The activation of the TGF-β/Smad2 signaling pathway by NEK2 further underscores its significance in driving CRC pathogenesis. Our findings highlight NEK2 as a potential prognostic marker and therapeutic target in CRC, with implications for enhancing treatment efficacy, particularly in combination with cisplatin. Further research into the precise mechanisms of NEK2-mediated signaling and its interactions with other pathways in CRC could uncover novel therapeutic strategies. Overall, our study contributes valuable insights into the understanding of CRC progression and the potential role of NEK2 in shaping the heterogeneous landscape of this disease.

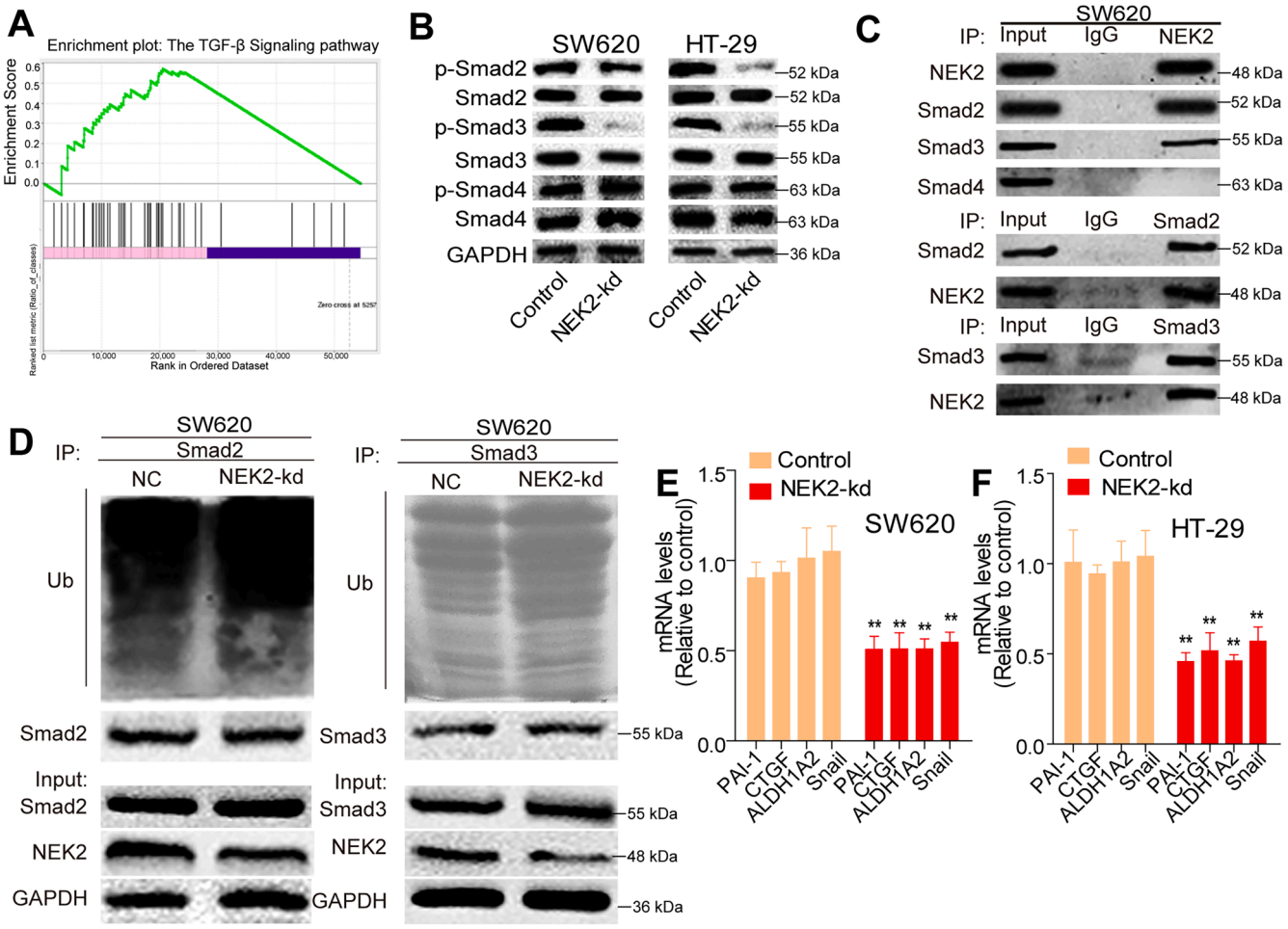


Fig. 6. NEK2 activates the TGF-β/Smad2 signaling pathway in CRC cells by phosphorylating Smad2. (A) GSEA analysis based on the genes with a positive correlation with NEK2 expression in CRC tissues. (B) The levels of p-Smad2/3/4 and Smad2/3/4 were examined in CRC cells with or without NEK2 knockdown. (C) The interaction between NEK2, Smad2, Smad3, and Smad4 was evaluated in CRC cells. (D) The ubiquitination levels of Smad2/3 were measured in CRC cells with or without NEK2 knockdown. (E and F) The expression of the downstream effectors (PAI-1, CTGF, ALDH1A2, Snail) was examined in CRC cells with or without NEK2 knockdown. $^{**}P < 0.01$ vs control group.

Ethics statement and informed consent

Approval of the research protocol by an institutional reviewer board

In vivo experiments were performed according to the protocol approved by the Ethics Committee of Beijing Jishuitan Hospital Guizhou Hospital.

Animal studies

Mice were operated and housed according to the protocols approved by the Ethics Committee of Beijing Jishuitan Hospital Guizhou Hospital.

Author contribution statement

Hai Qin and Yonghong Yang designed research; Yaqin Yuan analyzed data; Fengqiong Xia performed experiments; Hai Qin drafted the manuscript; Yonghong Yang reviewed the manuscript; All authors approved the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Funding information

This study received funding from the Health Commission of Guizhou Province (Nos. gzwkj2023–455).

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any respect of the work covered in this manuscript that has involved experimental animals has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

