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p19^{Arf} Inhibits the Invasion of Hepatocellular Carcinoma Cells by Binding to CtBP

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Abstract

The *INK4A/ARF* tumor suppressor locus is frequently inactivated in hepatocellular carcinoma (HCC), yet the consequences of this remain unknown. We recently described a HCC mouse model in which loss of the *Ink4a/Arf* locus accelerates the development of metastasis and enhances tumor cell migration and invasion in cell culture assays. We show here that knockdown of p19^{Arf} in a HCC cell line increases invasion in cell culture assays. Further, reintroduction of p19^{Arf} into HCC cell lines lacking *Ink4a/Arf* inhibits tumor cell invasion, without affecting cell proliferation, or cell transformation as measured by soft agar colony formation. Inhibition of cell invasion by p19^{Arf} was dependent on its C-terminal binding protein (CtBP) interaction domain, but independent of Mdm2 binding and nucleolar localization. Indeed, RNAi-mediated knockdown of CtBP1 or CtBP2 decreased cell invasion, and ectopic expression of CtBP2 enhanced tumor cell migration and invasion. Thus, our data indicate a novel role for the Arf tumor suppressor protein in regulating phenotypes associated with tumor progression and metastasis in HCC cells.

Keywords

Hepatocellular carcinoma; invasion; Arf; CtBP

Introduction

Worldwide, 626,000 new cases of hepatocellular carcinoma (HCC) are diagnosed each year, with a survival rate of less than 5%, and an average survival of less than one year after diagnosis (1). To date, surgery remains the only effective approach for treatment of small HCCs; unfortunately, there is currently no reliable therapy for most patients with advanced or metastatic HCC that has spread to the lymph nodes, portal vein, or lungs (2).

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Epidemiologic and molecular studies have determined that HCC development is associated with chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, alcoholic cirrhosis, metabolic disorders, and environmental factors such as aflatoxin B1 exposure (3). Furthermore, genetic alterations resulting in the activation of the c-myc oncogene; inactivation of tumor suppressor genes such as *TP53*, *RB1*, *INK4A/ARF*; and alterations that stimulate the Wnt signaling pathway are particularly important in hepatocarcinogenesis (4). However, little is known regarding the genes and molecular pathways that influence metastasis. Recent *in vivo* models and gene expression analyses have begun to shed some light on the tumor and host factors regulating metastasis in HCC (5–10).

To elucidate the mechanisms of hepatocarcinogenesis, several mouse models have been generated through the expression of oncogenes or viral genes, the inactivation of tumor suppressor genes, and chemical carcinogenesis. Given the impact of metastasis on the clinical management of HCC patients, models that allow the dissection of metastasis are of great importance. We have recently described a novel HCC mouse model induced by the somatic and sporadic activation of oncogenes specifically in the liver (8). We have further refined this model to include liver specific deletion of the *Trp53* and *Ink4a/Arf* tumor suppressor loci (Y-W.C. et al, submitted). Our recent data demonstrated that liver-specific *Trp53* deletion induces lung metastases, the formation of which can be accelerated by concomitant deletion of *Ink4a/Arf*. Furthermore, we showed that mouse HCC cell lines lacking both *Trp53* and *Ink4a/Arf* displayed enhanced migration and invasion capabilities, suggesting that the *Ink4a/Arf* locus may play a role in regulating these processes in liver cancer cells.

The *Ink4a/Arf* locus encodes two distinct tumor suppressors, the cyclin dependent kinase (Cdk) inhibitor $p16^{Ink4a}$ and a protein translated from an alternative reading frame, Arf, ($p14^{ARF}$ in human and $p19^{Arf}$ in mouse) (11). Through the inhibition of cyclin D/cdk4, p16 maintains pRb in the hypophosphorylated state and regulates the G_1 transition of the cell cycle (12). On the other hand, $p19^{Arf}$ inhibits the p53-ubiquitinating function of Mdm2, thereby stabilizing the p53 transcription factor, which regulates genes mediating G_1 cell cycle arrest and apoptosis (13). Arf also has p53-independent functions. It mediates a p53-independent cell cycle arrest by targeting both E2F1 and c-Myc to the nucleolus and preventing their transcription activation functions, and also induces p53-independent apoptosis by targeting the transcription factor CtBP2 for degradation (14–16). CtBP transcription factors have been previously shown to induce an epithelial to mesenchymal transition (EMT), and to stimulate cell migration (17–19). Thus, regulation of CtBP activity by Arf may represent an important tumor suppressor function. Significantly, the *INK4A/ARF* locus is frequently inactivated in HCC, yet its role in the pathogenesis of this disease remains unclear (20–22).

