hnRNP K suppresses apoptosis independent of p53 status by maintaining high levels of endogenous caspase inhibitors

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Hepatocellular carcinoma (HCC) is the third highest cause of cancer-related deaths globally. One of the cellular hallmarks of this disease is dysregulation of apoptosis, and a better understanding of this process is important if progress is to be made toward effectively treating HCC. Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a RNA-binding protein that is implicated in apoptosis and is upregulated in various cancers, including HCC. In this study, we report new evidence for a crucial role of hnRNP K in suppressing apoptosis in HCC cells. We used the chemotherapeutic agent 5-fluorouracil to induce apoptosis in HCC cell lines and found that hnRNP K was downregulated, independent of both p53 and caspases. Prolonged downregulation of hnRNP K using small interfering RNA (siRNA) significantly decreased cell viability and increased apoptosis in HCC cell lines in a p53-independent manner. Moreover, enhanced tumor necrosis factor-related apoptosis-inducing ligand potency, independent of BH3-interacting domain death agonist (BID) cleavage, was also observed in hnRNP K siRNA-treated cells. Examination of the underlying mechanism revealed that hnRNP K suppresses the activity of various caspases through controlling transcription of the caspase inhibitor XIAP. Taken together, this study establishes that hnRNP K plays an antiapoptotic role in HCC cell lines, independent of p53 status, via the maintenance of high levels of endogenous caspase inhibitors, and also identifies hnRNP K as a possible therapeutic marker for cancer treatment.

Introduction

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a highly conserved RNA-binding protein that was initially identified as part of the hnRNP complex (1). Apart from processing pre-mRNA, the ability of hnRNP K to reside in either the cytoplasm or the nucleus enables this protein to contribute to additional cellular functions including chromatin remodeling, transcription and translation (2). hnRNP K is frequently overexpressed in human cancers (3–8) and thought to play a role in tumor progression and malignancy, as high levels of expression correlate with poor clinical outcomes (3,4,9,10). hnRNP K has also been demonstrated to affect metastasis (11), positively regulate the expression of oncogene (12,13), bind to telomeric DNA (14) and serve as a transcriptional cofactor for p53 during the DNA damage response (15), further supporting its involvement in cancer development. Perhaps most importantly, however, hnRNP K has been implicated in apoptosis (15–20) but its precise role in this process has not been clearly defined.

Apoptosis is a regulated cell death process necessary to control cellular turnover, without which uncontrolled cellular proliferation can lead to the development of cancer. When apoptosis is triggered, it activates a signaling cascade involving sequential proteolytic cleavage of initiator and effector caspases that ultimately leads to the activation of caspase-3 and -7. To protect against unnecessary caspase activity, cells maintain the expression of various caspase inhibitors, such as cFLIP that prevents pro-caspase-8 activation and XIAP that sequesters activated caspase-3 and -7 (21). Reduction of caspase activity and upregulation of caspase inhibitors can dysregulate apoptosis, thereby contributing to the therapeutic resistance of many human malignancies (22). Therefore, understanding the underlying mechanism for altered apoptotic signaling would be instrumental in providing better cancer treatment strategies (23).

Hepatocellular carcinoma (HCC) is the fifth most common cancer and is the third highest cause of cancer-related death globally (24), with apoptosis dysregulation as one of its cellular hallmarks (25). Besides being overexpressed (26–28) and potentially serves as a marker for HCC (29), hnRNP K is also important for hepatitis B virus replication (30) and contributes to hepatitis C virus pathogenesis (31), both of which are important etiological factors contributing to this malignancy. 5-Fluorouracil (5-FU) is an antimetabolite commonly used as a first-line chemotherapeutic agent against HCC (32). Interestingly, hnRNP K is downregulated by 5-FU and this phenomenon has been suggested as a biomarker for chemosensitivity (33), although a mechanistic association between hnRNP K downregulation and chemosensitivity remains to be proven. Moreover, as 5-FU induces the expression of the tumor suppressor p53 (34), whether the cause and effect of hnRNP K downregulation is dependent on p53 status requires further clarification.

By using HepG2 and Hep3B HCC cell lines that express high endogenous levels of hnRNP K, we demonstrated that 5-FU-induced hnRNP K downregulation is independent of p53. hnRNP K was crucial for suppressing apoptosis, as specific knockdown using small interfering RNA (siRNA) resulted in reduced expression of the antiapoptotic mediators cFLIP and XIAP. This novel role of hnRNP K in suppressing caspase-3 and -7 activities through regulating XIAP expression suggests how elevated hnRNP K expression in malignancies can confer resistance to apoptosis. Thus, our results help in identifying hnRNP K as a strong candidate for a therapeutic marker in cancer treatment.

