S-phase kinase-associated protein 2 positively controls mitotic arrest deficient 2 in lung cancer cells

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Background. Mitotic arrest deficient 2 (Mad2) is a key component of spindle assembly checkpoint and overexpressed in human lung cancers, but the mechanism of the deregulation of Mad2 in lung cancer is largely unknown. We aim to investigate the regulation of Mad2 by S-phase kinase-associated protein 2 (Skp2) in human lung cancer cells.

Methods. Human lung cancer A549 and NCI-H1975 cells were transfected with MAD2 and SKP2 siRNAs or plasmids to silence or overexpress MAD2 and SKP2. Flavopiridol and HLM006474 were used to inhibit cyclin dependent kinases (CDKs) and E2F1, respectively. mRNA and protein levels were determined by real-time PCR and Western blot, respectively. Cell cycle progression was assayed by flow cytometery.

Results. Knockdown of Skp2 by siRNA decreased Mad2 mRNA and protein levels in A549 and NCI-H1299 cells, accompanied with upregulation of p27 but decrease of the phosphorylation of retinoblastoma (Rb). In contrast, ectopic overexpression of Skp2 increased Mad2 mRNA and protein levels and phosphorylation of Rb, while decreased p27. Pharmacological inhibition of CDK1/2 by flavopiridol or E2F1 with HLM006474 led to downregulation of Mad2 expression, and prevented the increase of Mad2 expression by Skp2. Accordingly, silencing of either Mad2 or Skp2 impaired the mitosis arrest in response to nocadazole.

Conclusion. SKP2 positively regulates the gene expression of MAD2 through p27-CDKs-E2F1 signaling pathway, suggesting that deregulation of Skp2 may lead to upregulation of Mad2 via enhancing the activity of CDKs in human lung cancers. Our findings may provide an explanation of the simultaneous upregulation of MAD2 and SKP2 in lung cancer and potential targets for the development of molecular targeted therapy for lung cancers.

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Running title: Regulation of MAD2 by SKP2

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Abstract

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Key words SKP2; MAD2; spindle assembly checkpoint; lung cancer; p27

Introduction

Spindle assembly checkpoint (SAC) controls the accurate and complete separation of sister chromatins during mitosis, and thereby plays pivotal role in the maintenance of chromosome stability in all eukaryotes (Holland and Cleveland, 2009). Chromosome instability is now recognized as a hallmark of human cancer cell, highlighting the important contribution of the deregulation of SAC during the multi-step processes of tumorigenesis (Hanahan and Weinberg, 2011). However, the molecular mechanism by which SAC dysregulation promotes tumorigenesis remains to be determined.

Deregulation of the components of SAC is a frequent characteristic of cancer, especially solid tumors (Kops et al., 2005). Mitotic arrest deficient 2 (Mad2) is an essential component of SAC and has been found highly expressed in a variety of human malignancies (Hisaoka et al., 2008; Rhodes et al., 2007; Schvartzman et al., 2011; Schuyler et al., 2012). In most lung cancers, Mad2 mRNA was found to be elevated. High-level Mad2 expression in human non-small-cell lung cancer (NSCLC) correlates with tumor progression and patients with tumors with elevated Mad2 expression demonstrate significantly shorter survival time (Kato et al., 2011). Similarly, overexpression of Mad2 in transgenic mice results in a wide variety of tumors (Yu et al., 2012). It was suggested that high-level Mad2 expression might be an independent prognostic factor for NSCLC (Kato et al., 2011). However, the mechanism by which Mad2 is deregulated in lung cancer is largely unknown.

S-phase kinase-associated protein 2 (Skp2) is an F-box protein of SCF ubiquitin ligase complex, which plays an important role in the regulation of cell cycle progression (Bashir et al.,

2004). One of the main targets of Skp2 is p27, an inhibitor of cyclin dependent kinases (CDKs) (Muth et al., 2010). It was reported that Skp2 is up-regulated in NSCLC and overexpression of Skp2 is correlated with a decrease of p27 (Hu et al., 2008). Moreover, it was found that Skp2 expression was significantly associated with tumor status, lymph node metastasis, stage, and vascular invasion (Takanami et al., 2005). Skp2 was also found to be an independent prognostic factor for survival in NSCLC (Osoegawa et al., 2004). These findings clearly indicate Skp2 plays an important role in the oncogenesis and development of NSCLC.