Guided by these findings in HCC and our mouse model, we sought to identify mechanisms by which Arf may regulate HCC metastasis. Using mouse HCC cell lines, we found that RNAi-mediated loss of Arf enhances cell invasion, and conversely, reintroduction of Arf into cell lines deficient for both *Trp53* and *Ink4a/Arf* inhibits tumor cell invasion. The ability of Arf to inhibit invasion is independent of its interaction with Mdm2 and nucleolar localization. However, it is dependent on Arf's ability to bind to CtBP. Consistent with this, RNAi-mediated depletion of CtBP1 or CtBP2 reduces cell invasion, and ectopic CtBP2 expression enhances tumor cell migration and invasion. Thus, our findings demonstrate a new role for the Arf tumor suppressor in HCC progression, one that may be applicable to other tumor types.

Material and Methods

Cell lines

Immediately after harvest, tumor tissue was minced and dissociated by pipetting in DMEM. Cells were washed once in sterile PBS and plated in DMEM supplemented with 10% fetal

bovine serum (FBS) and antibiotics. After 48 hours, non-adherent cells were removed, adherent cells washed with PBS and refed with fresh medium containing FBS.

To generate cells with knockdown of p19Arf, BL185 HCC cells were infected with MLP retroviral vectors encoding an shRNA targeted to Arf-specific exon 1β of the *Ink4a/Arf* locus (23). Infected cells were then placed in DMEM containing 8μg/ml puromycin (EMD Biosciences). Resistant cells were then analyzed by immunoblot.

To generate cell lines expressing either wild-type or mutant $p19^{Arf}$ proteins, HCC cell lines were infected with pBabe-puro retroviruses and then placed in DMEM containing 8μg/ml puromycin (EMD Biosciences). Resistant cells were then analyzed by immunoblot and immunofluoresence.

Cells with ectopic CtBP2 expression were generated by transfecting MM189 cells with pcDNA3-V5-CtBP2 or pcDNA3-V5-CtBP2, and selection of transfected cells in 4 mg/ml G418 (EMD Biosciences).

RNAi-mediated depletion of CtBP1 and CtBP2 was achieved by infecting HCC cells with pLKO-based lentiviruses encoding shRNAs targeting the appropriate mRNA. Infected cells were selected in 8μg/ml puromycin. To generate knockdown in HCC cells expressing the $p19^{Arf}$L46D mutant that were already puromycin resistant, infection with shRNA containing lentiviruses was performed twice within 24 hours. Knockdown was confirmed by immunoblot.

Immunoblotting

Cells were collected and lysed in RIPA buffer containing protease inhibitors. Protein concentration was determined with BCA assay kit (Pierce), and equal amounts of protein were loaded per lane of a polyacrylamide gel. After protein transfer, nitrocellulose membranes were blocked with 5% dry milk in tris-buffered saline, 0.1% Tween 20 (TBS-T) for 1 hour. Primary antibodies were incubated overnight at 4°C in 5% bovine serum albumin (BSA, Sigma) in TBS-T, and secondary antibodies for 1 hour at room temperature. Chemiluminescence was performed with Supersignal reagent (Pierce). Primary antibodies: anti-p19Arf (Abcam), anti-CtBP2 (Upstate Biotechnology), anti β-actin (1:5000, Santa Crutz), α-catenin (1:1000, Becton Dickinson (BD)), anti β-catenin (1:1000, BD), anti γ-catenin (1:1000, BD), anti E-cadherin (1:1000, BD), anti α smooth actin clone A14 (1:1000, Sigma), anti N-cadherin (1:1000, BD), anti fibronectin (1:1000, Santa Cruz), anti vimentin (1:1000, Lab Vision), and anti-V5 (1:2000, Covance).

Cell proliferation assay

10^3 cells were seeded in quadruplicate onto collagen coated 96-well plates and incubated at 37°C under 5% CO_2. After 24 hours, viable cell numbers were measured in quadruplicate every period for 4 days using CellTiter 96 Aqueous One Solution Cell proliferation assay (Promega) according to the manufacturer's instructions. The proliferation curves were constructed by calculating the mean value of optical density measurement at 490 nm using a 96-well plate reader.

Soft agar assay

Soft agar assays were performed as previously described (24). The number of colonies larger than 100 μm in diameter present within 20 microscopic fields was counted under a light microscope.

Migration and invasion assays

2.5×10^4 cells in 0.5 ml of serum-free DMEM were plated into either control or matrigel-coated invasion inserts (BD). Inserts were then placed in wells with 0.75 ml of DMEM containing 10% FBS as a chemoattractant. After culture for 20–24 hours at 37°C, cells were fixed with methanol for 8 minutes at room temperature and stained with Giemsa reagent diluted 5X in H_2O. Cells on the upper sides of the inserts were removed with a cotton swab, and the insert membranes removed and mounted on glass slides. Cell numbers for migration and invasion were then determined by counting the number of cells present in 10 microscope fields at 50X magnification per insert. The percent invasion was calculated as the number of invading cells divided by the number of migrating cells. The percent invasion was then normalized to get the invasion index, with the value for the control cell population set to 1. All experiments were performed in duplicate and repeated a minimum of 3 times. Data are shown for representative experiments.