We understand that the Corresponding Author is the sole contact for

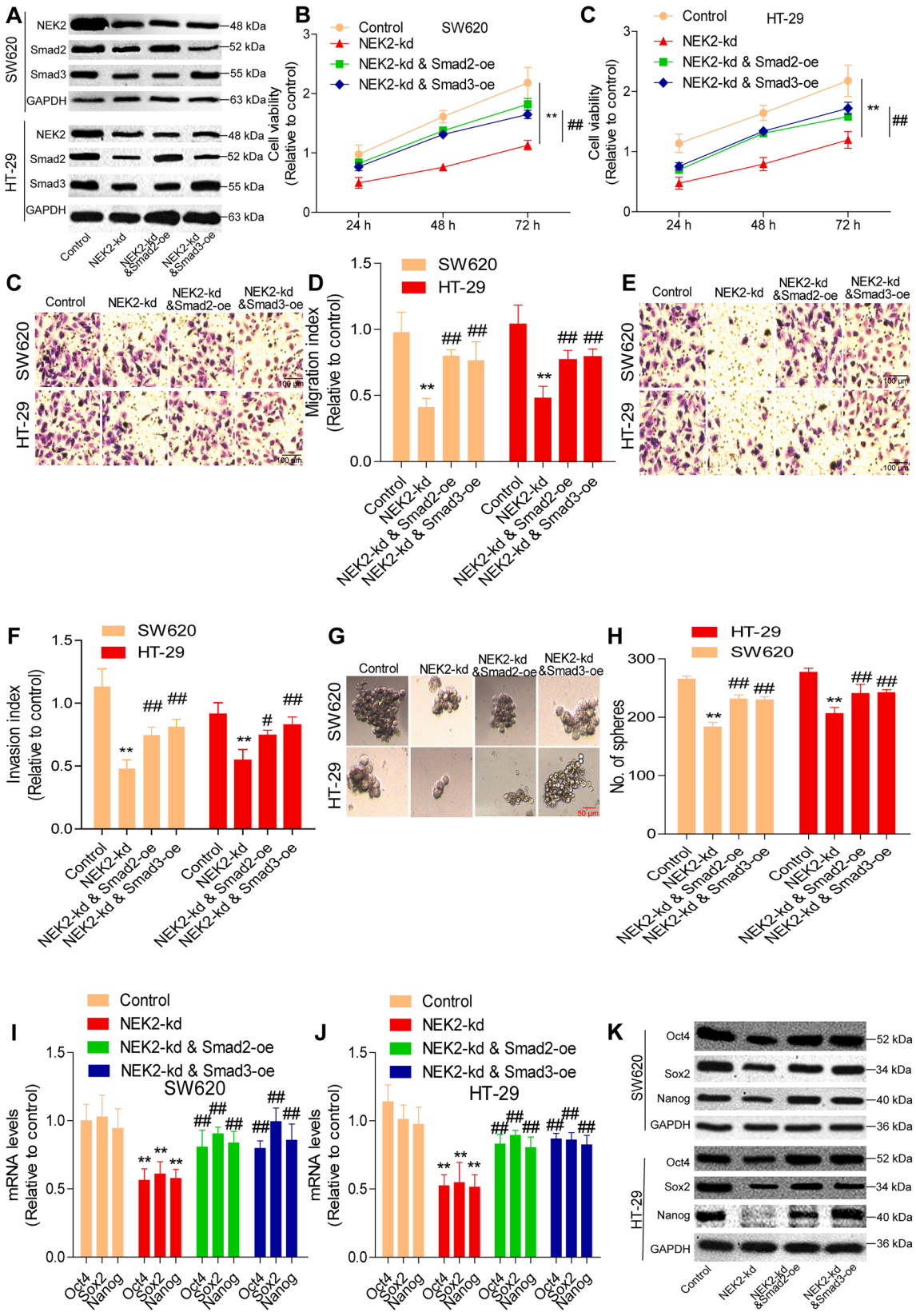


Fig. 7. NEK2 promotes the progression of CRC cells dependent on the TGF-β/Smad2 signaling pathway *in vitro*. (A) The protein levels of NEK2, Smad2, and Smad3 were examined in CRC cells with NEK2 knockdown as well as Smad2 or Smad3 overexpression. (B and C) Cell viability was detected in CRC cells with NEK2 knockdown as well as Smad2 or Smad3 overexpression. (C and D) Cell migration ability of CRC cells described in (A) was measured through Transwell-migration assay. (E and F) Cell invasion ability of CRC cells described in (A) was examined through transwell-invasion assay. (G and H) The sphere-formation ability was evaluated in CRC cells described in (A). (I - K) The expression of stemness markers was determined in CRC cells depicted in (A). $^{**}P < 0.01$ vs control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs NEK2-kd group.

