Overexpression of a Novel Activator of PAK4, the CDK5 Kinase–Associated Protein CDK5RAP3, Promotes Hepatocellular Carcinoma Metastasis

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Abstract

The CDK5 kinase regulatory subunit-associated protein 3 (CDK5RAP3 or C53/LZAP) regulates apoptosis induced by genotoxic stress. Although CDK5RAP3 has been implicated in cancer progression, its exact role in carcinogenesis is not well established. In this article, we report that CDK5RAP3 has an important prometastatic function in hepatocarcinogenesis. An examination of human hepatocellular carcinoma (HCC) samples revealed at least twofold overexpression of CDK5RAP3 transcripts in 58% (39/67) of HCC specimens when compared with corresponding nontumorous livers. CDK5RAP3 overexpression was associated with more aggressive biological behavior. In HCC cell lines, stable overexpression of CDK5RAP3 promoted, and small interfering RNA–mediated knockdown inhibited, tumorigenic activity and metastatic potential. We found that overexpression of CDK5RAP3 and p21-activated protein kinase 4 (PAK4) correlated in human HCCs, and that CDK5RAP3 was a novel binding partner of PAK4, and this binding enhanced PAK4 activity. siRNA-mediated knockdown of PAK4 in CDK5RAP3-expressing HCC cells reversed the enhanced cell invasiveness mediated by CDK5RAP3 overexpression, implying that PAK4 is essential for CDK5RAP3 function. Taken together, our findings reveal that CDK5RAP3 is widely overexpressed in HCC and that overexpression of CDK5RAP3 promotes HCC metastasis through PAK4 activation.

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Introduction

The CDK5 kinase regulatory subunit-associated protein 3 (CDK5RAP3, also called C53/LZAP) was first identified as a binding partner of cyclin-dependent kinase 5 activator, p35cdk5a, in yeast 2-hybrid screening (1). Northern analysis indicated that CDK5RAP3 is widely expressed in human tissues, and the expression level is relatively constant in the heart, brain, skeletal muscle, placenta, lung, liver, kidney, and pancreas (2). Overexpression of CDK5RAP3 has been shown to sensitize cells to apoptosis induced by genotoxic stress (3). CDK5RAP3 can interact with a well-known tumor suppressor, namely, the alternate reading frame (ARF; p14arf), by which it stabilizes and promotes the transcription activity of p53 (4). More recently, CDK5RAP3 has been found to be underexpressed in head and neck cancers, and forced expression of CDK5RAP3 can negatively regulate NF-κB activity (5), which suggests that CDK5RAP3 may function as a tumor suppressor. On the contrary, stable overexpression of the CDK5RAP3 isoform has been shown to promote hepatocellular carcinoma (HCC) and cardiac cell proliferation (6, 7), which indicates, furthermore, that CDK5RAP3 may enhance cell growth.

CDK5RAP3 is located at chromosome region 17q21.32, which has been reported to be amplified in HCC; however, the role of CDK5RAP3 in HCC has not been explored so far (8). In this study, we found that the expression of CDK5RAP3 was frequently upregulated in human HCCs at both transcript and protein levels. More importantly, we detected a remarkable enhancement of CDK5RAP3 expression in metastatic HCC. Although little information is available on how CDK5RAP3 regulates cancer metastasis, we found that CDK5RAP3 is a novel activator of p21-activated protein kinase 4 (PAK4) and activation of PAK4 can promote HCC cell migration. Therefore, we provided, in this study, a novel mechanism by which CDK5RAP3 contributes to the metastasis of HCC by activation of PAK4.

Materials and Methods

Cell culture

Human hepatoma cell lines PLC/PRF/5 and HepG2 were purchased from the American Type Culture Collection. The authentication of these cell lines was ensured by the provider through cytogenetic analysis. No additional test was conducted specifically for this study. The human HCC cell line
The bound proteins were then visualized by 2950 cells (5×10⁶) Nude mouse xenograft assay field. The experiments were carried out 3 times independently. Counted and data were shown as average number of colonies per colonies with diameter greater than 50 μm. Wong and colleagues (11) was adopted. For soft agar assay, invasion assays Cell proliferation, soft agar growth, cell migration, and statistical analysis of data. Tests were considered significant with P < 0.05.