Immunofluorescence

Cells on collagen coated culture slides were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 15 min at room temperature. After blocking with 10% goat serum in PBS for 1 hr, cells were incubated with primary anti-$p19^{Arf}$ antibody (1:200, Abcam) at 4°C overnight, followed by incubation with appropriate secondary antibodies and Rhodamine-Phalloidin at room temperature. Slides were mounted with antifade and viewed under a fluorescence microscope.

Results

$p19^{Arf}$ inhibits cell invasion

We have previously shown that mouse HCC cell lines lacking both *Trp53* and *Ink4a/Arf* have enhanced migration and invasion activity in Boyden chamber assays compared to a cell line that lacks *Trp53* alone (25). To determine whether loss of $p19^{Arf}$ influences tumor cell migration and invasion, we infected the BL185 HCC cell line which lacks *Trp53* but retains functional *Ink4a/Arf*, with retroviral vectors encoding shRNA against $p19^{Arf}$, or luciferase as a control (23). Knockdown of $p19^{Arf}$ was confirmed by immunoblot (Figure 1A). We then measured the migration and invasion activities of these cells using Boyden chamber assays. We found that depletion of Arf resulted in a 1.5-fold increase in the number of invading cells (Figure 1C), a result confirmed in independent infections (shRNA 1 and 2, Figure 1C), and multiple replications of the invasion assays. These data indicate that acute loss of Arf stimulates the *in vitro* invasion activity of *Trp53* null HCC cells, albeit to a lesser extent than that observed in HCC cells with genetic ablation of *Trp53* and *Ink4a/Arf* (data not shown).

To ascertain whether loss of $p19^{Arf}$ was required for the invasive phenotype of HCC cells lacking *Trp53* and *Ink4a/Arf*, double null HCC cell lines, MM189 and BL322, were infected with a retroviral vector encoding mouse $p19^{Arf}$, or empty vector as a control, and expression confirmed by immunoblot (Figure 1B). By immunohistochemistry with an anti-$p19^{Arf}$ antibody, positive signals were detected in nearly 70% of retrovirally infected MM189 cells, suggesting that $p19^{Arf}$ was re-expressed in most, but not all, cells (data not shown). Measurement of cell migration and invasion in Boyden chamber assays demonstrated that $p19^{Arf}$ expression reduced the invasion activity of MM189 cells by 80% (Figure 1D). $p19^{Arf}$ expression also led to a more modest decrease in cell migration in most, but not all, experiments (Figure 1D and data not shown). Consistent with this finding, $p19^{Arf}$ expression did not significantly impair cell migration in a wound healing assay (data not shown). Thus, $p19^{Arf}$ specifically inhibits the invasion of HCC cells.

Analysis of vector controls and $p19^{Arf}$-expressing MM189 cells demonstrated that $p19^{Arf}$ expression did not affect the proliferation of these cells (Figure 1E). Likewise, analysis of cell transformation by soft agar colony formation demonstrated no effect of $p19^{Arf}$ expression (Figure 1F and Supplementary Figure 1). Thus, in MM189 cells, $p19^{Arf}$ appears to regulate cell invasion without affecting any other features related to cell transformation. To confirm that this phenomenon was not restricted to a single HCC cell line, we performed the migration and invasion assays on BL322 HCC cells infected with either $p19^{Arf}$-expresing retrovirus or vector controls. We found a similar effect on migration and invasion in this cell line suggesting that this phenotype is not cell line specific (Figure 1G).

Tumor cell migration and invasion are often associated with reorganization of the actin cytoskeleton and a phenomenon called the epithelial to mesenchymal transition (EMT) (26, 27). To investigate whether $p19^{Arf}$-mediated inhibition of cell invasion occurs via the reversal of an EMT, we analyzed $p19^{Arf}$ expressing cells (MM189-WT) and vector controls (MM189-PB) by morphology and expression of epithelial and mesenchymal markers. Immunoblot experiments demonstrated that MM189 cells expressed epithelial proteins, such as α-catenin, β-catenin, γ-catenin and E-cadherin, and the expression of $p19^{Arf}$ did not enhance the levels of these proteins (Figure 2A). Examination of E-cadherin localization by immunofluorescent staining did not reveal any significant differences between MM189-PB and MM189-WT cells (data not shown). Consistent with these findings, MM189 cells did not express mesenchymal markers such as vimentin, a feature unaffected by $p19^{Arf}$ expression (Figure 2A). Analysis of mRNA levels for epithelial and mesenchymal markers demonstrated that these were similarly unaffected by $p19^{Arf}$ expression, although interestingly, the HCC cells expressed vimentin mRNA indicating that regulation occurred at the post-transcriptional level (Supplementary Figure 2). In agreement with the above data, analysis of the morphology of MM189-PB cells and MM189-WT cells showed that the expression of $p19^{Arf}$ did not alter cell morphology (Figure 2B). Finally, analysis of the actin cytoskeleton by phalloidin staining did not demonstrate any differences between $p19^{Arf}$-expressing cells and vector controls (Figure 2C).

