

A Novel MIF Signaling Pathway Drives the Malignant Character of Pancreatic Cancer by Targeting NR3C2

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Abstract

Pancreatic cancers with aberrant expression of macrophage migration inhibitory factor (MIF) are particularly aggressive. To identify key signaling pathways that drive disease aggressiveness in tumors with high MIF expression, we analyzed the expression of coding and noncoding genes in high and low MIF-expressing tumors in multiple cohorts of pancreatic ductal adenocarcinoma (PDAC) patients. The key genes and pathways identified were linked to patient survival and were mechanistically, functionally, and clinically characterized using cell lines, a genetically engineered mouse model, and PDAC patient cohorts. Here, we report evidence of a novel MIF-driven signaling pathway that inhibits the orphan nuclear receptor NR3C2, a previously undescribed tumor suppressor that impacts aggressiveness and survival in PDAC. Mechanistically, MIF upregulated miR-301b that targeted NR3C2

and suppressed its expression. PDAC tumors expressing high levels of MIF displayed elevated levels of miR-301b and reduced levels of NR3C2. In addition, reduced levels of NR3C2 expression correlated with poorer survival in multiple independent cohorts of PDAC patients. Functional analysis showed that NR3C2 inhibited epithelial-to-mesenchymal transition and enhanced sensitivity to the gemcitabine, a chemotherapeutic drug used in PDAC standard of care. Furthermore, genetic deletion of MIF disrupted a MIF-miR-301b-NR3C2 signaling axis, reducing metastasis and prolonging survival in a genetically engineered mouse model of PDAC. Taken together, our results offer a preclinical proof of principle for candidate therapies to target a newly described MIF-miR-301b-NR3C2 signaling axis for PDAC management. *Cancer Res*; 76(13); 3838–50. ©2016 AACR.

Introduction

Pancreatic cancer is a lethal malignancy and ranked as fourth leading cause of cancer-related death (1). Majority of the pancreatic cancer patients are diagnosed at an advanced stage of the

disease and are refractory to the available treatments. Therefore, identification of novel therapeutic targets and accelerated assessment of their clinical relevance are desperately needed to improve outcome.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine and was initially characterized as a key regulator of innate immune response (2, 3). However, recent investigations have described the presence of a high level of MIF in tumors and provided evidence indicating a role of MIF in human cancer (4, 5). A high level of MIF is found in tumors from pancreatic ductal adenocarcinoma (PDAC) patients (6, 7). MIF-overexpressing tumors are highly aggressive and an elevated MIF in pancreatic tumors predicted poor survival in patients with PDAC, indicating its potential biologic relevance in disease aggressiveness (7). MIF is produced by a variety of cell type, including immune, inflammatory, and epithelial cells, and can either bind to the cell surface receptor CD74 and CD44 or enter through endocytosis (2, 8, 9). In addition, tumor cells itself secrete high levels of MIF and thus induce protumorigenic signaling pathway through its autocrine function (10). One of the mechanisms for the enhanced intratumoral expression of MIF in malignant cells is its stabilization by HSP90 chaperone complex by protecting it from degradation (11). On the basis of the evidence involving MIF in immune regulation and its association with tumor progression, it is hypothesized that MIF is a molecular link between

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inflammation and cancer (5, 12). However, the exact mechanism is not clear.

Here, we comprehensively investigated the role of MIF in pancreatic cancer progression by applying an integrative analysis of coding and noncoding genes in tumor biology. On the basis of the analysis of multiple independent cohorts of human PDAC, we identified key characteristics of tumors with aberrantly high MIF expression that link these tumors to enhanced disease aggressiveness and mortality. We also performed functional characterization using *in vitro* and *in vivo* models with clinical validation of our findings, which led to the discovery of a novel MIF-driven signaling pathway, involving MIF-miR-301b-NR3C2 axis with potential therapeutic significance in PDAC.

Materials and Methods

Cell lines, human samples, and mouse model

All the cell lines used in this study were purchased from ATCC in 2009 and were authenticated by short tandem repeat analysis (STR) analysis in 2015.