Materials and methods

Reagents, siRNAs and hnRNP K expression constructs

5-FU and actinomycin D were purchased from Sigma–Aldrich, whereas pan-caspase inhibitor Z-VAD-fmk (Z-Val-Ala-Asp(OMe)-CH2F) was purchased from R&D Systems (Minneapolis, MN) and TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) (SuperKillerTRAIL™) was purchased from Enzo Life Sciences (Farmingdale, NY). Stealth select RNAi duplex for human HNRPK, TP53 and non-specific control siRNA were purchased from Invitrogen (Carlsbad, CA). The open reading frames of hnRNP K isoforms a and b were first amplified from hnRNP K expression constructs generated in previous study (30) with open reading frame cloning primers listed in Supplementary Table 1, available at Carcinogenesis Online, and subsequently cloned into pXJ40-HA vector using Xhol and Smal restriction sites. To generate hnRNP K isoforms a and b expression plasmids that harbor four silent mutations (HA-hnRNP K-a and HA-hnRNP K-b) for hnRNP K expression level restoration analyses, four successive rounds of site-directed mutagenesis using QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) were carried out using resultant expression plasmids from each round as template for the subsequent PCR with site-directed mutagenesis primers listed in Supplementary Table 1, available at Carcinogenesis Online.

Cell culture and transfection

Human HCC cell lines Hep3B and HepG2 were purchased from the ATCC (Manassas, VA). The human colorectal carcinoma isogenic HCT116 cell lines were kindly provided by Dr Bert Vogelstein (Johns Hopkins University).
Cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (Invitrogen) and kept at 37°C in 5% CO₂ conditions. For 5-FU treatment, the drug was first dissolved in dimethyl sulfoxide (DMSO) and further diluted in medium before use. Cells were treated with 20ng/ml 5-FU unless otherwise stated. To examine whether hnRNP K downregulation by 5-FU was due to caspase cleavage, caspase activity was inhibited by pretreating cells with 10 μM of Z-VAD-fmk, prior to drug treatment. Transection of siRNA (10–40nM/10cm² well) and/or expression plasmids (2μg/10cm² well) were considered 50–60% confluent cells was mediated by Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions.

Western blotting
Cells were lysed on ice with lysis buffer (50mM Tris–HCl, pH 7.8, 150mM NaCl, 1% NP-40) containing 1mM phenylmethylsulfonyl fluoride (Sigma–Aldrich) and 1× complete mini protease inhibitor mixture (Roche Applied Science, Mannheim, Germany). Equal amounts of proteins were separated by 10–12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto Immobilon-P® polyvinylidine difluoride membranes (Merck Millipore, Billerica, MA). Antibodies against hnRNP K (1-DNA Biotechnology Pte Ltd, Singapore), HA-tag (Sigma–Aldrich), p53, caspase-8 (Santa Cruz Biotechnology, Santa Cruz, CA), cleaved poly (ADP ribose) polymerase 1 (PARP-1), BAX-interacting domain death agonist (BID) (Cell Signaling, Danvers, MA) and XIAP (Abcam, Cambridge, MA) were utilized as primary antibodies in this study for protein detection and were visualized with appropriate horseradish peroxidase-conjugated secondary antibodies (Dako, Denmark) using SuperSignal West FemtoChemiluminescent Substrate reagents (Thermo Fisher Scientific, Rochester, NY) for detection. Loading of protein samples was verified with antibodies to β-actin (Merck Millipore).

Caspase activity assay
To determine caspase-3/7 or caspase-8 activity in differentially transfected or treated cells, Caspase Glo® 3/7 or 8 assay (Promega, Fitchburg, WI) was utilized according to manufacturer's instructions. Briefly, the total cell lysates of the cells were first collected in lysis buffer. Thereafter, Caspase Glo® 3/7 or 8 reagent was added to 10 μg of total cell lysate per reaction in triplicates in 96-well Flat Bottom Black Polystyrene Plates (Corning, Linlfield, Australia) and incubated for 1 h at room temperature before caspase activity was measured with GloMax® 96 Microplate Luminometer (Promega) with manufacturer's predefined settings.