Results

CDK5RAP3 was overexpressed in human HCCs
To elucidate the role of CDK5RAP3 in human HCCs, we

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qPCR. We found that CDK5RAP3 transcripts were frequently (58%, 39/67) overexpressed (≥2-fold) in the HCCs as compared with their corresponding nontumorous livers (Fig. 1A). Moreover, the transcript levels of CDK5RAP3 in the tumor samples were significantly higher than those of the nontumorous livers (P < 0.001, Mann–Whitney test). To understand the clinicopathologic significance of CDK5RAP3 in HCC, we correlated the overexpression of CDK5RAP3 mRNA with the patients’ clinicopathologic features (Table 1). Overexpression of CDK5RAP3 was found to be significantly associated with a more aggressive phenotype, namely, the presence of tumor microsatellite formation (P = 0.024) and poorer cellular differentiation (P = 0.023). However, for the survival analysis, overexpression of CDK5RAP3 was not associated with the overall and disease-free survival rates among patients (Table 1).

To examine the expression level of the protein CDK5RAP3 in human HCCs, we generated an antibody that specifically recognized the CDK5RAP3 protein (Supplementary Fig. S1A). Using the purified antibody, we showed that CDK5RAP3 was ubiquitously expressed in a panel of HCC cell lines (Supplementary Fig. S1B). To confirm that CDK5RAP3 was upregulated in human HCCs, immunohistochemical staining was carried out. As shown in Fig. 1B, there was a strong cytoplasmic staining of CDK5RAP3 in the tumor cells but not in the corresponding nontumorous hepatocytes, confirming that CDK5RAP3 was overexpressed in HCCs. Because overexpression of CDK5RAP3 mRNA in HCC samples was associated with a more metastatic phenotype, we examined the expression of CDK5RAP3 in patients with extrahepatic metastatic HCC. A tissue microarray consisting of 25 cases each, containing the primary HCC, extrahepatic metastasis, and nontumorous liver from the same patient, was examined for CDK5RAP3 expression. Quantification of CDK5RAP3 staining was scored by an experienced pathologist (I. Oi-Lin Ng). Eleven of the 25 (44%) cases had higher expression levels of CDK5RAP3 in primary HCCs than in the corresponding nontumorous livers. Among the 11 cases with overexpression of CDK5RAP3, 55% (6/11) had higher levels of CDK5RAP3 in tumor metastases than in the corresponding primary HCCs (Fig. 1C), indicating upregulation of CDK5RAP3 during HCC progression.

CDK5RAP3 enhanced cell proliferation and tumorigenicity of HCC cells

The expression of the human CDK5RAP3 isoform (IC53-2) has previously been shown to promote the proliferation of HCC cells (7). To study the effect of the full-length (FL) form of CDK5RAP3 on growth properties of HCC cells, we conducted a colony formation assay in human HCC cell lines with transient overexpression and knockdown of CDK5RAP3. PLC/PRF/5 cells transiently transfected with CDK5RAP3 expression plasmid formed more colonies than the vector control (P = 0.005; Fig. 2A), whereas transient knockdown of CDK5RAP3 using short hairpin RNA (shRNA) specific to CDK5RAP3 suppressed colony formation in PLC/PRF/5 cells (P = 0.005; Fig. 2B), indicating that CDK5RAP3 promotes HCC cell growth. To further examine the effect of CDK5RAP3 in HCC tumorigenesis, we established 2 CDK5RAP3 stably overexpressing HepG2 cells. The CDK5RAP3 stable overexpression was confirmed by immunoblotting (Fig. 2C). A cell proliferation assay using the stable clones showed that the doubling time of both CDK5RAP3 stably expressing clones 1 and 2 (CDK5RAP3#1, 29.28 hours; CDK5RAP3#2, 28.56 hours) was remarkably shorter than the vector control (31.6 hours), indicating that stable clones grew faster than the vector (Fig. 2C). Because there is little information about the tumorigenic activity of CDK5RAP3, a soft agar growth assay was conducted to evaluate the change in the anchorage-independent growth property of the CDK5RAP3 stably expressing HCC cells. The results showed that 3-fold more colonies were formed in stable clones CDK5RAP3#1 (P = 0.027) and CDK5RAP3#2...
Table 1. Association of overexpression of CDK5RAP3 mRNA with clinicopathologic features in human HCCs