Human primary pancreatic tumor tissues from PDAC patients were collected at the University of Maryland Medical System at Baltimore (Baltimore, MD) and University of Medicine (Gottlingen, Germany). The characteristics of the patients in test cohort ($N = 69$) are described in Supplementary Table S1. Patients' characteristics in validation cohorts ($N = 41$, $N = 64$), and the cohort in The Cancer Genome Atlas (TCGA; $N = 69$) are shown in Supplementary Tables S2–S4. The tissue microarray was prepared from additional 173 PDAC cases at the Institute of Pathology, University of Heidelberg (Heidelberg, Germany; Supplementary Table S5). Use of clinical specimens was reviewed and permitted by the NCI-Office of Human Subject Research Protection (OHSRP, Exempt# 4678) at the NIH (Bethesda, MD).

MIF-deficient *LSL-Kras^{G12D};LSL-Trp53^{R172H/+};Pdx-1-Cre* (MKPC) pancreatic cancer mouse model was generated, through crossbreeding of KPC (13) and MIF knockout mice (14) with an approved animal study protocol by Animal Care and Use Committee at National Cancer Institute (NCI, Frederick, MD).

Microarray expression profiling

miRNA and mRNA expression profiling were performed at the microarray core facility, NCI. The miRNA and mRNA microarray expression data have been deposited in the National Center for Biotechnology Information's (NCBI's) Gene Expression Omnibus with accession numbers GSE 62498 and GSE 62452. Details are provided in Supplementary Methods.

Cell proliferation, migration and invasion, colony formation, and drug cytotoxicity assays.

These assays were performed using kits from various manufacturers. Details are provided in Supplementary Methods.

Statistical analysis

Student *t* test was used to assess the difference in the miRNA and mRNA expression and other endpoints including, migration and invasion, colony-forming ability, and cell survival among different comparison groups. When more than two groups were compared, a one-way ANOVA, followed by a Scheffe multiple comparison test was used. Kaplan–Meier analysis and log-rank test were performed to compare the difference in survival between two groups of patients using GraphPad Prism 6.0. Pearson correlation

was used for determining any correlation between the expressions of two genes. $P < 0.05$ was considered statistically significant.

Results

miRNA profiling of MIF-high and MIF-low PDAC revealed MIF-associated miRNAs with prognostic significance

Molecular characterization of MIF-high and MIF-low tumors was performed using miRNA profiling in a test cohort ($N = 69$) of patients with PDAC, using Nanostring nCounter miRNA Expression Array, and testing the hypothesis that MIF regulates miRNAs, associated with tumor progression and patient survival. Stratification of PDAC cases into MIF-high and MIF-low groups was based on the MIF expression above the median (high) and below the median (low) values as determined by qRT-PCR. Unsupervised principal component analysis (PCA) of miRNA expression profile revealed distinct groupings for MIF-high and MIF-low tumors, indicating a systematic change in global miRNA expression profile by MIF in PDAC (Fig. 1A). Further analysis identified 53 differentially expressed miRNAs in MIF-high versus MIF-low tumors and a higher expression of five of these miRNAs, miR-301b, miR-15b, miR-10b, miR-93, and miR-590-5p, in MIF-high tumors were also associated with poor survival in PDAC cases (Fig. 1A and Supplementary Fig. S1A and S1B; Supplementary Table S6). Differentially expressed miRNAs in MIF-high and MIF-low tumors also included miR-200b, which is consistent with the earlier report describing its inhibition by MIF. However, the expression of miR-200b does not show any association with patient survival. miRNAs, found to be both differentially expressed in MIF-high versus MIF-low tumors, as well as associated with patient survival, were then validated by using qRT-PCR. Expression of the above mentioned 5 miRNAs (miR-301b, miR-15b, miR-10b, miR-93, and miR-590-5p), as determined by qRT-PCR, and their prediction of prognosis were consistent with the findings from array analysis (Fig. 1B and C). Furthermore, the miRNA expression obtained by miR-array and qRT-PCR was highly correlated (Supplementary Fig. S1C). To ensure that these findings are not restricted to just one group of PDAC used as test cohort, we further validated these results in an independent validation cohort-1 ($n = 41$; Supplementary Table S2) of PDAC. The cases were grouped as MIF-high and MIF-low based on the MIF expression in tumors and dichotomized based on the median value. Expression of miR-301b was elevated in MIF-high tumors as compared with MIF low tumors and a higher miR-301b and miR-590-5p was also associated with poor survival, which is consistent with the findings in the test cohort (Fig. 1D and E). However, the expression of miR-15b, miR-10b, miR-93, and miR-590-5p did not show significant difference between MIF-high and MIF-low groups in the validation cohort. Therefore, we continued our investigation to examine the association between MIF and miR-301b. Examining a panel of eight pancreatic cancer cell lines revealed a positive correlation between MIF and miR-301b expression ($r = 0.67$, $P = 0.014$; Supplementary Fig. S2A). To assess a potential MIF-mediated regulation of miR-301b, MIF expression was knocked down using lentiviral-mediated shRNA in pancreatic cancer cell lines, which resulted in a significant reduction in miR-301b (Supplementary Fig. S2B and S2C). These findings were further confirmed by a different approach involving the lentiviral-mediated stable overexpression of MIF, which resulted in a significant increase in miR-301b expression in these pancreatic cancer cells (Supplementary Fig. S2D and S2E). These findings