Cell proliferation and quantification assay
To examine the effect of 5-FU on cell viability, cells were plated at 60% confluency in 96-well plates in triplicate, treated with 20ng/ml 5-FU, and proliferation of viable cells was measured daily using the modified 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay, WST-1 reagent (Roche, according to manufacturer's instructions. Similarly, to examine the effect of hnRNP K knockdown on HCC cell viability, cells were transiently transfected with siRNA K-specific siRNA or non-specific control siRNA in 10 cm² wells before replating into 96-well plates in triplicate, at 24h post-transfection, and proliferation of viable cells was analyzed with WST-1 reagent at each time point indicated. Absorbance was measured using the Infinite® 200 PRO plate reader (Tecan, Männedorf, Switzerland) at 450 nm with reference at 690 nm.

RNA isolation and real-time quantitative reverse transcription–polymerase chain reaction
Total RNA was extracted from cells using NucleoSpin® RNA II with in-column DNase-I treatment (Machery-Nagel) and the first stand cDNA was synthesized using Maxima® First Strand cDNA Synthesis Kit (Thermo). The real-time quantitative reverse transcription–polymerase chain reaction was performed using KAPA SYBR® FAST Roche LightCycler®480 2× qPCR Master Mix (KapaBiosystems, Woburn, MA) in the Roche LightCycler® 480 System (Roche). HPRT was used for normalization in all real-time quantitative reverse transcription–polymerase chain reactions performed and primers utilized are listed in Supplementary Table 2, available at Carcinogenesis Online.

Terminal deoxynucleotidyl transferase dUTP nick end labeling assay
Terminal deoxynucleotidyl transferase dUTP nick end labeling assay (TUNEL) assay was carried out with in situ Cell Death Detection Kit, Fluorescein (Roche), according to manufacturer’s instructions. Briefly, at 48h post-transfection, Hep3B cells were replated into 4-well culture slides (BD Biosciences, Franklin Lakes, NJ) and cultured for an additional 48h. Cells on culture slides were rinsed with phosphate-buffered saline and fixed with 4% paraformaldehyde. Fixed cells were then permeabilized with 1% Triton X-100 in 0.1% sodium citrate and washed with phosphate-buffered saline again before TUNEL labeling and subsequent mounting with Prolong Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Cells were observed under the Zeiss Fluorescence Microscope (Carl Zeiss AG) with the fluorescein isothiocyanate and DAPI channels and imaged with the attached Canon Powershot camera (Canon, Tokyo, Japan). Fluorescent cells on the images captured were counted using Image-Pro Software (Media Cybernetics, Bethesda, MD).

Flow cytometric analysis
Hep3B cells transiently transfected with hnRNP K-specific siRNA or negative control siRNA for 72 and 96h were collected and fixed with ice-cold 70% ethanol overnight. Fixed cells were treated with 100 μg/ml RNase A (Sigma–Aldrich) for 5 min prior to staining with 50 μg/ml of propidium iodide (Sigma–Aldrich) at room temperature for 1h in the dark. DNA content of stained cells was subsequently analyzed with FACS Calibur and CellQuest software (BD Biosciences).

Statistical analyses
Analysis of variance with F-test was carried out to determine differences between the means, followed by the two-tailed Student’s t-test with equal or unequal variance to determine the statistical significance of results. Results are shown as mean ± SD for representative experiments. The P values <0.05 were considered significant:

Results
5-FU downregulates hnRNP K expression independent of both p53 and caspases
It has been suggested that hnRNP K expression is regulated by p53 (35,36) and that 5-FU induces upregulation of p53 (34). Thus, to determine whether 5-FU-mediated downregulation of hnRNP K is dependent on p53 status, we subjected HCC cell lines with different p53 statuses. HepG2 (p53+/+) and Hep3B (p53−/−), to 5-FU treatment. hnRNP K protein levels were lowered in both HCC cell lines in a 5-FU dose-dependent manner by 12h and more so at 48h post-treatment. In addition, increased expression of p53 was observed only in HepG2 following 5-FU treatment (Figure 1A). Apart from these two HCC cell lines, 5-FU-induced hnRNP K reduction was also observed in various other HCC cell lines with different p53 statuses (Supplementary Figure 1, available at Carcinogenesis Online), further confirming the independence from p53 in hnRNP K down regulation following 5-FU treatment. Furthermore, the mRNA expression levels of hnRNP K in both Hep3B and HepG2 were also significantly downregulated (P <0.05) following 5-FU treatment (Figure 1B), suggesting that the reduction in hnRNP K protein level was driven by a decrease in mRNA.