| Parameters                        | Category          | CDK5RAP3 not overexpressed | CDK5RAP3 overexpressed | P value |
|----------------------------------|-------------------|-----------------------------|------------------------|---------|
| Overall survival                 |                   | 23                          | 37                     | 0.264   |
| Disease-free survival            |                   | 23                          | 37                     | 0.701   |
| Sex                              | Male              | 16                          | 27                     | 0.026*  |
|                                  | Female            | 10                          | 4                      |         |
| Age, y                           | <40               | 2                           | 4                      | 0.678   |
|                                  | >40               | 24                          | 27                     |         |
| HBV surface antigen              | Absent            | 9                           | 8                      | 0.267   |
|                                  | Present           | 18                          | 30                     |         |
| HBV core antigen                 | Absent            | 14                          | 18                     | 0.582   |
|                                  | Present           | 2                           | 1                      |         |
| Liver cirrhosis                  | Absent            | 8                           | 16                     | 0.139   |
|                                  | Present           | 18                          | 16                     |         |
| Resection margin                 | Absent            | 25                          | 37                     | 0.642   |
|                                  | Present           | 3                           | 2                      |         |
| Venous invasion                  | Absent            | 17                          | 18                     | 0.239   |
|                                  | Present           | 11                          | 21                     |         |
| Tumor microsatellite             | Absent            | 20                          | 17                     | 0.024*  |
|                                  | Present           | 8                           | 22                     |         |
| Liver invasion                   | Absent            | 16                          | 20                     | 0.192   |
|                                  | Present           | 7                           | 18                     |         |
| Tumor encapsulation              | Absent            | 16                          | 27                     | 0.322   |
|                                  | Present           | 11                          | 11                     |         |
| Cellular differentiation         | I–III             | 19                          | 15                     | 0.023*  |
| (Edmondson’s grading)            | IV–VI             | 9                           | 23                     |         |
| Tumor size (cm)                  | ≤5                | 9                           | 17                     | 0.159   |
|                                  | >5                | 17                          | 15                     |         |
| Tumor stage                      | I–II              | 12                          | 13                     | 0.672   |
|                                  | III–V             | 14                          | 19                     |         |
| Number of tumor nodules          | Single            | 22                          | 27                     | 1.000   |
|                                  | Multiple          | 4                           | 5                      |         |

*Statistically significant.

(P = 0.002) than in the vector control (Fig. 2D). To address the loss-of-function effect of CDK5RAP3 in HCC cells, we established CDK5RAP3 stable knockdown clones in PLC/PRF/5 and the highly motile SMMC-7721 HCC cells by using shRNA (3). Thus, 2 different cell lines were used for establishing stable clones to eliminate cell-line–specific effect. Two CDK5RAP3 stable knockdown clones were selected (shCDK5RAP3#1 and shCDK5RAP3#2) in each cell line, and the knockdown of CDK5RAP3 was confirmed by Western blotting (Fig. 2E and Supplementary Fig. S2A). As compared with vector control cells, the loss of CDK5RAP3 significantly reduced both the cell-proliferation rate (Fig. 2E) and anchorage-independent growth of PLC/PRF/5 cells (Fig. 2F, P < 0.005). Furthermore, a similar result was observed in CDK5RAP3 stable knockdown SMMC-7721 clones (Supplementary Fig. S2). Together with the data in CDK5RAP3 stably overexpressing HepG2 cells, these results consistently suggest that CDK5RAP3 enhanced the tumorigenicity of HCC cells and the effect was not cell-line specific.