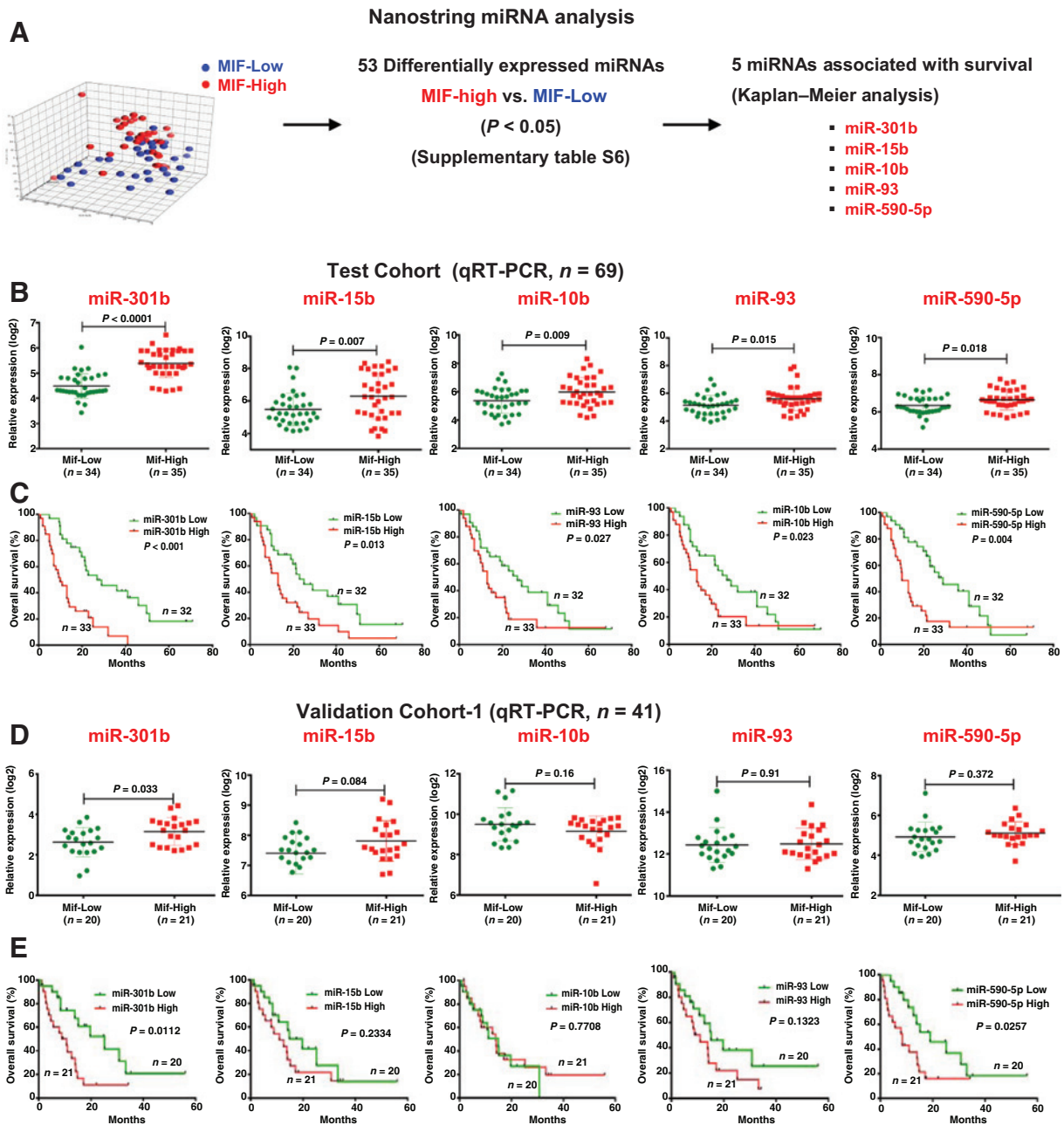


Figure 1. miRNA profiling of MIF-high and MIF-low tumors identified MIF-associated miRNAs with prognostic significance. A, unsupervised principal components analysis, differentially expressed miRNAs, and their association with survival in MIF-high and MIF-low tumors. B–E, miRNA expression as determined by qRT-PCR, and Kaplan–Meier survival analysis in test and validation cohorts.