Thus, both Mad2 and Skp2 are upregulated in lung cancers, and high-level expression of either Mad2 or Skp2 is associated with tumor progression and predicts poor survival of NSCLC patients, suggesting that there might be a functional link between Mad2 and Skp2 in the promotion of the tumorigenesis of lung cancer. It has been reported that Skp2 is an E2F target gene and Rb directly binds Skp2 to repress its ability to mediate p27 degradation and to bring it to APC/C for ubiquitination and degradation (Zhang et al., 2006). Recently, it was found that Mad2 is positively regulated by Rb-E2F1 at the transcriptional level (Sotillo et al, 2007; Wang et al., 2012; Hao et al., 2015). Rb-E2F1 is controlled by CDKs, while Skp2 negatively regulates p27, an inhibitor of CDKs. These facts led us to hypothesize that Skp2 might promote the gene expression of Mad2 via p27-CDKs-E2F1 signaling axis.

In this study, we investigated the expression of Mad2 by silencing Skp2 with siRNA and ectopic overexpression of Skp2 in human lung cancer A549 and NCI-H1975 cells. We further assessed the gene expression of Mad2 following pharmacological inhibition of CDK1/2 and E2F1.

Materials and Methods

Drugs

CDKs inhibitor flavopiridol was purchased from Selleck Chemicals (Houston, TX). E2F inhibitor HLM006474 was from Millipore. Nocodazole was from Sigma (St. Louis, MO).

Cells and cell transfection

Human NSCLC cell lines A549 and NCI-H1975 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI 1640 medium (GIBCO), supplemented with 10 % fetal bovine serum (FBS) (GIBCO), 100 units/mL penicillin, 100 mg/mL streptomycin and 2 mmol/L L-glutamine; the cells were incubated at 37 °C with a 5 % CO2 atmosphere. Control and ON-TARGETplusSMARTpool siRNAs of SKP2 and MAD2 were purchased from Dharmacon (Chicago, IL). Plasmid pcDNA-SKP2 was from Addgene (Cambridge, MA). Plasmid pcDNA-MAD2 was constructed by cloning the open reading frame of MAD2 gene from A549 cells into vector pcDNA3.1. Lipofectamine 2000 was from Invitrogen (Carlsbad, CA) and transfection of siRNA or plasmids in A549 or NCI-H1975 cells was performed according to the manufacture's instructions.

Flow cytometry

Cells were harvested with 0.25% trypsin following treatment, washed twice with PBS, fixed with 70% ethanol and saved at 4˚C. Following washing twice with PBS, propidium iodide (PI) was added. The analysis was performed with a FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA) to analyzed DNA content.

Cell proliferation assay

Cell proliferation was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at 4×10^3/well in 96-well plates the day before transfection. Following transfection of siRNAs for 24 h, cells were treated with nocodazole for

24 h. 20μL of 5mg/mL MTT solution was added to wells and cells were cultured for additional 4 h. The culture medium was removed and 150μL dimethylsulfoxide (DMSO) was added to dissolve formazan. Cell viability was quantified by measuring absorbance at 492nm using a microplate spectrophotometer to calculate the optical density (OD) values.

Western blotting

Total protein was extracted from cells using lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, 1% DTT, and 1% protease inhibitor cocktail (Roche). Equal amounts of protein extracts (40 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane. Membranes were blocked with 5% w/v non-fat dry milk dissolved in Tris buffered saline plus Tween-20 (TBS-T; 0.1% Tween-20; pH 8.3) at room temperature for 1 h, then incubated with primary antibodies at 4°C overnight. The primary antibodies used were rabbit anti-Mad2 (Abcam, Cambridge, MA), rabbit anti-Skp2 and GADPH (Santa Cruz Biotech, Santa Cruz, CA), rabbit anti-Rb, pRb-Ser807/811 and pRb-S780 (Cell Signaling Biotechnology, Boston, MA). After washing with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (Santa Cruz Biotech, Santa Cruz, CA) for 1 h at room temperature. Immunobands were visualized using enhanced chemiluminescence (ECL) kit (GE Healthcare, Waukesha, WI, USA) according to manufacture's instructions and exposed to X-ray films.