The viability of both Hep3B and HepG2 cells treated with 5-FU decreased with time and was similarly independent of p53 status (Figure 1C). This is consistent with previous reports where 5-FU-induced apoptosis occurred in p53 mutant cell lines, though it has also been reported to be p53 dependent (37). As hnRNP K has been identified as a caspase substrate in large-scale screening studies (38,39), we went on to ask whether the decrease in hnRNP K protein in response to 5-FU was caspase dependent. Hep3B and HepG2 cells were treated with 5-FU alone or in combination with pan-caspase inhibitor (Z-VAD-fmk) for 48h. Pan-caspase inhibitor treatment did not affect 5-FU-induced hnRNP K protein downregulation (Figure 1D). To confirm the validity of these results, inhibition of caspase activity following Z-VAD-fmk treatment was demonstrated using a caspase-3/7 activity assay. 5-FU treatment had significantly increased caspase-3/7 activity in both Hep3B and HepG2 (P <0.05) by ~2.5- and 10-fold, respectively, compared with DMSO-treated controls, and this increase was abrogated by the pan-caspase inhibitor. The phenomenon is not limited to HCC cell lines, as similar results were obtained with the colorectal cancer cell line HCT116 and its isogentic p53-null cell line (Supplementary Figure 2, available at Carcinogenesis Online).These data show that 5-FU causes apoptosis in cells independent of p53 status, though the presence of p53 augments 5-FU-induced apoptosis, as reflected by the higher caspase-3/7 activity recorded in HepG2 compared with Hep3B cells (Figure 1E).

In summary, 5-FU-induced hnRNP K downregulation is independent of both caspases and p53, which intriguingly correlates with the observation that 5-FU-induced apoptosis is also p53 independent.
hnRNP K is required for HCC cell viability

To investigate the role of endogenous hnRNP K in HCC cells, we transiently knocked down hnRNP K in HCC cell lines using siRNA. Silencing of hnRNP K expression with siRNA in Hep3B and HepG2 was verified at the protein (Figure 2A) and mRNA (Figure 2B) levels. As 5-FU-induced hnRNP K downregulation correlated well with the decrease in HCC cell viability (Figure 1C), we measured HCC cell viability in hnRNP K-silenced cells with a modified 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrazolium bromide assay using WST-1 reagent. Knockdown of hnRNP K reduced HCC cell viability monitored with time compared with HCC cells transfected with negative control siRNA (Figure 2C). Moreover, silencing of hnRNP K resulted in a reduction in cell number compared with control (Figure 2D). These data suggest that hnRNP K plays an important role in the viability of HCC cells.

hnRNP K reduction induces apoptosis independent of p53 status

We further evaluated whether the reduction of HCC cell viability observed with the knockdown of hnRNP K was a result of the induction of apoptosis in these cell cultures. hnRNP K was first knocked down with siRNA in p53-null Hep3B cells, then cell lysates were probed for the presence of cleaved PARP-1, which indicates apoptosis. Cleaved PARP-1 was evident in hnRNP K-silenced Hep3B cells 72 h post-transfection (Figure 3A), indicating that apoptosis was induced. Consistent with the detection of cleaved PARP-1, caspase-3/7 activity was also significantly increased (P < 0.01) at the same time point (Figure 3A), as was the percentage of TUNEL-positive cells (P < 0.01) at 96 h post-transfection (Figure 3B). These data in Hep3B cells indicate that an extended duration of hnRNP K reduction alone is capable of inducing apoptosis, without the need for exogenous cytotoxic agents or any contribution from p53.

Interestingly however, downregulation of hnRNP K also reduced the viability of HepG2 cells, which possess wild-type p53 (Figure 2C). Therefore, we next investigated the role of p53 in the apoptosis induced by hnRNP K downregulation by simultaneously knocking down p53 and hnRNP K with siRNAs in HepG2 cells. Silencing of both hnRNP K and p53 was validated with quantitative real-time PCR at 48 h post-transfection (Figure 3C), as well as with western blot at 72 h post-transfection (Supplementary Figure 3, available at Carcinogenesis Online), and resulted in a significant increase in caspase-3/7 activity 72 h post-transfection that was similarly observed.
hnRNP K suppresses apoptosis