Inhibition of cell invasion by $p19^{Arf}$ requires the CtBP interaction domain

To determine the functional domains of $p19^{Arf}$ that are required for inhibition of cell invasion, we introduced retroviral vectors encoding various $p19^{Arf}$ mutant proteins into MM189 cells. The introduced mutants were Δ2–10 that impairs Mdm2 binding; Δ8–32 that impairs Mdm2 binding and nucleolar localization; Δ46–53 that blocks CtBP binding; and the L46D point mutant that also blocks CtBP binding. The locations of these mutations are shown schematically in Figure 3A. The amino terminal deletions also affect Arf's interaction with c-Myc, E2F1 and Foxm1b. We confirmed the expression of the $p19^{Arf}$ mutants by immunoblotting with Arf specific antibodies (Figure 3B), and demonstrated that the majority of cells expressed the mutant $p19^{Arf}$ proteins by immunostaining (data not shown).

Introduction of the Δ2–10 and Δ8–32 mutants into MM189 cells led to the efficient inhibition of cell invasion (Figure 3C). However, introduction of the Δ46–53 and L46D mutants failed to appreciably impair cell invasion, particularly in comparison to the number of migrating cells (Figure 3C). This resulted in an increased invasion index for cells expressing these two $p19^{Arf}$ mutants (Figure 3C). To rule out the possibility that some effects were due to the random integration of retroviruses, retroviral infections and invasion assays were repeated with independent retroviral stocks, several pools of infected cells with varying levels of $p19^{Arf}$ expression examined, and consistent results obtained. As was observed with expression of wild type $p19^{Arf}$, expression of the Arf mutants did not affect proliferation of MM189 cells (data not shown). Thus, $p19^{Arf}$-mediated inhibition of tumor cell invasion is independent of Mdm2 binding and nucleolar localization, but is dependent on the CtBP interaction domain. As was observed after reintroduction of wild-type $p19^{Arf}$, introduction of mutant Arf proteins did not

alter the expression of epithelial and mesenchymal proteins (Figure 2A). Thus, $p19^{Arf}$-mediated inhibition of cell invasion does not occur via the reversal of an EMT.

CtBP regulates tumor cell invasion

Our finding that the $p19^{Arf}$ Δ46–53 and L46D mutants fail to impair tumor cell invasion suggested that functional inhibition of CtBP proteins by $p19^{Arf}$ might be critical in this process. We therefore determined whether inactivation of CtBP reduces tumor cell invasion. Lentiviral shRNA vectors targeting CtBP1 or CtBP2, or vector control, were introduced into MM189 cells, the cells selected for puromycin resistance, and their invasion activity measured. By immunoblotting, we identified two shRNAs targeting each of the CtBP family members that displayed efficient knockdown (Figure 4A). Measurement of the proliferation of CtBP knockdown cells demonstrated that loss of CtBP proteins did not affect cell proliferation (Supplementary Figure 3). Analysis of the invasion activity demonstrated that cells with CtBP knockdown had lower invasion activity relative to vector controls (Figure 4C). We next determined whether depletion of CtBP1 and CtBP2 in MM189 cells expressing the Arf L46D mutant would similarly result in reduced cell invasion. We introduced shRNAs targeting either CtBP1 or CtBP2 into these cells and confirmed knockdown by immunoblotting with anti-CtBP antibodies (Supplementary Figure 4). We found that depletion of either family member led to a reduced cell invasion index (Figure 4D). Thus, CtBP family members are required for HCC tumor cell invasion.

To determine whether ectopic expression of CtBP could enhance cell invasion, we introduced a V5-tagged CtBP2 expression construct, or vector control, into MM189 cells. Detection of the introduced CtBP was confirmed by immunoblotting with anti-V5 antibodies (Figure 4B). Interestingly, the levels of the ectopic CtBP appeared to be less than the endogenous levels of CtBP2 as determined by immunoblotting with CtBP2-specific antibodies (data not shown). In cell invasion assays, exogenous CtBP2 expression led to a 1.5-fold increase in invasion activity relative to vector controls, supporting a role for CtBP2 in tumor cell invasion (Figure 4E). Importantly, introduction of a NADH-binding deficient CtBP2 mutant (G189A) (28) failed to stimulate cell invasion (Figure 4E) even though it was expressed at levels similar to wild-type CtBP2 (Figure 4B). Interestingly, in MM189 cells with enforced $p19^{Arf}$ expression, ectopic CtBP2 failed to significantly enhance tumor cell migration and invasion suggesting a dominant effect of $p19^{Arf}$ on the invasion phenotype (data not shown).