RNA Isolation, cDNA Synthesis and RT-PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen, USA). Reverse transcription was performed using the First-strand cDNA Synthesis System (Invitrogen). Real-time PCR was performed on the 7900HT Fast Real-Time PCR System using the TaqMan®

Universal Mastermix II. Human *MAD2 and SKP2* expression was quantified in real-time with *MAD2 and SKP2* specific FAM dye-labeled MGB-probes and normalized to *GAPDH*. *GAPDH* was used as internal control. Each experiment was repeated twice in triplicate. The relative expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data were analyzed using SPSS19.0 statistical software. Measurement data are expressed as mean±SEM. Comparison was made by *t* test between two groups. A *P* value of <0.05 was considered statistically significant.

Results

Downregulation of Skp2 by siRNA results in decrease of Mad2

The upregulation of both MAD2 and SKP2 in human lung cancer suggests that Mad2 might be regulated by Skp2. To test this hypothesis, we knocked down Skp2 by siRNA in human lung cancer A549 and NCI-H1975 cells and determined the mRNA and protein levels of Mad2 by RT-QPCR and Western blotting, respectively. In comparison to control siRNA, Skp2 siRNA decreased Skp2 protein levels 48 hr after transfection in both A549 and NCI-H1975 cells (Fig. 1A). As expected, the Mad2 protein levels were drastically decreased by Skp2 siRNA (Fig. 1A). Consistent with the decrease of Mad2 protein, the mRNA levels of Mad2 were also significantly downregulated by Skp2 siRNA in both A549 and NCI-H1975 cells (Fig. 1B). These results indicate the gene transcription of Mad2 is controlled by the Skp2-mediated signaling pathway.

Ectopic overexpression of Skp2 increases the expression of Mad2

To further support the above observation that Mad2 is under control of Skp2 signaling, we transfected A549 and NCI-H1975 cells with SKP2 plasmid to ectopically overexpress SKP2, and determined the mRNA and protein levels of MAD2 by RT-QPCR and Western blotting, respectively. In comparison to control vector pcDNA3.1, transfection of pcDNA-SKP2 obviously increased Skp2 protein levels 24 hr after transfection, and apparently after 48 and 72 hr in both A549 and NCI-H1975 cells (Fig. 1C). The mRNA levels of Mad2 were also significantly increased by pcDNA-SKP2 in both A549 and NCI-H1975 cells (Fig. 1D). Together, these results clearly demonstrated that Skp2 signaling controls Mad2 expression at transcriptional level in A549 and NCI-H1975cells.