To demonstrate that the apoptosis induced in HCC cells was hnRNP K specific, we restored the declining endogenous levels of hnRNP K brought about by siRNA with expression constructs encoding the two known major hnRNP K isoforms, isoforms a and b (30). Four silent mutations T1182A, A1188G, T1197A and T1203A were introduced into the hnRNP K isoforms nucleotide sequence such that their mRNA would not be targeted by our hnRNP K-specific siRNA. These constructs were subsequently cotransfected individually with the siRNAs into Hep3B. Our results demonstrate that the levels of hnRNP K isoforms were successfully restored even in the presence of hnRNP K-specific siRNA, by 24 h post-transfection (Figure 4A). Subsequent flow cytometric analysis allowed quantification of hypodiploid cells (sub G1/G0) in the differentially transfected cells with hnRNP K levels restored, revealing that restoration of either isoform a or b was able to reduce the percentage of hypodiploid cells from 31.3 to 21.6% and 25.4%, respectively, at 72 h post-transfection and from 53.9 to 29.4% and 35.2%, respectively, at 96 h post-transfection (Figure 4B). Similarly, when the degree of apoptosis in the differentially transfected cells was visualized using the TUNEL assay, restoration of hnRNP K isoform a or b reduced the percentage of TUNEL-positive cells from 40.8% to 23.6% (P < 0.01) and 30.7% (P < 0.05), respectively (Figure 4C). These results confirmed our findings presented above that the apoptosis observed in HCC cell lines is hnRNP K specific, and restoration of hnRNP K levels reduces the amount of apoptosis induced. Furthermore, it appears that hnRNP K can function as a genuine antiapoptotic protein.

hnRNP K reduction sensitizes HCC cells to TRAIL-induced apoptosis

To address the role of hnRNP K functioning as an antiapoptotic protein, we assessed caspase-3/7 activity in hnRNP K-silenced HCC cells following stimulation of apoptosis. As we have shown that hnRNP K reduction-induced apoptosis is p53 independent, and it is known that the extrinsic apoptotic pathway is also independent of p53 (40), we asked what the relationship was between hnRNP K and apoptosis via the extrinsic pathway. We addressed this question using TRAIL, which activates various cell death receptors (41). HCC cell lines were pretransfected with hnRNP K-specific siRNA for 24 h, when apoptosis is not yet detectable (Figure 3A and C), prior to the addition of 2 ng/ml of TRAIL for another 8 h and subsequent analysis. Consistent with the results above, HCC cells with or without hnRNP K silenced for 32 h revealed no significant difference in caspase-3/7 activity (Figure 5A). However, caspase-3/7 activity measured 8 h following TRAIL treatment was significantly elevated in cells with hnRNP K silenced, compared with controls, for both Hep3B and HepG2 by ~1.7-fold (P < 0.01) and 5.4-fold (P < 0.01), respectively. The results thus revealed that downregulation of hnRNP K sensitizes cells to apoptosis induced by TRAIL.

To confirm that HCC cells were sensitized to TRAIL-induced apoptosis specifically by hnRNP K, an increasing concentration of hnRNP K-specific siRNA was transfected into both HCC cell lines to generate a gradient of hnRNP K knockdown for 24 h prior to 8 h of

(mean ± SD) percentage absorbance relative to the absorbance of cells from each treatment group at Day 1 post-transfection. (D) Images of HCC cells, Hep3B and HepG2, by phase contrast microscopy at 96 h post-hnRNP K downregulation. si-ctrl, non-specific control siRNA; si-K, hnRNP K-specific siRNA.
hnRNP K reduction induces apoptosis independently of p53. (A) hnRNP K downregulation induces apoptosis from 72 h post-transfection in p53-null Hep3B cells. hnRNP K and cleaved PARP-1 levels were examined in total cell lysates of Hep3B transfected with si-K (40 nM) across the different time points indicated; polyclonal antibody specifically recognizing the 89 kDa fragment of caspase-cleaved PARP-1 was utilized (upper panel). Caspase-3/7 activity in Hep3B cells transfected with 40 nM of si-K was measured at the time points indicated. Data expressed as normalized caspase-3/7 activity relative to cells transfected with si-ctrl at each time point (lower panel). (B) Differentially transfected Hep3B cells were subjected to the TUNEL assay at 96 h post-transfection; representative images of fields under the fluorescence microscope are depicted (left panel), where DAPI-stained cells and TUNEL-labeled cells represent total cell population and those undergoing apoptosis, respectively, which were quantified using Image-Pro software. Data were expressed as percentage of TUNEL-positive cells from Hep3B transfected with si-ctrl and si-K in the total cell population obtained from triplicates. (C) Apoptosis induced by hnRNP K downregulation does not involve p53. Single knockdowns of hnRNP K (si-K) or p53 (si-p53) and simultaneous knockdowns of hnRNP K and p53 (si-K + si-p53) were carried out in HepG2, which possess wild-type p53. Knockdown of both genes was verified with real-time quantitative PCR at 48 h post-transfection; data expressed as relative fold expression compared with cells transfected with non-specific control siRNA (si-ctrl) after normalization to HPRT expression level. (D) Caspase-3/7 activity for these differentially transfected HepG2 cells was also determined with the caspase-3/7 assay; data expressed as normalized caspase-3/7 activity relative to cells transfected with si-ctrl at each time point indicated. **P < 0.01. si-ctrl, non-specific control siRNA; si-K, hnRNP K-specific siRNA; si-p53, p53-specific siRNA.