To further show the tumorigenic properties of CDK5RAP3 in vivo, a nude mouse xenograft assay was conducted using CDK5RAP3 stable knockdown PLC/PRF/5 cells. Although the tumor incidences for the PLC/PRF/5-shCDK5RAP3#2 (4/5) and vector control (5/5) were similar, tumors generated from PLC/PRF/5-shCDK5RAP3#2 had remarkably lower weights and slower growth rates than the control (Fig. 2G), suggesting that loss of CDK5RAP3 inhibited tumor growth in vivo.

Knockdown of CDK5RAP3 inhibited cell migration and invasiveness of HCC cells

Clinical data for HCCs in this study reveal that CDK5RAP3 protein levels increased with HCC progression.
CDK5RAP3 Activates PAK4 in HCC

and that overexpression of CDK5RAP3 was statistically correlated with clinicopathologic features of a more metastatic phenotype. To examine whether the loss of CDK5RAP3 inhibits cell motility, Transwell migration assay was conducted using SMMC-7721 cells treated with small interfering RNA (siRNA) targeting CDK5RAP3 (siCDK5RAP3). The number of migrated SMMC-7721 cells transfected with siCDK5RAP3 was reduced by 36% as compared with the cells transfected with siRNA control (Fig. 3A). Furthermore, we then queried whether loss of CDK5RAP3 suppressed the invasiveness of HCC cells. Compared with parental and siRNA-control transfected cells, the number of invaded cells was reduced by 36% as compared with the cells transfected with siCDK5RAP3 (Fig. 3B). In contrast, the CDK5RAP3 stably overexpressing HepG2 cells showed an increase in migration rate by 37% in the Transwell assay (Fig. 3C). Thus, both loss- and gain-of-function assays indicated that CDK5RAP3 promoted the cell migration and invasiveness of HCC cells.

Association analysis and physical correlation between CDK5RAP3 and PAK4 in HCCs

From our functional assays and clinicopathologic correlation data, we inferred that CDK5RAP3 was closely associated with HCC cell invasiveness. We then analyzed the correlation of the overexpression of CDK5RAP3 transcripts with the expression levels of metastasis-related genes (e.g., PAK4, PAK1, NF-κB, and p53) in our database (9). Interestingly, we found that CDK5RAP3 overexpression was significantly correlated with PAK4 overexpression in 63 cases of HCC (P < 0.001, Pearson correlation; Fig. 4A).

To delineate the physiologic implication of this correlation, we first examined the interaction of PAK4 and CDK5RAP3 by coimmunoprecipitation assay. GFP-PAK4, but not GFP-PAK1, was coprecipitated with Myc-CDK5RAP3 in transfected HEK293T cells, indicating that CDK5RAP3 is specifically associated with PAK4 (Fig. 4B). Furthermore, the endogenous CDK5RAP3 and PAK4 proteins were shown, by
coimmunoprecipitation assay, to interact with each other in HepG2 cells (Fig. 4C). Moreover, a GST affinity pull-down assay showed a direct interaction between CDK5RAP3 and PAK4 (Fig. 5A). By confocal microscopy, CDK5RAP3 was found to be significantly colocalized with PAK4 (Fig. 4D). The subcellular localization of CDK5RAP3, which was mainly nuclear, was altered by coexpression with PAK4 and became more concentrated at peripheral regions (Fig. 4D).