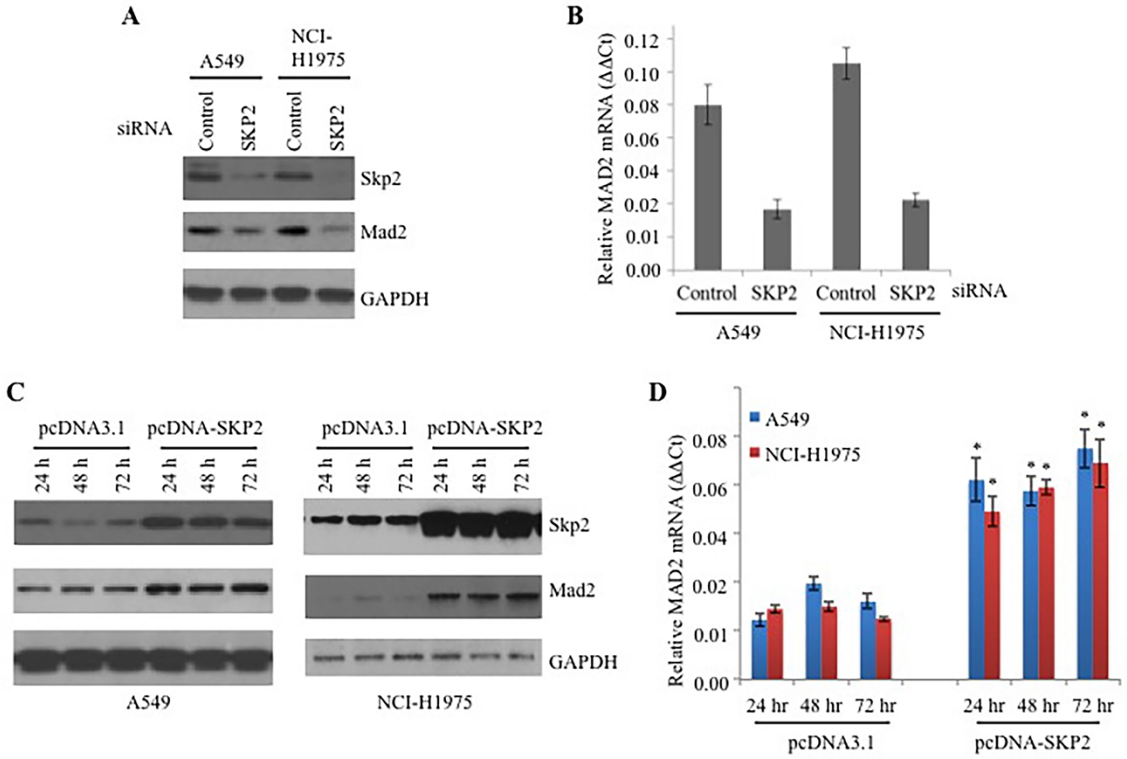


Figure 1. Silencing of SKP2 by siRNA led to decrease of MAD2 expression in A549 and NCI-H1975 cells.

(A) Human lung cancer A549 and NCI-H1975 cells were transfected with 50 nM control or SKP2 specific siRNA with lipofectamine 2000. 48 hr later, total proteins were extracted for the detection of the protein levels Skp2 and Mad2 by Western blotting. GAPDH served as loading control.

(B) Human lung cancer A549 and NCI-H1975 cells were transfected with 50 nM control or SKP2 specific siRNA with lipofectamine 2000. 48 hr later, total RNA was extracted for the detection of the mRNA levels MAD2 by RT-QPCR with GAPDH as internal control. Quantitative analysis are expressed as mean ± SEM. n =3, *P <0.01 vs. control siRNA-transfected cells.

(C) Human lung cancer A549 and NCI-H1975 cells were transfected with 2 μg of vector pcDNA3.1 or pcDNA-SKP2 for 24, 48, and 72 hr. Total proteins were extracted for the detection of the protein levels Skp2 and Mad2 by Western blotting. GAPDH served as loading control.

(D) Human lung cancer A549 and NCI-H1975 cells were transfected with 2 μg of vector pcDNA3.1 or pcDNA-SKP2 for 24, 48, and 72 hr. Total RNAs were extracted for the detection of the mRNA levels MAD2 by RT-QPCR with GAPDH as internal control. Mean value of triplicate is shown. Quantitative analysis are expressed as mean ± SEM. n =3, *P <0.01 vs. control pcDNA3.1-transfected cells.

Downregulation of Skp2 decreases while ectopic overexpression of Skp2 increases the phosphorylation of Rb

To investigate the underlying mechanism by which Skp2 regulates Mad2 expression, we first assessed the protein level of p27, a well-known downstream target of Skp2, after silencing or

overexpression of Skp2 in A549 and NCI-H1975 cells by immunoblotting. As shown in Figure 2A, knockdown of Skp2 by siRNA led to an increase of p27 in both A549 and NCI-H1975 cells. p27 is a potent inhibitor of CDKs. We further tested the phosphorylation of Rb at Ser780 and Ser807/811, a marker of the activation of CDKs. Consistent with the upregulation of p27, pRb-S780 and pRb-S807/811 signals were apparently decreased following SKP2 siRNA transfection in A549 and NCI-H1975 cells (Fig. 2A). In contrast, ectopic overexpression of SKP2 resulted in decrease of p27 while increase of the phosphorylation of Rb at Ser780 and Ser807/811 in these cells (Fig. 2B). These results showed that Skp2 signaling positively regulates the activity of CDKs by decreasing p27 in A549 and NCI-H1975 cells, supporting the conclusion that Skp2 promotes cell cycle progression by degrading p27.