Discussion

The *Ink4a/Arf* locus is regarded as one of the most important anti-tumoral defenses in mammalian systems, and the two protein-coding genes at this locus are frequently independently inactivated in HCC. Studies of human tumors and mouse models have suggested a role for the Arf tumor suppressor in constraining tumor initiation (22,29,30). We have recently shown that deletion of the *Ink4a/Arf* locus enhances the progression of liver tumors induced by polyoma middle T antigen and *Trp53* deletion, consistent with findings in a skin carcinogenesis mouse model that indicated that Arf may additionally play a role in tumor progression (31). Further, we showed that mouse HCC cell lines that lacked both *Trp53* and *Ink4a/Arf* displayed enhanced migration and invasion capabilities in cell culture assays compared to a cell line that lacks *Trp53* alone (25). We have now shown that $p19^{Arf}$ specifically impairs tumor cell invasion without affecting either proliferation or soft agar colony formation, pointing to a potential role for Arf in regulating tumor metastasis.

$p19^{Arf}$ induces cell cycle arrest in a p53-dependent manner, and relays signals to p53 after oncogene-induced stress that stimulates the onset of cellular senescence, a potent anti-tumorigenic checkpoint. However, accumulating data indicate that Arf may additionally have p53-independent tumor suppressor functions (32). Our data show that Arf-mediated inhibition

of tumor cell invasion is independent of its interaction with Mdm2, suggesting that regulation of tumor invasion may be another p53-independent tumor suppressor function of Arf. Importantly, this function is also independent of Arf's ability to bind to other interacting proteins such as E2F1, c-Myc, and Foxm1b, as deletion mutants that interfere with the ability of Arf to bind to these proteins still effectively block invasion.

Instead, our data demonstrate that the effect of $p19^{Arf}$ on tumor cell invasion occurs via a p53-independent mechanism involving CtBP. Mutant Arf proteins that fail to bind to CtBP do not inhibit cell invasion, and shRNA-mediated ablation of CtBP reduces invasion in HCC cells expressing CtBP-binding-deficient Arf proteins. Guo et al. have previously shown that genetic disruption of *Arf* in mouse embryonic fibroblasts (MEFs) enhances cell motility in a p53-dependent manner through the stimulation of Rac1 activity (33). Our data indicate that Arf expression in HCC cells does not alter cell morphology or the actin cytoskeleton, as assessed by phalloidin staining. This suggests that the activity of Rac1, or other Rho family GTPases involved in cytoskeletal remodeling, may not be affected by Arf expression, although this has not be formally tested.

Additional studies demonstrated that activated Rho GTPases could stimulate invasion by *Trp53* null, but not *Arf* null, MEFs suggesting that $p19^{Arf}$ regulates cell migration but not invasion (34). Thus, our experiments are the first to show that Arf inhibits tumor cell invasion via a p53-indpendent pathway. The differences between the previous studies and ours may reflect the different cell types utilized - epithelial-derived tumor cell lines versus transformed mouse fibroblasts. Importantly, in a related study, we have also shown that $p14^{Arf}$ inhibits the hypoxia-induced migration of H1299 human lung carcinoma cells in a p53-independent and CtBP-dependent manner (35), and previous studies have indicated that CtBP can regulate the migration of this cell line, although cell invasion was not assayed in these studies (19). Significantly, the leucine 46 residue that is critical for the Arf-CtBP interaction is one of six invariant residues conserved across several species including human, mouse, chicken, pig and opossum (32). Thus, the Arf-CtBP interaction likely mediates a highly conserved tumor suppressor function of Arf, and may have implications for the dissemination of human tumors.

CtBPs play a critical role in cellular regulation by binding to a variety of transcriptional repressors important for development and tumorigenesis (36). CtBPs have also been suggested to play a role in inhibiting anoikis (18). However, the mechanisms by which Arf inhibits CtBP function remain unclear. Our findings suggest that Arf may impede CtBP function, yet the mechanisms by which this might occur remain unclear, although potential links are beginning to emerge. While we have observed that Arf expression does not influence the steady state levels of CtBP, Arf, but not a mutant defective for CtBP binding, stimulates the degradation of CtBP in response to cellular stresses such as UV irradiation [Supplemental Figure 2 and (16)]. Therefore, it is possible that Arf stimulates the degradation of CtBP under stress conditions relevant during cell migration and invasion, such as detachment from the basement membrane. In addition, recent findings from the Grossman lab indicate that Arf can inhibit CtBP-mediated transcriptional repression of a target promoter (R. Kovi and S.R.G., unpublished observations).