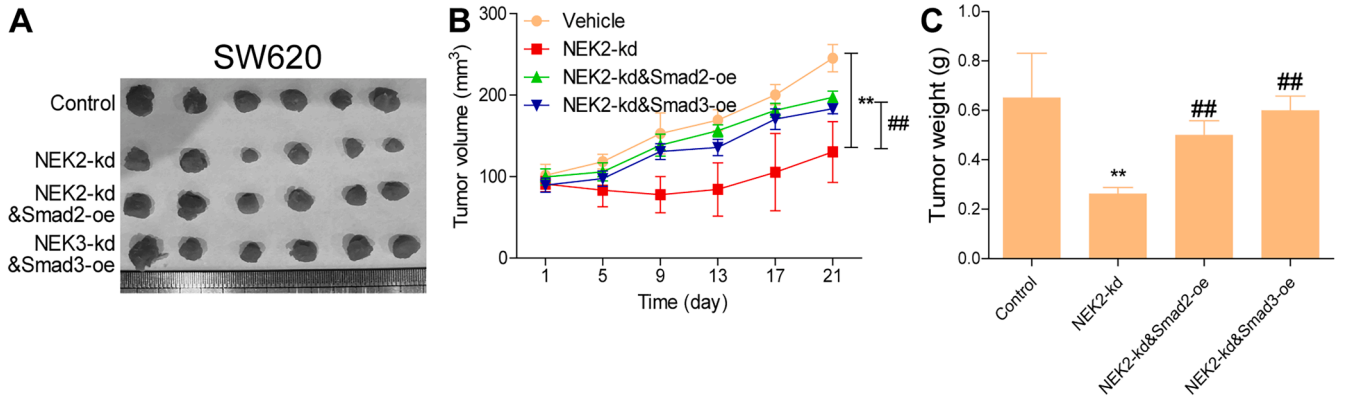


Fig. 8. NEK2 promotes the progression of CRC cells dependent on the TGF-β/Smad2 signaling pathway *in vivo*. (A) Tumor images derived from CRC cells with NEK2 knockdown as well as Smad2 or Smad3 overexpression. (B) Tumor volume was measured at the timepoints as indicated in (A). (C) Tumor weight was measured for the tumors described in (A). $^{**}P < 0.01$ vs control group; $^{\#\#}P < 0.01$ vs NEK2-kd group.

the Editorial process (including Editorial Manger and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirmed that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from.

Acknowledgements

The authors declare that artificial intelligence is not used in this study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2024.102186.