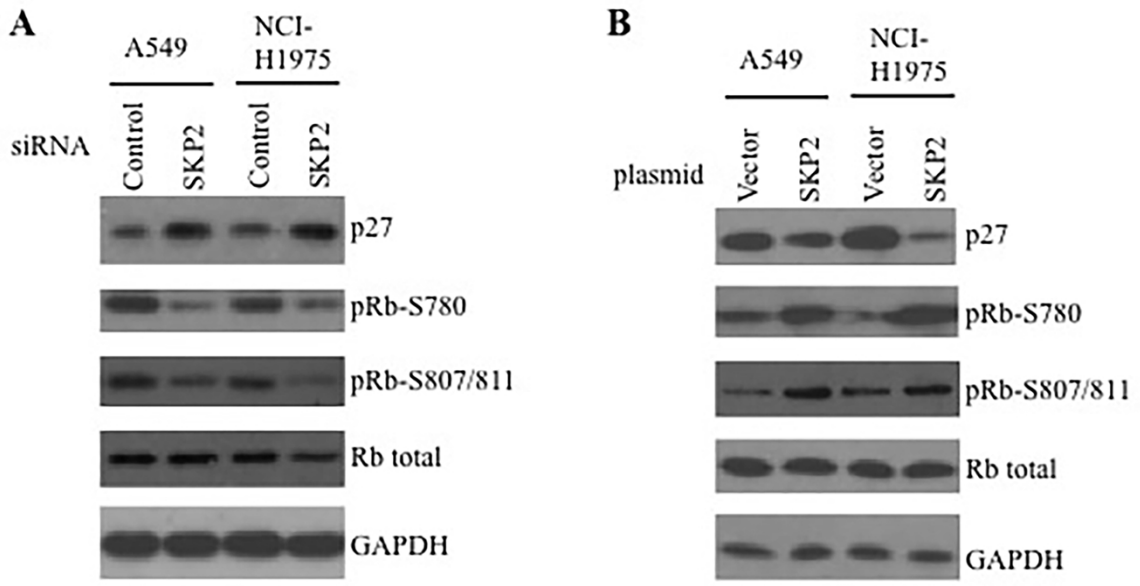
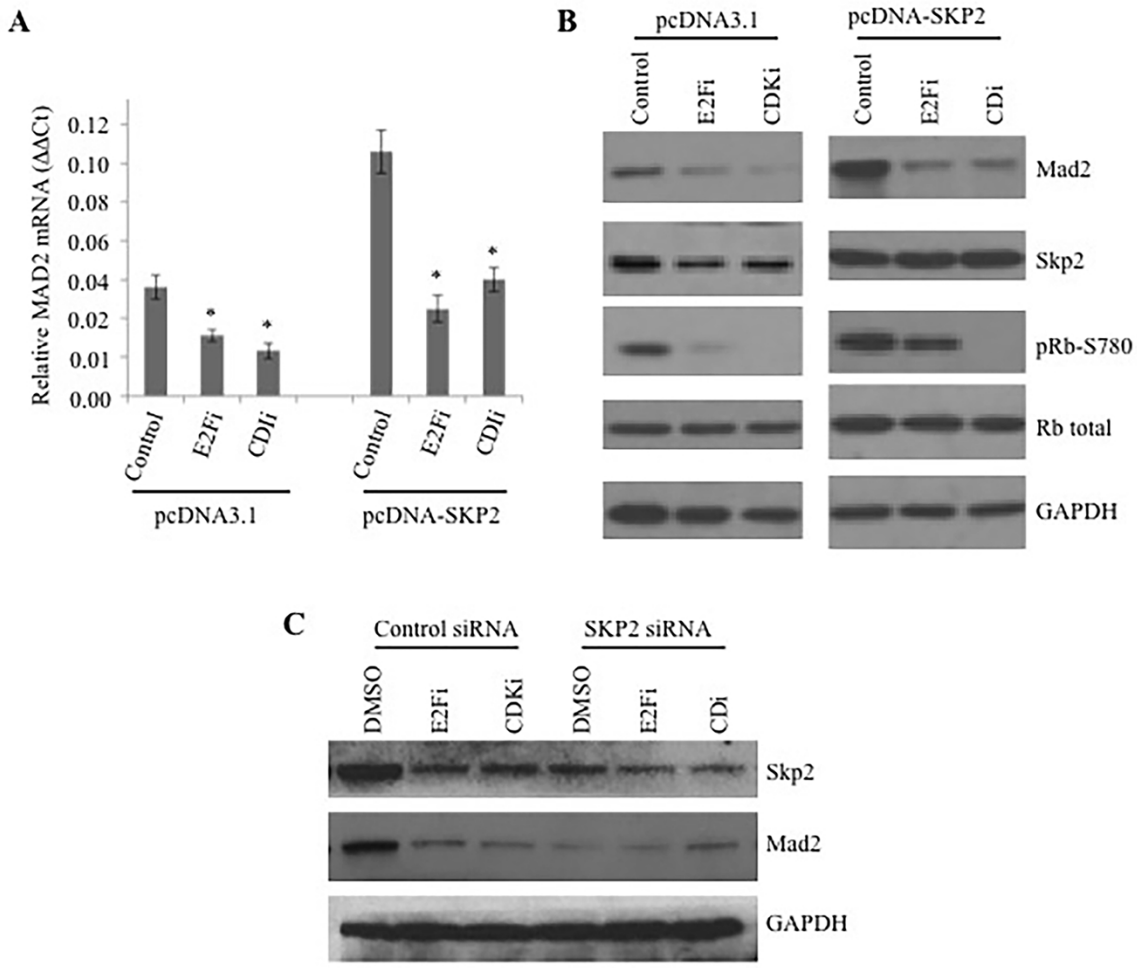