As high levels of hnRNP K enable cells to resist apoptosis, as exemplified by its ability to impede TRAIL-induced apoptosis, we set out to define the mechanism underlying its antiapoptotic function. We first assessed whether the enhanced TRAIL-induced caspase-3/7 activity by hnRNP K reduction was independent of enhanced BID cleavage. It was observed that with obvious BID cleavage, 50 ng/ml of TRAIL produced caspase-3/7 activity (Supplementary Figure 4, available at Carcinogenesis Online) of comparable magnitude generated by 2 ng/ml of TRAIL in hnRNP K-silenced HepG2 (Figure 5A). However, the levels of full-length BID were comparable in differentially transfected HCC cells treated with 2 ng/ml of TRAIL regardless of hnRNP K reduction (Figure 6A). This strongly suggested that the observed enhancement to TRAIL-induced caspase-3/7 activity by hnRNP K knockdown was independent of enhanced BID cleavage.

As such, we hypothesized that the enhancement of TRAIL-induced caspase-3/7 activity in HCC cells by hnRNP K downregulation could occur through either, or both, of two distinct mechanisms: the reduction of endogenous caspase-3/7 inhibitor or enhanced upstream caspase-8 signaling. We first addressed the possible contribution of enhanced caspase-8 signaling to the enhanced TRAIL-induced caspase-3/7 activity observed in hnRNP K knockdown cells. Caspase-8 activity was indeed significantly increased in TRAIL-treated HCC cells with hnRNP K silenced compared with those transfected with negative control siRNA (P < 0.01 for both Hep3B and HepG2) (Figure 6B).

Moreover, the possibility that increased caspase-8 expression contributed to its enhanced activity observed can be discounted, as endogenous levels of caspase-8 were comparable between HCC cells with or without hnRNP K knocked down (Figure 6B). As the caspase-8 inhibitor cFLIP was recently shown to be transcriptionally activated by hnRNP K (16), it could therefore be inferred that enhanced caspase-8 signaling plays a role in contributing to the heightened caspase-3/7 activity observed in TRAIL-treated and hnRNP K-silenced HCC cells.
The possibility of a reduction in an endogenous caspase-3/7 inhibitor was also assessed. We next determined the expression level of XIAP, a well-known potent effector caspase inhibitor (43), in HCC cell lines with hnRNP K silenced. Our results demonstrated a decrease in XIAP expression level following hnRNP K knockdown in both HCC cell lines tested (Figure 6C) and therefore suggested that hnRNP K knockdown reduces the protein expression level of XIAP, thereby contributing to enhanced TRAIL-induced caspase-3/7 activity through the reduction of effector caspase inhibition.