confirmed that MIF positively regulates miR-301b in pancreatic cancer.

MIF-induced miR-301b targets nuclear receptor subfamily 3, group C, member 2

As a next step in our effort to examine MIF-induced signaling involved in the tumor progression and disease aggressiveness in PDAC, we performed a comprehensive analysis to identify potential targets of MIF-induced miR-301b by using an integrative

strategy, which combines miRWalk-target prediction module (15) with global gene expression profile and Cox regression analysis in the same cohort of PDAC cases (Fig. 2A). Putative miR-301b target genes predicted by miRWalk were compared with genes that showed inverse correlation with miR-301b through transcriptomic profiling, and genes that were associated with survival by Cox regression analysis in our PDAC cohort. This approach identified 109 potential miR-301b target genes (Supplementary Table S7). These potential target genes were further

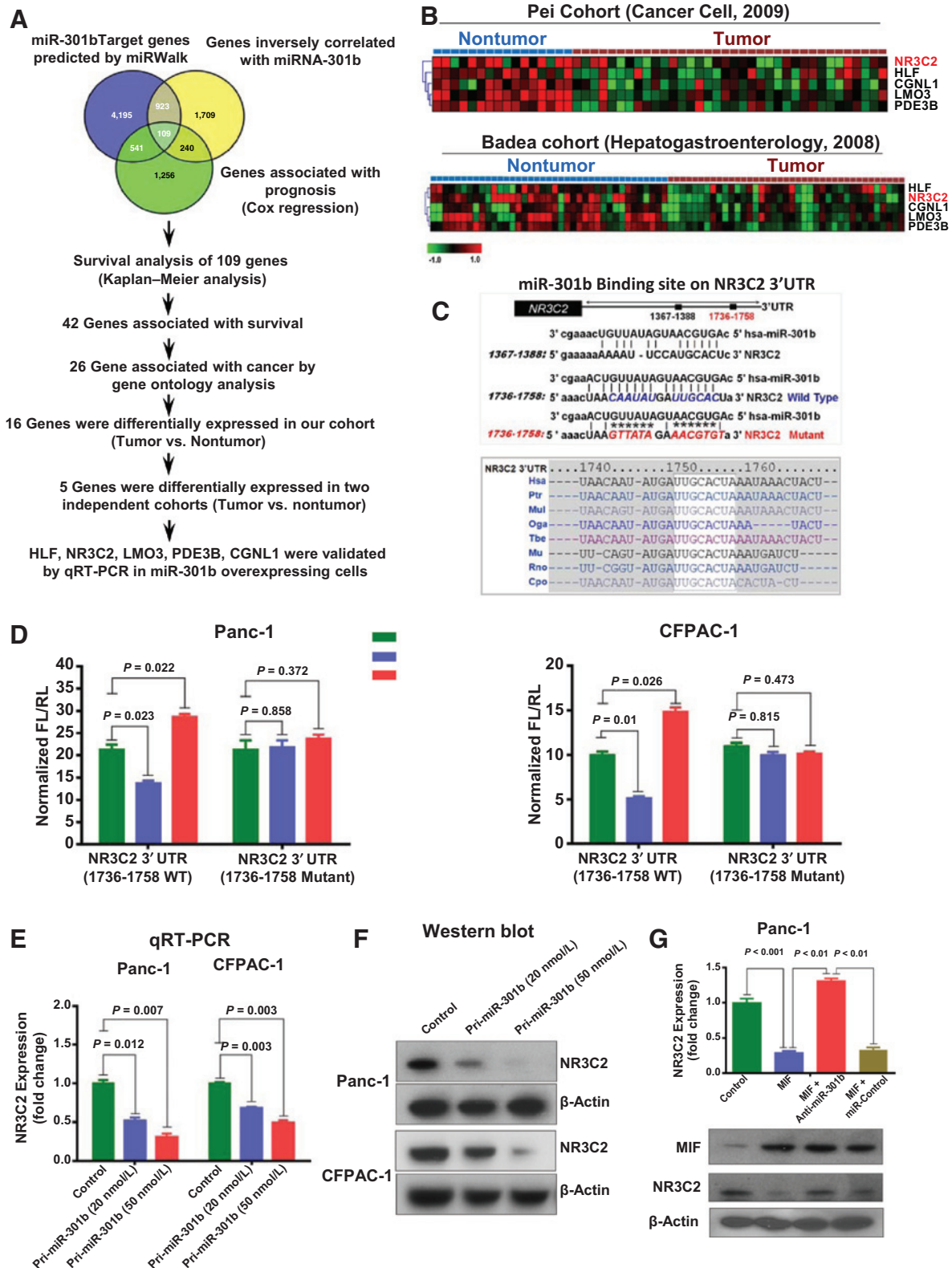


Figure 2. MIF-induced miR-301b targets NR3C2. A, schema depicting integrative approach to identify putative miR-301b target genes. B, a lower expression of predicted targets of miR-301b in tumors in the publicly available datasets (16, 17). C, two miR-301b target sites reside at nucleotides spanning 1367–1388 and 1736–1758 of NR3C2 3'UTR, and 1736–1758 region was highly conserved in different species. D, Luciferae reporter assay showing the targeting of 3'UTR sequence of NR3C2 by miR-301b. E and F, a dose-dependent decrease in endogenous NR3C2 expression by miR-301b in pancreatic cancer cell lines. G, MIF suppressed NR3C2 expression, which is rescued by coexpression of anti-miR-301b in pancreatic cancer cells.