CDK5RAP3-regulated PAK4 kinase activity

PAK4 has been shown to induce tumorigenesis and cell adhesion by phosphorylating its substrates (15), and, therefore, we wondered whether CDK5RAP3 promotes HCC formation by regulating PAK4 activity. To evaluate this, we constructed a panel of CDK5RAP3 truncated mutants (Fig. 5A) and assessed their interaction with PAK4 as well as their effect on PAK4 activity. The results of the GST affinity pull-down assay revealed that the GST control, 434-506 and 1-304 mutants of CDK5RAP3, did not bind to PAK4. However, FL and 255-436 mutant (M2) showed strong binding affinity to PAK4, whereas the 256-506 mutant (M3) displayed a weaker binding affinity to PAK4 (Fig. 5A). It is possible that the additional aa 437-506 may have attenuated the binding of M3 to PAK4. To further investigate how CDK5RAP3 interacted with PAK4, we constructed 3 deletion mutants of PAK4: 2 of them (aa 1–201 and 1–325) contained the N-terminal p21-binding domain (PBD) and one contained the C-terminal kinase domain (aa 325–591). Using a GST affinity pull-down assay, we found that both the FL and M2 of CDK5RAP3 were associated with N-terminal region of PAK4, which contained the PBD domain but not the C-terminal kinase domain (Fig. 5B). To further examine whether CDK5RAP3 regulated PAK4 activity, a PAK4 kinase assay was conducted. The result revealed that incubation of FL, M2, and M3 of CDK5RAP3 with PAK4 not only promoted the PAK4 activity measured by a specific GST-PAK4tide substrate but also promoted the autophosphorylation of PAK4 (Fig. 5C, lanes 2–4). In contrast, the 434-506 mutant (M4) of CDK5RAP3 showed no PAK4 activation, similar to the GST control (Fig. 5C, lane 5). This result indicated that the aa sequence 255–436 of CDK5RAP3 contains an activation domain of PAK4. Moreover, as revealed by kinase assay, CDK5RAP3 was a good substrate for PAK4 (Fig. 5C). Using a PAK4 peptide kinase assay, we further illustrated that the FL and M2 of CDK5RAP3 significantly enhanced the activity of PAK4, but not the 1-304 mutant (M1), M4, and GST control, in a dose-dependent manner (Fig. 5D). Together with the interaction data, our results implied that the direct binding of CDK5RAP3 to PAK4 elicited the activation of PAK4. Furthermore, the result from Fig. 5B indicated that CDK5RAP3 may interact with the N-terminal domain, possibly the PBD, thus leading to the activation of PAK4.

Knockdown of PAK4 abolished the promotion of cell invasiveness in CDK5RAP3 overexpression cells

To investigate whether the overexpression of CDK5RAP3 activated PAK4 in HCCs, the correlation between CDK5RAP3

Figure 3. Knockdown of CDK5RAP3 suppressed HCC cell migration and invasiveness. SMMC-7721 cells were transfected with siCDK5RAP3 and siRNA control, and nontransfected cells were subjected to (A) the Transwell migration assay and (B) the invasion assay. *, P = 0.05 compared with siControl (Student t test). Immunoblotting showed the CDK5RAP3 expression. Representative photomicrographs are shown. C, CDK5RAP3 stably overexpressing HepG2 cells were used for the Transwell migration assay. *, P < 0.05 compared with HepG2–vector control cells (Student t test). Error bars, mean ± SD.
and p-PAK4 expression levels in the same set of tissue microarray was assessed by immunohistochemical staining. Phosphorylated PAK4 showed strong nuclear staining, and the staining was higher in the primary HCCs than in the corresponding nontumorous liver tissues (10/20 cases); however, the expression levels of CDK5RAP3 and p-PAK4 positively correlated with one another (Fig. 1C; \( P = 0.024 \), Pearson correlation). In addition, 3 of 5 cases with higher CDK5RAP3 expression in the metastases than in their primary HCCs showed a higher level of p-PAK4 in metastases than in primary HCCs (Fig. 1C), suggesting a positive correlation of CDK5RAP3 and PAK4 activity during HCC progression. In addition, PAK4 activity, as revealed by p-PAK4 staining, was quantitatively reduced in the tumor tissues obtained from the CDK5RAP3-knockdown, nude mouse xenograft (Supplementary Fig. S3).