References

[1] L. Li, Y.J. Fang, A. Abulimiti, C.Y. Huang, K.Y. Liu, Y.M. Chen, C.X. Zhang, Educational level and colorectal cancer risk: the mediating roles of lifestyle and dietary factors, Eur. J. Cancer Prev. 31 (2) (2022) 137–144.
[2] D. Sun, X.P. Xie, X. Zhang, Z. Wang, S.F. Sait, S.V. Iyer, Y.J. Chen, R. Brown, D. R. Laks, M.E. Chipman, et al., Stem-like cells drive NF1-associated MPNST functional heterogeneity and tumor progression, Cell Stem Cell 28 (8) (2021) 1397–1410.e1394.
[3] Y. Liu, Y. Chen, Q. Zhao, T. Xie, C. Xiang, Q. Guo, W. Zhang, Y. Zhou, Y. Yuan, Y. Zhang, et al., A positive TGF-β/miR-9 regulatory loop promotes the expansion and activity of tumour-initiating cells in breast cancer, Br. J. Pharmacol. 180 (17) (2023) 2280–2297.
[4] H. Chen, H. Li, W. Shi, H. Qin, L. Zheng, Roles of m6A RNA methylation modification in cancer stem cells: new opportunities for cancer suppression, Cancer Insight 1 (2) (2020) 1–18.
[5] N.K. Panchal, Evan Prince S: the NEK family of serine/threonine kinases as a biomarker for cancer, Clin. Exp. Med. 23 (1) (2023) 17–30.
[6] Z. Gu, J. Xia, H. Xu, I. Frech, G. Tricot, F. Zhan, NEK2 promotes aerobic glycolysis in multiple myeloma through regulating splicing of pyruvate kinase, J. Hematol. Oncol. 10 (1) (2017) 17.
[7] X. Zhang, X. Huang, J. Xu, E. Li, M. Lao, T. Tang, G. Zhang, C. Guo, X. Zhang, W. Chen, et al., NEK2 inhibition triggers anti-pancreatic cancer immunity by targeting PD-L1, Nat. Commun. 12 (1) (2021) 4536.
[8] W. Su, H. Hu, Q. Ding, M. Wang, Y. Zhu, Z. Zhang, Z. Geng, S. Lin, P. Zhou, NEK2 promotes the migration and proliferation of ESCC via stabilization of YAP1 by phosphorylation at Thr-143, Cell Communicat. Signal.: CCS 20 (1) (2022) 87.
[9] Y.R. Zhang, P.S. Zheng, NEK2 inactivates the Hippo pathway to advance the proliferation of cervical cancer cells by cooperating with STRIPAK complexes, Cancer Lett. 549 (2022) 215917.
[10] Y. Takahashi, T. Iwaya, G. Sawada, J. Kurashige, T. Matsumura, R. Uchi, H. Ueo, Y. Takano, H. Eguchi, T. Sudo, et al., Up-regulation of NEK2 by microRNA-128 methylation is associated with poor prognosis in colorectal cancer, Ann. Surg. Oncol. 21 (1) (2014) 205–212.
[11] C.P. Neal, A.M. Fry, C. Moreman, A. McGregor, G. Garcea, D.P. Berry, M. M. Manson, Overexpression of the Nek2 kinase in colorectal cancer correlates with beta-catenin relocalization and shortened cancer-specific survival, J. Surg. Oncol. 110 (7) (2014) 828–838.
[12] K. Luo, Signaling Cross Talk between TGF-β/Smad and Other Signaling Pathways, Cold Spring Harb. Perspect. Biol. 9 (1) (2017).
[13] T. Zhang, X. He, L. Caldwell, S.K. Goru, L. Ulloa Severino, M.F. Tolosa, P.S. Misra, C.M. McEvoy, T. Christova, Y. Liu, et al., NUAK1 promotes organ fibrosis via YAP and TGF-β/SMAD signaling, Sci. Transl. Med. 14 (637) (2022) eaaz4028.
[14] B. Győrffy, Discovery and ranking of the most robust prognostic biomarkers in serous ovarian cancer, Geroscience 45 (3) (2023) 1889–1898.
[15] J.T. Fekete, B. Győrffy, ROCplot.org: validating predictive biomarkers of chemotherapy/hormonal therapy/anti-HER2 therapy using transcriptomic data of 3,104 breast cancer patients, Int. J. Cancer 145 (11) (2019) 3140–3151.
[16] Á. Bartha, B. Győrffy, TNMplot.com: a web tool for the comparison of gene expression in normal, tumor and metastatic tissues, Int. J. Mol. Sci. 22 (5) (2021).