CtBP has also been previously shown to induce an epithelial to mesenchymal transition (EMT) (17,18). EMT, first identified as a critical process during normal embryonic development, involves the downregulation of epithelial cell markers such as E-cadherin and the induction of mesenchymal markers such as vimentin, and is postulated to be involved in tumor cell invasion and metastasis (27). However, our data indicate that our HCC cell lines invade without undergoing EMT, and E-cadherin protein levels are not significantly altered in cells by reintroduction of $p19^{Arf}$. A similar phenomenon has been recently described by Christofori and colleagues in connection to tumor cell invasion stimulated by the mucin-like protein

podoplanin (37). EMT has been postulated to a transient process. Thus, an alternate possibility is that Arf may prevent the transient induction of CtBP, and the consequent transient reduction of E-cadherin levels, in response to pro-invasion stimuli that would allow a tumor cell to initiate invasion. A similar model has been postulated for metastasis of colorectal carcinomas (38).

Thus, our findings indicate a potential new role for Arf in tumor invasion. Further exploration of the pathways involved in this process may yield promising new targets for therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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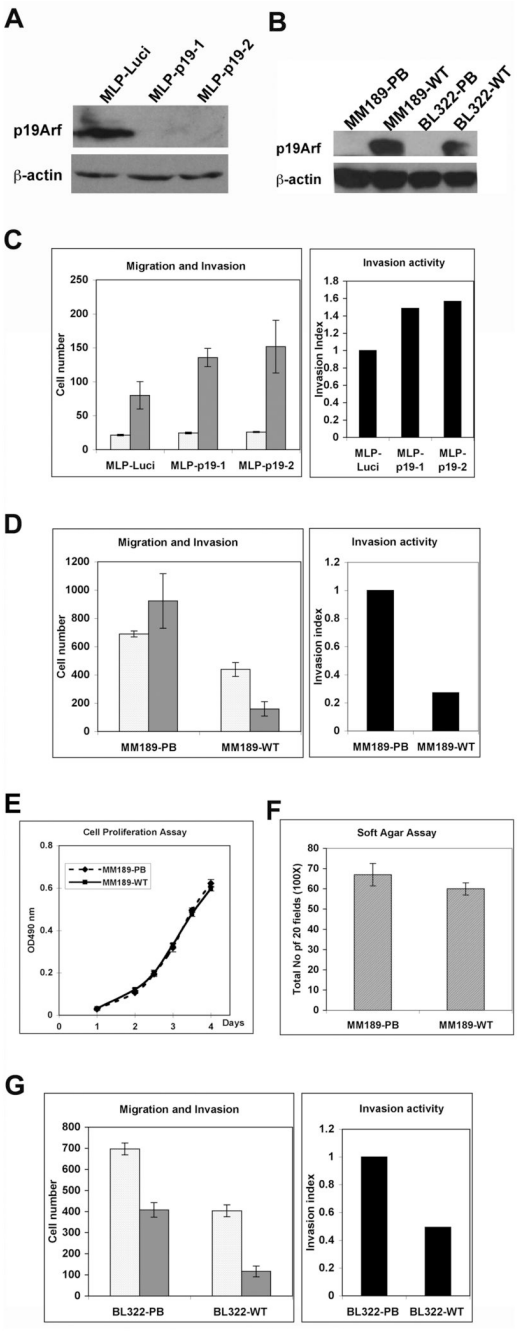

Figure 1.

p19^{Arf} inhibits tumor cell invasion. (A) Immunoblot analysis of BL185 HCC cells infected with retroviral vectors targeting luciferase or p19^{Arf}. β-actin serves as a control. (B) Immunoblot detection of p19^{Arf} in HCC cell lines infected with either pBabe puro retrovirus (PB) or pBabe puro retrovirus encoding p19^{Arf} (WT). β-actin serves as a control. (C) Migration (clear bars) and invasion (grey bars) activity of BL185-MLP-Luci and BL185-MLP-p19 cells. Data are from a representative experiment performed in triplicate. Error bars represent standard error of the mean (S.E.M.). The cell number is the number of migrated or invaded cells in five 50X microscopic fields per insert. The invasion index was calculated as described in the methods. (D) Migration (clear bars) and invasion (grey bars) activity of MM189-PB and

MM189-WT cells. Data are from a representative experiment performed in duplicate. Error bars represent S.E.M. (E) Cell proliferation assay for MM189-PB and MM189-WT cells. (F) Anchorage-independent growth assay for MM189-PB and MM189-WT cells. The colony number is the total number for twenty 100 X microscopic fields in every plate. Data are from a representative experiment performed in duplicate. Error bars are S.E.M. (G) Migration and invasion assay for BL322-PB and BL322-WT cells. Error bars are S.E.M.