Figure 2. Silencing of SKP2 resulted in increase while overexpression of SKP2 led to decrease of p27.

(A) Human lung cancer A549 and NCI-H1975 cells were transfected with 50 nM control or SKP2 specific siRNA with lipofectamine 2000. 48 hr later, total proteins were extracted for the

211 detection of the protein levels p27, the phsophorylation of Rb at Ser780 (pRb-S780) and
212 Ser807/811 (pRb-S807/811) by Western blotting. GAPDH served as loading control.
213 (B) Human lung cancer A549 and NCI-H1975 cells were transfected with 2 μg of vector
214 pcDNA3.1 or pcDNA-SKP2 for 48 hr. Total proteins were extracted for the detection of the
215 protein levels p27, the phsophorylation of Rb at Ser780 (pRb-S780) and Ser807/811 (pRb-
216 S807/811) by Western blotting. GAPDH served as loading control.
217
218 **Pharmacological inhibition of CDK1/2 or E2F1 abolishes the promotion of the expression**
219 **of Mad2 by Skp2.**
220 MAD2 gene transcription is regulated by Rb-E2F1, which is controlled by CDKs. Our
221 observation that Skp2 promotes the activity of CDKs by downregulating p27 suggest that Skp2
222 may positively regulate MAD2 expression via CDKs-E2F1 axis. To test this hypothesis, we
223 treated A549 cells transfected with pcDNA-SKP2 with CDK1/2 inhibitor flavopiridol or E2F1
224 inhibitor HLM006474, which is a small molecule pan-E2F inhibitor and has been shown to
225 specifically inhibit E2F target genes in melanoma cells and synergizes with paclitaxel lung
226 cancer cells (Ma et al., 2008; Kurtyka et al., 2014). Then, we determined MAD2 expression by
227 RT-QPCR and immunoblotting. In comparison untreated control, flavopiridol or HLM006474
228 alone decreased the mRNA levels of MAD2; Transient transfection of SKP2 plasmid resulted in
229 elevation of the mRNA level of MAD2; whereas, either flavopiridol or HLM006474
230 significantly abolished the increase of MAD2 mRNA by SKP2 overexpression (Fig. 3A). In
231 agreement with the alteration of MAD2 mRNA levels, flavopiridol or HLM006474 alone
232 decreased the protein levels of Mad2 compared with control; ectopic overexpression of SKP2 led
233 to increase of the protein levels of Skp2 and Mad2 and phosphorylation of Rb at Ser780;

234 however, either flavopiridol or HLM006474 apparently prevented the increase of Mad2 protein
235 and pRb-S780 signal but not the level of Skp2 protein (Fig. 3B). In addition, CDK or E2F1
236 inhibitor treatment did not further decrease Mad2 expression in Skp2 knockdown cells (Fig. 3C).
237 Thus, Skp2 promotes the gene transcription of MAD2 via p27-CDKs-E2F1 signaling.



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239 **Figure 3. Pharmacological inhibition of either CDK1/2 or E2F1 prevented the induction of**
240 **the expression of MAD2 by SKP2 overexpession.**

241 (A) Human lung cancer A549 cells were transfected with 2 μg of vector pcDNA3.1 or pcDNA-
242 SKP2 for 48 hr, then treated with CDK1/2 inhibitor flavopiridol or E2F1 inhibitor HLM006474
243 for additional 24 hr. Total RNAs were extracted for the detection of the mRNA levels MAD2 by

RT-QPCR with GAPDH as internal control. Quantitative analysis are expressed as mean ± SEM. n =3, *P <0.05 vs. control.

(B) Human lung cancer A549 cells were transfected with 2 μg of vector pcDNA3.1 or pcDNA-SKP2 for 48 hr, then treated with CDK1/2 inhibitor flavopiridol or E2F1 inhibitor HLM006474 for additional 24 hr. Total proteins were extracted for the detection of the protein levels Skp2 and Mad2, and the phsophorylation of Rb at Ser780 (pRb-S780) by Western blotting. GAPDH served as loading control.