As hnRNP K also acts as a transcription factor, we next asked whether the reduction of XIAP occurred at the mRNA level. XIAP mRNA expression levels were examined in Hep3B and HepG2 with hnRNP K silenced and cFLIP was utilized as a positive control. As expected, cFLIP mRNA expression levels were reduced in both HCC...
cell lines (Figure 6D), which is consistent with a previous study (16) as well as providing further support for the contribution of enhanced caspase-8 signaling toward augmented TRAIL-induced caspase-3/7 signaling in hnRNP K-silenced cells. Notably, the results also revealed a significant reduction in XIAP mRNA expression level in Hep3B (P < 0.01) and HepG2 (P < 0.01) cells with hnRNP K silenced, indicating that the observed reduction in XIAP is likely due to a reduction in its transcription. Progressive decrease in XIAP mRNA expression levels was further detected in hnRNP K-silenced Hep3B over time (Supplementary Figure 5A, available at Carcinogenesis Online) and treating hnRNP K-silenced Hep3B cells with 5 μg/ml of actinomycin D to block de novo mRNA synthesis revealed that the regulation of XIAP by hnRNP K does not occur at the post-transcriptional level, but likely at the transcriptional level (Supplementary Figure 5B, available at Carcinogenesis Online). Taken together, our findings lead us to conclude that the underlying mechanism for hnRNP K’s antiapoptotic function is likely mediated via its ability to maintaining constant levels of endogenous caspase inhibitors.

Discussion
The frequent detection of elevated hnRNP K expression in various cancers such as those in the liver, breast, colon and prostate (3–8) suggests that it plays a role in tumor development. In support of this, hnRNP K has been implicated in several biological functions crucial for cancer development (11,14) including apoptosis (15–20), which this study aimed to examine in greater detail. With the use of HCC cell lines, our results demonstrated that extended duration of hnRNP K downregulation using siRNA was sufficient to induce apoptosis (Figure 3) and that this effect was hnRNP K specific, as the restoration of declining endogenous levels of hnRNP K reduced the degree of apoptosis (Figure 4). Moreover, though silencing hnRNP K at earlier time points (i.e. 24 and 48 h post-transfection) did not induce apoptosis (Figure 3A and D), it was able to sensitize HCC cells to TRAIL-induced apoptosis as exemplified by the dramatic enhancement in TRAIL-induced caspase-3/7 activity (Figure 5A and B). The induction of apoptosis after sustained hnRNP K downregulation in different cells might be dependent on the availability and type of apoptotic stimuli present in each cell type, thus accounting for the apparent lack of direct effects on cell viability and apoptosis in some studies involving silencing of hnRNP K (15,16,20). Nevertheless, the results from this study are concordant with those from recent reports that similarly knocked down hnRNP K in various other cell types (17–19). More importantly, our data showed that hnRNP K could directly suppress TRAIL-induced caspase-3/7 activity (Figure 5C), providing confirmation for hnRNP K’s function as an antiapoptotic protein. Of note, our results indicate that apoptosis induced via hnRNP K reduction does not involve p53 (Figures 3A and B and 6D). As p53 is mutated in more than half of all cancers and inactivated by indirect mechanisms in a large proportion of the remainder (44), pathways mediating p53-independent apoptosis, such as that involving hnRNP K as demonstrated here, are appealing for therapeutic intervention.

hnRNP K participates in RNA and DNA processes such as transcription, mRNA splicing and stability (2). We revealed for the first time that hnRNP K positively regulates the caspase-3/7 inhibitor XIAP at the mRNA level (Figure 6D and Supplementary Figure 5, available at Carcinogenesis Online) and consistent with previous findings (16), our results also showed that hnRNP K can regulate the expression of caspase-8 inhibitor cFLIP (Figure 6D). Hence in response to TRAIL-induced apoptosis, the alleviation of caspase-8 and -3/7 inhibition by the combined reduction in cFLIP and XIAP expression when hnRNP K is downregulated reflects that hnRNP K is important for maintaining high levels of endogenous caspase inhibitors. With the results from our study, we proposed a mechanistic model for the antiapoptotic function of hnRNP K (Figure 6E), where high levels of hnRNP K maintain the expression of endogenous caspase inhibitor XIAP. This may be applied to human HCC, where tumor cells have elevated levels of hnRNP K (29) and XIAP (45–47) when compared with peripheral non-tumor tissues. The model also accounts for the loss of
hnRNP K suppresses apoptosis