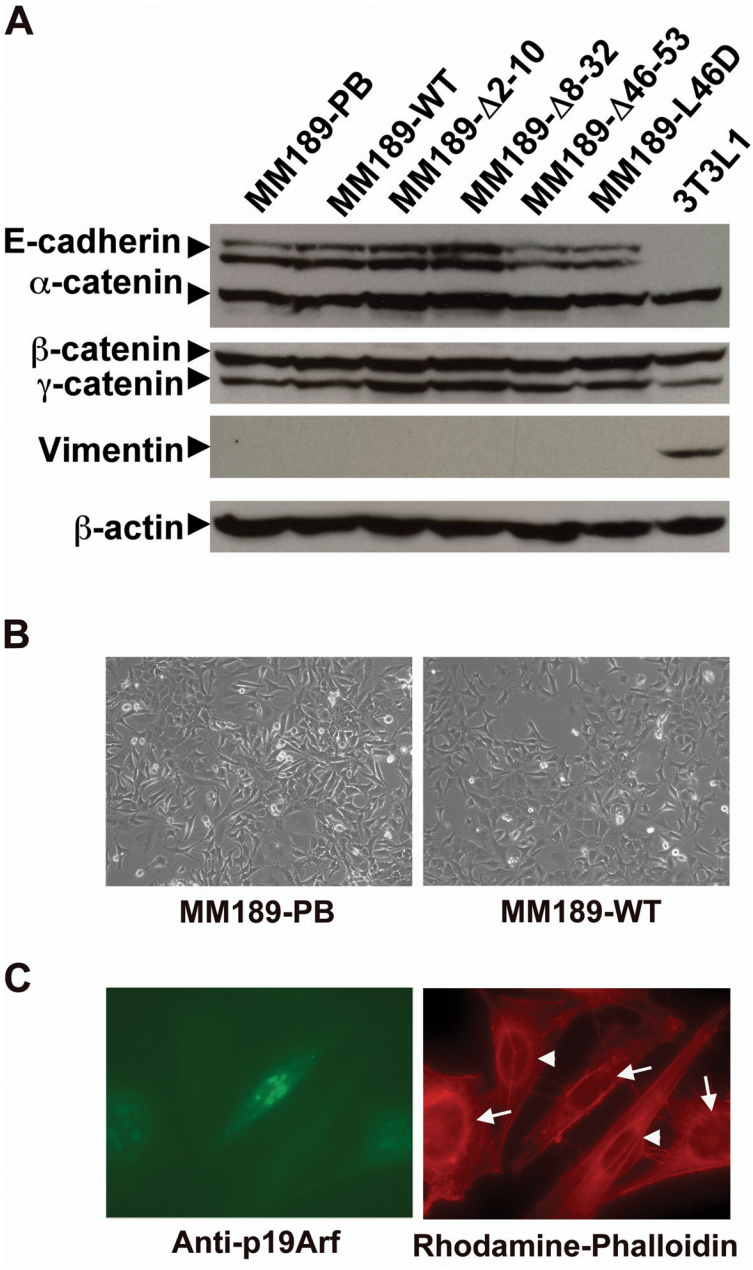

Figure 2.

p19^{Arf} does not influence cell morphology or EMT. (A) Immunoblot analysis of epithelial and mesenchymal proteins in MM189 cells expressing either wild-type p19^{Arf} or the indicated p19^{Arf} mutant proteins. 3T3L1 serves as a positive control for expression of the mesenchymal marker vimentin. β-actin serves as a loading control. (B) Phase contrast image of MM189-PB and MM189-WT cells grown for 3 days on a collagen matrix. (C) Immunofluorescent images of MM189 cells expressing p19^{Arf} and vector controls. Left panel: p19^{Arf} positive cells (arrows) are labeled with FITC-conjugated antibodies, while p19^{Arf} negative cells (arrowheads) are not. Right panel: Rhodamine-conjugated phalloidin labels the actin cytoskeleton.

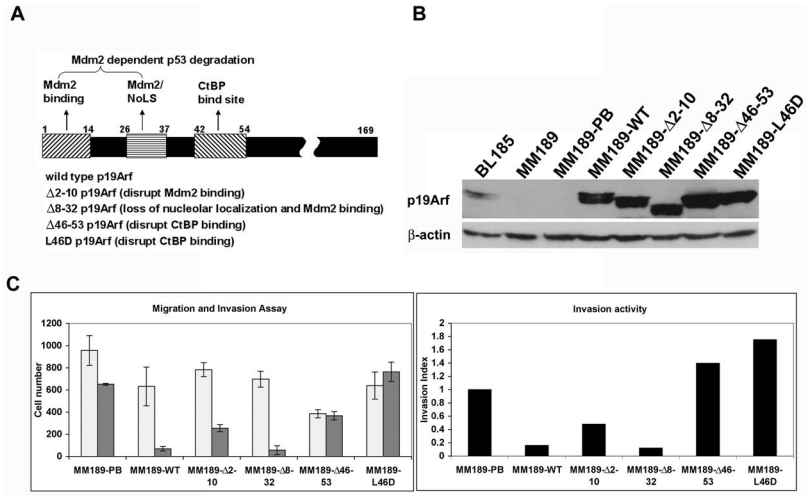

Figure 3.

p19^{Arf} inhibition of cell invasion is dependent on the CtBP binding domain. (A) Schematic illustration of the p19^{Arf} mutants used in this study. (B) Immunoblot detection of expression of wild-type or mutant p19^{Arf} proteins in MM189 HCC cells. (C) Migration (clear bars) and invasion (grey bars) activity of MM189 cells infected with retroviruses encoding the indicated p19^{Arf} mutant proteins.