To further confirm the specific activation of PAK4 by CDK5RAP3 within cells, PAK4, in combination with increasing doses of CDK5RAP3, was cotransfected into HepG2 cells and p-PAK4 was determined by immunoblotting. As shown in Fig. 6A, increased expression of CDK5RAP3 promoted the total protein and phosphorylation levels of PAK4 in HepG2 cells in a dose-dependent manner. The upregulation of phosphorylated PAK4 was functionally active, as indicated by the increase in PAK4 activity in CDK5RAP3 cotransfected cells (Fig. 6A). Consistently, the phosphorylation of PAK4 by CDK5RAP3 was observed in CDK5RAP3 stably overexpressing HepG2 cells (Fig. 6B). To examine whether CDK5RAP3 promoted HCC-cell invasiveness through activation of PAK4, we used siRNA to specifically knock down PAK4 in CDK5RAP3 stably overexpressing HepG2 cells and conducted the cell-invasion assay. The result showed that loss of PAK4, as compared with the vector control cells, significantly reduced the invasiveness of both CDK5RAP3 stably expressing clones (Fig. 6C) and, thus, strongly suggests that CDK5RAP3 enhanced HCC-cell invasiveness through activation by PAK4.

**Discussion**

Several studies have investigated the roles of CDK5RAP3 in carcinogenesis by using various cancer models; however, so far, its definite roles remain contradictory. CDK5RAP3 has been found to promote apoptosis induced by genotoxic stress in HeLa cells by triggering G2/M arrest (3). In addition, CDK5RAP3 has been proposed to be a tumor suppressor because CDK5RAP3 inhibits the NF-\( \kappa \)B cell-survival pathway and its protein level is significantly reduced in head and neck squamous cell carcinomas (5). However, a similar NF-\( \kappa \)B-suppressive effect of CDK5RAP3 was not observed in our study using HCC cells, and this could probably be attributed to some tissue-specific effects. In contrast, overexpression of the CDK5RAP3 isoform has been reported to promote cell proliferation of HCC cells (7). Recently, CDK5RAP3 has been shown to be overexpressed in lung adenocarcinoma (16). In the present study, using qPCR and immunohistochemical staining assays, we showed that both transcripts and protein of CDK5RAP3 were frequently and significantly overexpressed in human HCCs (Fig. 1). The mechanism of such overexpression is currently unclear, but it has been reported that the chromosomal region 17q, which contains the CDK5RAP3, is frequently amplified in HCCs (8). Although CDK5RAP3 has...
been hypothesized to possess tumor-suppressor activity, several lines of evidence here indicate that overexpression of CDK5RAP3 is causally associated with HCC tumorigenicity. We have used 3 different HCC cell lines, specifically PLC/PRF/5, SMMC-7721, and HepG2, to show the transforming ability of CDK5RAP3. Results from both gain-of-function and loss-of-function approaches concurred to indicate that CDK5RAP3 enhanced cell-proliferation rate, anchorage-independent growth, cell migration, and cell invasiveness, suggesting that CDK5RAP3 plays an oncogenic role in hepatocarcinogenesis.

In our clinicopathologic analysis, overexpression of CDK5RAP3 was significantly correlated with more aggressive tumor behavior in terms of the presence of tumor microsatellite formation and poorer HCC differentiation (Table 1). Consistently, our immunohistochemical staining in clinical HCCs indicated that CDK5RAP3 is highly expressed in metastatic HCC cells (Fig. 1C). Furthermore, CDK5RAP3 stably overexpressing HCC cells showed an increase in invasion rate (Fig. 3C) whereas knockdown of CDK5RAP3 suppressed the invasion (Fig. 3B). Taken together, these data strongly suggested that CDK5RAP3 is involved in cancer metastasis.

Although a previous report has shown that overexpression of CDK5RAP3 suppressed cancer invasion in head and neck cancers (5), our results have suggested that loss of CDK5RAP3 suppressed HCC-cell invasion. The reason for the discrepancy remains unclear, but the possibility that the effect of CDK5RAP3 is tissue specific cannot be completely ruled out.