(C) Human lung cancer A549 cells were transfected with 50 nM control or SKP2 specific siRNA with lipofectamine 2000 for 48 hr, then treated with CDK1/2 inhibitor flavopiridol or E2F1 inhibitor HLM006474 for additional 24 hr. Total proteins were extracted for the detection of the protein levels Skp2 and Mad2 by Western blotting. GAPDH served as loading control.

Silencing of Skp2 impairs mitotic checkpoint

Mitotic checkpoint arrests cells in metaphase in response to mitotic spindle damage. Moreover, it has been well documented that increased expression of MAD2 enhances mitotic checkpoint and leads to mitotic arrest (Schuyler et al., 2012). Our results showed that silencing Skp2 resulted in apparent decease of Mad2, implying that downregulation of SKp2 may impair the function of mitotic checkpoint under mitotic spindle damage. To assess this possibility, we transfected A549 cells with SKP2 or MAD2 siRNA for 24 hr, followed by nocodazole treatment for additional 24 hr, and determined cell cycle progression by flow cytometry. Either MAD2 or SKP2 siRNA decreased Mad2 in the presence or absence of nocodazole (Fig. 4A). Nocodazole treatment resulted in apparent elevation of the phosphorylation of histone H3 at Ser10, which was attenuated by either MAD2 or SKP2 siRNA. In comparison to control, either MAD2 or SKP2

siRNA did not apparently altered cell cycle progression in the absence of nocodazole. In contrast, most cells were arrested in G2/M phase with 4N DNA content by nocodazole. Intriguingly, nocodazle-induced G2/M phase arrest was partially reduced by MAD2 siRNA as well as SKP2 siRNA (Fig. 4B). Thus, Skp2 is required for the proper functions of mitotic checkpoint in response to spindle damage.

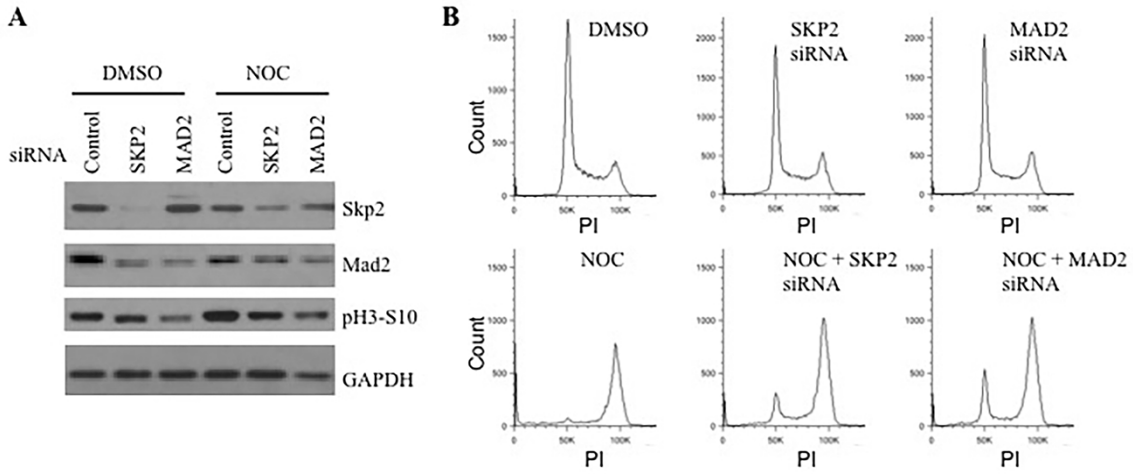

Figure 4. Silencing of SKP2 impaired the G2/M phase cell cycle arrest by nocodazole in A549 cells.

(A) A549 cells were transfected with SKP2 or MAD2 siRNA for 24 hr, followed by nocodazole treatment for additional 24 hr. Total proteins were extracted for the detection of the protein levels Skp2, Mad2 and pH3-S10 by Western blotting. GAPDH served as loading control.

(B) A549 cells were transfected with SKP2 or MAD2 siRNA for 24 hr, followed by nocodazole treatment for additional 24 hr. Cells were stained with DAPI and analyzed by flow cytometry. 2N, diploid DNA; 4N, tetraploid DNA.