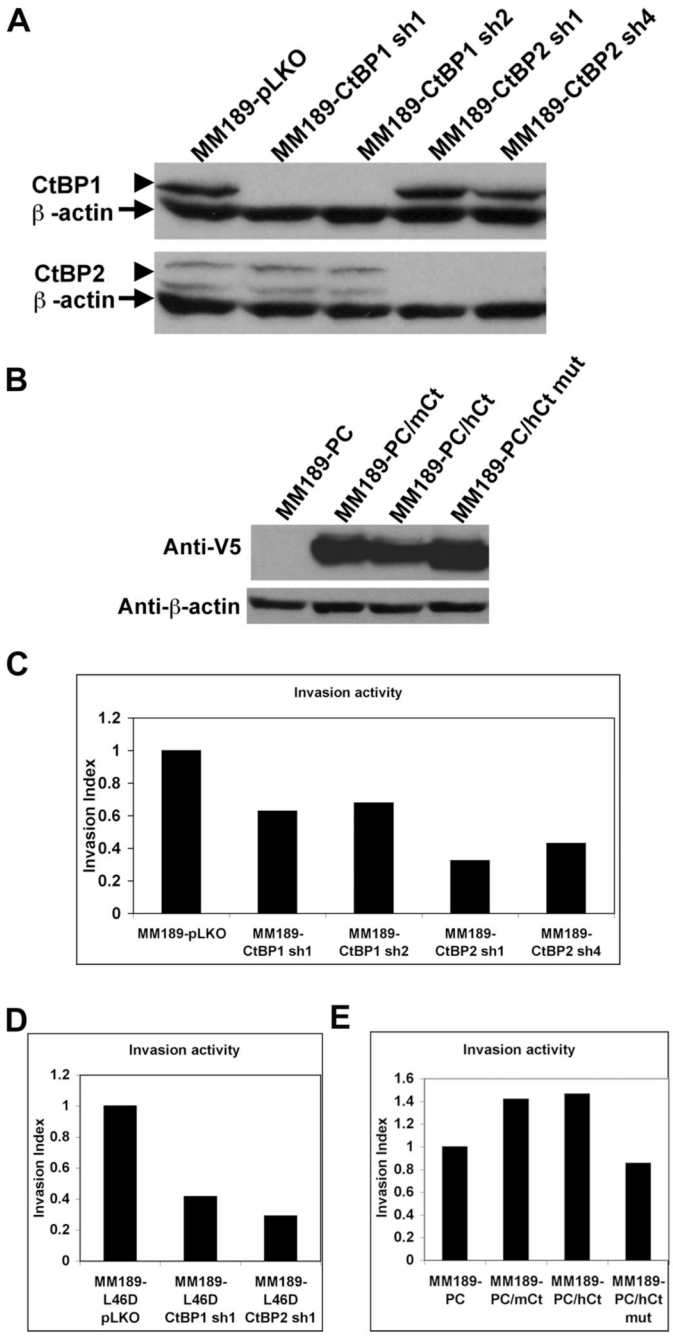

Figure 4.

CtBPs regulate tumor cell invasion. (A) Detection of CtBP1 and CtBP2 by immunoblot after introduction of lentiviral vectors encoding shRNAs specific for either family member. Empty pLKO vector was introduced as a control. Protein bands corresponding to CtBP1 or CtBP2 are indicated by arrowheads. β-actin serves a a loading control (indicated by arrows). (B) Detection of ectopic V5 epitope-tagged CtBP2 by immunoblot with anti-V5 antibodies in MM189 cells transfected with pcDNA3-CtBP2 (PC/Ct) or empty vector control (PC). β-actin serves as a loading control. The band shown indicated by the arrow is non-specific. (C) Invasion activity, represented by invasion index (see methods) for MM189 cells infected with either control lentivirus or lentiviruses encoding shRNAs targeting either CtBP1 or CtBP2. (D) Invasion

activity of MM189 cells expressing the $p19^{Arf}$ L46D mutant and infected with either control lentivirus or lentiviruses encoding shRNAs specific for either CtBP1 or CtBP2. (E) Invasion activity of MM189 cells transfected with pcDNA3-CtBP2 or empty vector.