[17] Y. Wang, C. Wei, Y. Yang, A. Luo, X. Zhang, D. Zheng, X. Lu, K. Zhang, X. Duan, X. Xu, Hepatocyte nuclear factor-1β suppresses the stemness and migration of colorectal cancer cells through promoting miR-200b activity, Mol. Carcinog. 59 (8) (2020) 989–999.
[18] Y. Hu, G.K. Smyth, ELDA: extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays, J. Immunol. Methods 347 (1–2) (2009) 70–78.
[19] S.B. Jeon, P.S. Jeong, H.G. Kang, M.J. Kim, J.H. Yun, K.S. Lim, B.S. Song, S.U. Kim, S.K. Cho, B.W. Sim, NEK2 plays an essential role in porcine embryonic development by maintaining mitotic division and DNA damage response via the Wnt/β-catenin signalling pathway, Cell Prolif. 57 (8) (2024) e13626.
[20] D. Guo, W. Yao, X. Du, J. Dong, X. Zhang, W. Shen, S. Zhu, NEK2 promotes esophageal squamous cell carcinoma cell proliferation, migration and invasion through the Wnt/β-catenin signaling pathway, Discov Oncol 14 (1) (2023) 80.
[21] M. McCrury, K. Swafford, S.L. Shuttleworth, S.H. Mehdi, B. Acharya, D. Saha, K. Naceanceno, S.D. Byrum, A.J. Storey, Y.Z. Xu, et al., Bifunctional inhibitor reveals NEK2 as a therapeutic target and regulator of oncogenic pathways in lymphoma, Mol. Cancer Ther. 23 (3) (2024) 316–329.
[22] Q. Lei, J. Xia, X. Feng, J. Guo, G. Li, W. Zhou, NEK2 promotes the progression of liver cancer by resisting the cellular senescence, Zhong Nan Da Xue Xue Bao Yi Xue Ban 47 (2) (2022) 153–164.
[23] M.X. Zhang, X.M. Xu, P. Zhang, N.N. Han, J.J. Deng, T.T. Yu, Y.Y. Gan, X.Q. He, Z. X. Long, Effect of silencing NEK2 on biological behaviors of HepG2 in human hepatoma cells and MAPK signal pathway, Tumour Biol. 37 (2) (2016) 2023–2035.
[24] Z. Xing, M. Zhang, X. Wang, J. Liu, G. Liu, K. Feng, X. Wang, Silencing of Nek2 suppresses the proliferation, migration and invasion and induces apoptosis of breast cancer cells by regulating ERK/MAPK signaling, J. Mol. Histol. 52 (4) (2021) 809–821.
[25] W.D. Fan, T. Chen, P.J. Liu, NIMA related kinase 2 promotes gastric cancer cell proliferation via ERK/MAPK signaling, World J. Gastroenterol. 25 (23) (2019) 2898–2910.
[26] M.J. Ko, Y.R. Seo, D. Seo, S.Y. Park, J.H. Seo, E.H. Jeon, S.W. Kim, K.U. Park, D. B. Koo, S. Kim, et al., RPL17 promotes colorectal cancer proliferation and stemness through ERK and NEK2/β-catenin signaling pathways, J. Cancer 13 (8) (2022) 2570–2583.
[27] K. Suzuki, T. Kokuryo, T. Senga, Y. Yokoyama, M. Nagino, M. Hamaguchi, Novel combination treatment for colorectal cancer using Nek2 siRNA and cisplatin, Cancer Sci. 101 (5) (2010) 1163–1169.
[28] Y. Zhang, P.B. Alexander, X.F. Wang, TGF-β family signaling in the control of cell proliferation and survival, Cold. Spring Harb. Perspect. Biol. 9 (4) (2017).
[29] A. Tiwari, S. Swamynathan, N. Alexander, J. Gnalian, S. Tian, P.R. Kinchington, S. K. Swamynathan, KLF4 regulates corneal epithelial cell cycle progression by suppressing canonical TGF-β signaling and upregulating CDK Inhibitors P16 and P27, Invest. Ophthalmol. Vis. Sci. 60 (2) (2019) 731–740.
[30] J. Saltis, TGF-beta: receptors and cell cycle arrest, Mol. Cell. Endocrinol. 116 (2) (1996) 227–232.
[31] C. Prunier, D. Baker, P. Ten Dijke, L. Ritsma, TGF-β family signaling pathways in cellular dormancy, Trends Cancer 5 (1) (2019) 66–78.