Discussion

In this study, we demonstrated that silencing of SKP2 by siRNA led to decrease while ectopic overexpression of SKP2 resulted in increase of MAD2 expression in A549 and NCI-H1975 cells. Moreover, knockdown of SKP2 resulted in elevation of p27 and downregulation of phosphorylation of Rb; whereas overexpression of SKP2 led to downreguation of p27 and upregulation of phosphorylation of Rb. Furthermore, Pharmacological inhibition of either CDK1/2 or E2F1 reduced the increase MAD2 expression by SKP2 overexpession. Finally, silencing of SKP2 impaired the G2/M phase cell cycle arrest by nocodazole in A549 cells. Our findings indicate that Skp2 positively regulates Mad2 via p27-CDKs-E2F1 signaling axis, suggesting that deregulation of SKP2 may at least in part contribute to chromosome instability through MAD2.

Chromosomal instability is a hallmark of cancer cell and may promote chromosome translocations, aneuploidy, gene dosage change and other chromosomal chaos of cancer cells (Holland and Cleveland, 2009). More than 100 years ago, Theodor Boveri stated that chromosomal instability drives tumorigenesis and recent studies demonstrated that chromosomal instability drives a mutation phenotype both in yeast and human cancers (Sheltzer et al., 2011; Solomon et al., 2011). It was once believed that inactivation of SAC (also called mitotic checkpoint) promotes chromosomal instability. However, genetic inactivation mutation of the components of SAC was rarely found in human cancers (Holland and Cleveland, 2009). In contrast, increasing evidence implies that overexpression but not downregulation of the components of SAC results in missegregation of chromosomes and hence genome instability (Holland and Cleveland, 2009; Schvartzman et al., 2011; Sotillo et al., 2007 and 2010; van Deursen, 2007). Oncogene activation leads to chromosomal instability but the underlying mechanism is largely unknown.

It was well documented that MAD2 overexpression promotes tumorigenesis (van Deursen, 2007; Malumbres, 2011). MAD2 is frequently overexpressed in chromosomally unstable tumors (Pérez de Castro et al., 2007). Moreover, MAD2 overexpression is frequently observed in various tumors including liver cancer (Zhang et al., 2008), breast cancer (Scintu et al., 2007), soft-tissue sarcoma (Hisaoka et al., 2008), and NSCLC (Kato et all., 2011). However, the mechanism by which Mad2 is upregulated in lung cancer is largely unknown. Our observation that Skp2 positively regulates Mad2 via the p27-CDKs-E2F1 signaling pathway, suggest that oncogene activation such as SKP2 may promote chromosome instability through deregulating MAD2.

MAD2 was identified as a direct target of E2F1 and hence Rb inactivation leads to overexpression of MAD2 (Hernando et al., 2004). Moreover, overexpression of MAD2 leads to chromosomal instability and enhances tumorigenesis in mouse models (Sotillo et al., 2007). Recently it was shown that increase of MAD2 is essential for the induction of aneuploidy by Rb inactivation (Schvartzman et al., 2011). As Rb signaling circuit is deregulated in most tumors and SKP2 is highly expressed in NSCLC, our data suggest that oncogene induced-chromosomal instability is probably through 'oncogene-induced mitotic stress' (Malumbres, 2011). It will be important to elucidate the mechanisms of the deregulation of SKP2 in NSCLC.

Consistence with mitotic checkpoint function of MAD2, knockdown of MAD2 by siRNA resulted in premature mitosis progression following nocodazole treatment. It is well established that MAD2 is controlled by CDKs (Sotillo et al., 2007). In consequence, inhibition of either CDKs or E2F by specific inhibitors led to precocious inactivation of mitotic checkpoint. With a long history of clinical application, mitotic spindle-targeting agents including Vinca alkaloids, taxanes and epothilones are the most classical and reliable anticancer drugs (Chan et al., 2012).

We showed that inhibition of CDKs or E2F by specific inhibitors resulted in decrease of MAD2 expression, it will be important to investigate whether CDKs or E2F specific inhibitors sensitize NSCLC cells to mitotic spindle-targeting chemotherapies.

Conclusions

In summary, we found that SKP2 positively regulated the gene expression of MAD2 through p27-CDKs-E2F1 signaling pathway. Our findings may provide an explanation of the simultaneous upregulation of MAD2 and SKP2 in lung cancer.

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Competing financial interests

The authors declare that they have no competing financial interests.

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