subjected to Kaplan–Meier analysis, gene ontology analysis (Supplementary Fig. S3A), assessment of their differential expressions in tumors as compared with nontumor pancreas, and validation in two separate cohorts (16, 17) in OncoPrint gene expression database. This highly stringent approach identified nuclear receptor subfamily 3, group C, member 2 (*NR3C2*), *HLF*, *LMO3*, *PDE3B*, and *CGNLI* as the most predicted target of MIF-induced miR-301b in pancreatic cancer (Fig. 2A and B). We then validated these putative targets by overexpressing miR-301b in Panc-1 and Capan-2 pancreatic cancer cell lines. Overexpression of miR-301b significantly reduced *NR3C2* expression (Supplementary Fig. S3B). Furthermore, examining 3'UTR of *NR3C2* revealed two miR-301b-binding sites in the sequence spanning 1367–1388 and 1736–1758 nucleotides with the latter found to be highly conserved in different mammalian species (Fig. 2C). Luciferase reporter-based activity assay showed the interaction of miR-301b to 3'UTR sequence 1736–1758 of *NR3C2* resulting in a decrease of reporter activity following the expression of miR-301b. Furthermore, cotransfection with anti-miR-301b enhanced the *NR3C2* luciferase reporter activity. However, these effects were abolished by the mutation of miR-301b-binding site (Fig. 2D; and Supplementary Fig. S3C–S3E). Consistently, the overexpression of pri-miR-301b suppressed both mRNA and protein expression of *NR3C2* in a dose-dependent manner confirming miR-301b-mediated suppression of *NR3C2* (Fig. 2E and F). We then examined whether MIF can downregulate *NR3C2* and whether this inhibition is dependent on miR-301b. As shown in Fig. 2G, MIF overexpression significantly reduced *NR3C2* mRNA and protein levels. However, the MIF-induced suppression of *NR3C2* was abolished by the inhibition of miR-301b. Furthermore, endogenous *NR3C2* expression was negatively correlated with MIF and miR-301b in a panel of human pancreatic cancer cell lines (Supplementary Fig. S4A and S4B). Taken together these findings show that MIF-induced increase in miR-301b targets and reduces *NR3C2* level.

NR3C2 inhibits proliferation, colony formation, migration, invasion, and enhances sensitivity of pancreatic cancer cells to gemcitabine.