[32] D. Dana, T. Das, A. Choi, A.I. Bhuiyan, T.K. Das, T.T. Talele, S.K. Pathak, Nek2 kinase signaling in malaria, bone, immune and kidney disorders to metastatic cancers and drug resistance: progress on Nek2 inhibitor development, Molecules 27 (2) (2022).
[33] Q. Chen, H. Li, L. Yang, S. Wen, X. Huang, J. Liu, X. Guo, B. Hu, G. Li, M. He, Preparation of an anti-NEK2 monoclonal antibody and its application in liver cancer, BMC Biotechnol. 21 (1) (2021) 62.
[34] S.L. Miller, G. Antico, P.N. Raghunath, J.E. Tomaszewski, C.V. Clevenger, Nek3 kinase regulates prolactin-mediated cytoskeletal reorganization and motility of breast cancer cells, Oncogene 26 (32) (2007) 4668–4678.
[35] N.H. Ding, L. Zhang, Z. Xiao, Z.X. Rong, Z. Li, J. He, L. Chen, D.M. Ou, W.H. Liao, L. Q. Sun, NEK4 kinase regulates EMT to promote lung cancer metastasis, J. Cell. Mol. Med. 22 (12) (2018) 5877–5887.
[36] L. Chen, G. Wu, Y. Li, Q. Cai, Anesthetic propofol suppresses growth and metastasis of lung adenocarcinoma *in vitro* through downregulating circ-MEMO1-miR-485-3p-NEK4 ceRNA axis, Histol. Histopathol. 37 (12) (2022) 1213–1226.