Fig. 6. Downregulation of hnRNP K reduces the level of endogenous caspase inhibitors. (A) Enhanced TRAIL-induced apoptosis by hnRNP K downregulation is independent of enhanced BID cleavage. hnRNP K and full-length BID protein levels with β-actin as loading control in HepG2 and Hep3B transfected with 40 nM si-ctrl or si-K for 24 h prior to 8 h incubation with 2 ng/ml of TRAIL were examined with western blot. (B) Knockdown of hnRNP K augments TRAIL-induced caspase-8 activity. Hep3B and HepG2 transfected with 40 nM of si-ctrl or si-K for 24 h were treated with 2 ng/ml of TRAIL for additional 8 h prior to caspase-8 activity measurement. Normalized caspase-8 activity was expressed relative to untreated cells transfected with si-ctrl. hnRNP K and pro-caspase-8 protein levels with β-actin as loading control were examined with western blot for total cell lysates from Hep3B and HepG2 transfected with 40 nM of si-K (+) or si-ctrl (−) for 24 h. (C) Reduction of XIAP protein levels following hnRNP K downregulation. XIAP and hnRNP K protein levels with β-actin as loading control were examined in total cell lysates from Hep3B and HepG2 transfected with 40 nM of si-K (+) or si-ctrl (−) for 24 h (left panel) with corresponding densitometry data of XIAP expression relative to the β-actin (right panel). (D) hnRNP K regulates mRNA expression of cFLIP and XIAP. cFLIP and XIAP mRNA expression levels were assessed with real-time quantitative PCR in Hep3B and HepG2 transfected with 40 nM of si-ctrl or si-K for 24 h, expressed as relative fold expression compared with cells transfected with si-ctrl after normalization to HPRT expression level. (E) Mechanistic model underlying the antiapoptotic function of hnRNP K. Elevated expression levels of hnRNP K in malignancies such as HCC result in high expression levels of the endogenous caspase inhibitor, XIAP, which can suppress the activity of caspase-3/7 (left panel). However, with a reduction of hnRNP K expression levels such as during treatment with chemotherapeutic drug 5-FU, expression of XIAP thus decreases and subsequently results in a reduction in caspase activity inhibition. Coupled with the enhanced caspase-8 activity observed with hnRNP K reduction, these explain the sensitization to death-ligand-induced apoptosis (right panel). *P < 0.05, **P < 0.01. si-ctrl, non-specific control siRNA; si-K, hnRNP K-specific siRNA.
XIAP expression following hnRNP K reduction in sensitizing cells to death-ligand-induced apoptosis, for which the loss of XIAP expression alone in hepatocytes has been shown to deliver the same effect without the need for BID cleavage (48). Similarly, this model could also be applicable to CFLIP, which has been shown to be transcriptionally activated by hnRNP K (16) and found to be upregulated in human HCC (49). Reduced caspase activity and dysregulated expression of caspase inhibitors are indeed examples of mechanisms cancer cells acquire to evade apoptosis (23), which is one of the hallmarks of cancer (22) including HCC (25,50), and the cause of enormous challenges in treating the disease.

Downregulation of hnRNP K has often been observed together with decreased cell survival in cancer cell lines treated with various chemotherapeutic agents such as mitomycin C (36), RITA (51), docetaxel (52) and bicalutamide (3). In this study, we have showed the physiological relevance of hnRNP K downregulation during chemotherapy-drug treatment, where 5-FU treatment reduces the expression level of hnRNP K. Our results demonstrated that this effect is independent of p53 as well as caspases and instead depends on the reduction of hnRNP K mRNA expression (Figure 1 and Supplementary Figures 1 and 2, available at Carcinogenesis Online). In agreement with previous studies (51,53), 5-FU also reduces HCC cell viability (Figure 1C) via apoptosis in both p53-dependent and -independent manners as indicated by the different degree of caspase-3/7 activity induced in HepG2 and Hep3B (Figure 1E). Hence, taking into account that hnRNP K exerts an antiapoptotic effect via the maintenance of endogenous caspase inhibitors’ levels, it could be hypothesized that its reduction contributes to the chemotherapeutic effect of 5-FU observed in HCC cells. Furthermore, hnRNP K downregulation has been implicated in several other cell death pathways involving proteasomes (17), calpain (54) as well as granzymes (19). Collectively, these observations have positioned hnRNP K as an important antiapoptotic protein in cancer cells and indicated that its downregulation is a common mechanism in ensuring the execution of cell death.

In conclusion, this study describes the antiapoptotic function of hnRNP K, where its high expression in HCC cell lines maintains high levels of endogenous caspase inhibitors to suppress apoptosis independently of p53 status. This, thus, shed light on how elevated hnRNP K expression in malignancies confronting resistance to apoptosis and identifies hnRNP K as a potential therapeutic target for the inhibition of tumor progression as well as a therapeutic marker for cancer treatment to indicate chemotherapeutic response.

Supplementary material

Supplementary Figures 1–5 and Tables 1 and 2 can be found at http://carcin.oxfordjournals.org/

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References

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