The mechanism by which CDK5RAP3 enhances cancer metastasis is not completely understood, but one of the possible mechanisms is through the activation of PAK4. Previous studies have shown that the constitutively active form of PAK4 induces anchorage-independent growth, cell-rounding phenotype, and defect in cell spreading onto a fibronectin-coated surface, all of which are related to

![Figure 5. CDK5RAP3-regulated PAK4 kinase activity. A (top), schematic diagram of CDK5RAP3 deletion mutants. The numbers represent the corresponding amino acid residues of the sequence; bottom, result of GST pull-down assay. B (top), schematic diagram of PAK4 deletion mutants. PBD, p21-binding domain; KD, kinase domain; bottom, CDK5RAP3 FL and 255–436 mutant (M2) were mixed with PAK4 FL 1–591, 1–201, and 1–325 mutants for GST pull-down assay. C, CDK5RAP3 FL and mutants were incubated with His-PAK4, and in vitro gel kinase assay was conducted using GST-PAK4tide as substrate; GST was included as a negative control; index of relative band intensity is shown. D, different amounts of GST-CDK5RAP3 proteins were incubated with His-PAK4, and peptide kinase assay was conducted using PAK4tide as a substrate; the incorporation rate of radioactivity (counts per minute) was plotted against the amount of GST-CDK5RAP3. WB, Western blotting.](image-url)

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cancer-cell migration (17, 18). With regard to the upstream signaling, PAK4 is found to be activated by hepatocyte and keratinocyte growth factors during cell migration (19, 20). Our data indicate that overexpression of CDK5RAP3 in HCC cells was significantly associated with enhanced expression and activity of PAK4 through Ser474 phosphorylation (Figs. 1C, 4A, 6A and B). With regard to the mechanism by which the overexpression of CDK5RAP3 could increase the expression of PAK4 in HCCs, we hypothesized that CDK5RAP3 might stabilize PAK4 through inhibition of PAK4 ubiquitination. Furthermore, recent reports have shown that CDK5RAP3 stably overexpressing HCC cells were transfected with siPAK4, as indicated for invasion assay; top, Western blotting; bottom, graph showing number of invaded cells after transfection of PAK4 and control siRNA. *P = 0.005 (Student t test); representative photomicrographs are shown.

Figure 6. CDK5RAP3 promoted HCC-cell invasiveness mediated through activation by PAK4 kinase activity. A, increasing amounts of CDK5RAP3 were transiently cotransfected with PAK4 in HepG2 cells; expression of GFP-PAK4 and Myc-CDK5RAP3 and phosphorylation of PAK4 were determined by immunoblotting; bottom, graph showing number of invaded cells after transfection of PAK4 and control siRNA. B, cell lysates from vector and CDK5RAP3 stably expressing HepG2 cells (CDK5RAP3#1 and CDK5RAP3#2) were analyzed by Western blotting. C, CDK5RAP3 stably overexpressing HepG2 cells were transfected with siPAK4, as indicated for invasion assay; top, Western blotting; bottom, graph showing number of invaded cells after transfection of PAK4 and control siRNA.
that overexpression of PAK4 in prostate cancer promotes cell migration and invasion (15). Thus, we hypothesized that the activation of PAK4 by CDK5RAP3 may promote HCC-cell invasion. For this purpose, we used siRNA to specifically knock down PAK4 in CDK5RAP3 stably overexpressing HepG2 cells and showed that the loss of PAK4 attenuated the invasiveness of stable clones back to similar level as the vector control, indicating that PAK4 plays a key role in CDK5RAP3-mediated HCC cancer metastasis (Fig. 6C). Our findings have established that upregulation of CDK5RAP3 may occur in HCC progression and metastasis through the regulation of PAK4.

In summary, we provide the first evidence that CDK5RAP3 is overexpressed in human HCCs and that overexpression of CDK5RAP3 promotes metastasis of HCC. We also provide evidence that supports a single mechanism wherein CDK5RAP3 enhances metastasis of HCC through the activation of PAK4. Therefore, inhibition of CDK5RAP3 can potentially be used to suppress HCC formation, providing a new molecular target for therapeutic intervention in HCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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