NR3C2 gene encodes a mineralocorticoid receptor and functions as a ligand-dependent transcription factor mediating aldosterone action on salt and water balance. Interestingly, a decrease in *NR3C2* expression is associated with poor prognosis in lung and colorectal cancer (18, 19). However, the role of *NR3C2* has not been described in pancreatic cancer. We investigated the function of *NR3C2* in pancreatic cancer by first screening its expression in a panel of pancreatic cancer cell lines. We then generated lentiviral-mediated *NR3C2*-overexpressing stable pancreatic cancer cell lines, Panc-1 and MiaPaCa-2, which had the lowest endogenous *NR3C2* expression (Supplementary Fig. S5A). *NR3C2* overexpression resulted in a significant decrease in the proliferation index of these cells (Fig. 3A). The effect of *NR3C2* on the growth characteristics was measured by clonogenicity assay, which showed 50% to 75% reduction in the colony formation (Fig. 3B). We further investigated the potential role of *NR3C2* in the mobility of tumor cells, which is essential for invasion and metastasis. *NR3C2* overexpression significantly reduced the migration and invasion ability of pancreatic cancer cells (Fig. 3C). Gemcitabine has been used as a first-line standard chemotherapeutic drug against PDAC for more than a decade and

recently is being used in combination with other compounds with some success. Therefore, it was examined whether an enhanced level of *NR3C2* improves the sensitivity of pancreatic cancer cells to gemcitabine. *NR3C2*-overexpressing cells showed a significant reduction in survival as compared with control cells following gemcitabine treatment (Supplementary Fig. S5B). These findings were further supported by clonogenicity assay, which showed significantly lower colony forming ability of *NR3C2*-overexpressing pancreatic cancer cells as compared with control cells following gemcitabine treatment (Supplementary Fig. S5C). On the contrary, stable knockdown of *NR3C2* in Capan-2 cells significantly enhanced proliferation and invasiveness, and reduced sensitivity to gemcitabine (Supplementary Fig. S6A–S6D). Taken together, these findings support a potential tumor-suppressive role of *NR3C2* in pancreatic cancer.

NR3C2 inhibits epithelial-to-mesenchymal transition

Acquiring features of epithelial-to-mesenchymal transition (EMT) are a critical component in the ability of cancer cells to metastasize. Inflammatory mediators enhance metastatic ability of pancreatic tumors and preneoplastic cells (20) and MIF-overexpressing pancreatic tumors are highly metastatic (7). To further examine the role of *NR3C2* in pancreatic cancer, we hypothesized that *NR3C2* inhibits EMT. A lower endogenous *NR3C2* expression was associated with EMT phenotype (Supplementary Fig. S7A and S7B). We further showed that *NR3C2* inhibition by lentiviral-mediated shRNA (Supplementary Fig. S8A) resulted in the acquisition of mesenchymal features in pancreatic cancer cells. *NR3C2* inhibition led to the reduction in E-cadherin and increase in zeb1, N-cadherin, and vimentin mRNA, and protein expression in pancreatic cancer cell lines (Fig. 3D and E and Supplementary Fig. S8B–S8E). These findings show that *NR3C2* inhibits EMT in pancreatic cancer cells and support its potential tumor-suppressive role in pancreatic cancer.

MIF enhances tumor cell invasiveness by targeting NR3C2 through the upregulation of miR-301b

MIF-induced signaling leading to enhanced tumor cell aggressiveness is not clearly understood. An enhanced migration and invasion in MIF-overexpressing stable pancreatic cancer cells were attenuated, when miR-301b was inhibited (Fig. 4A and Supplementary Fig. S9). However, this inhibition was abrogated by siRNA-mediated depletion of *NR3C2* (Fig. 4A). These results showed that MIF-induced migration and invasion are mediated by targeting *NR3C2* through an increase in miR-301b. We then examined whether the MIF-induced suppression of *NR3C2* and the resulting enhancement of migration and invasion in pancreatic cancer cells is achieved through EMT. Pancreatic cancer cells with stable overexpression of MIF showed a decrease in E-cadherin and increase in N-cadherin, vimentin, and zeb1 expression (Fig. 4B). However, inhibition of miR-301b in these cells enhanced E-cadherin and reduced the expression of N-cadherin, vimentin, and zeb1. We then examined whether this reversion in the expression of EMT marker proteins following miR-301b inhibition is due to an increase in *NR3C2*. Suppressing both miR-301b and *NR3C2* resulted in a decrease in E-cadherin and increase in N-cadherin, vimentin, and zeb1 expression. These results showed that inhibition of *NR3C2* by MIF through upregulation of miR-301b mediated the MIF-induced invasiveness and EMT (Fig. 4B). We then validated these findings using a

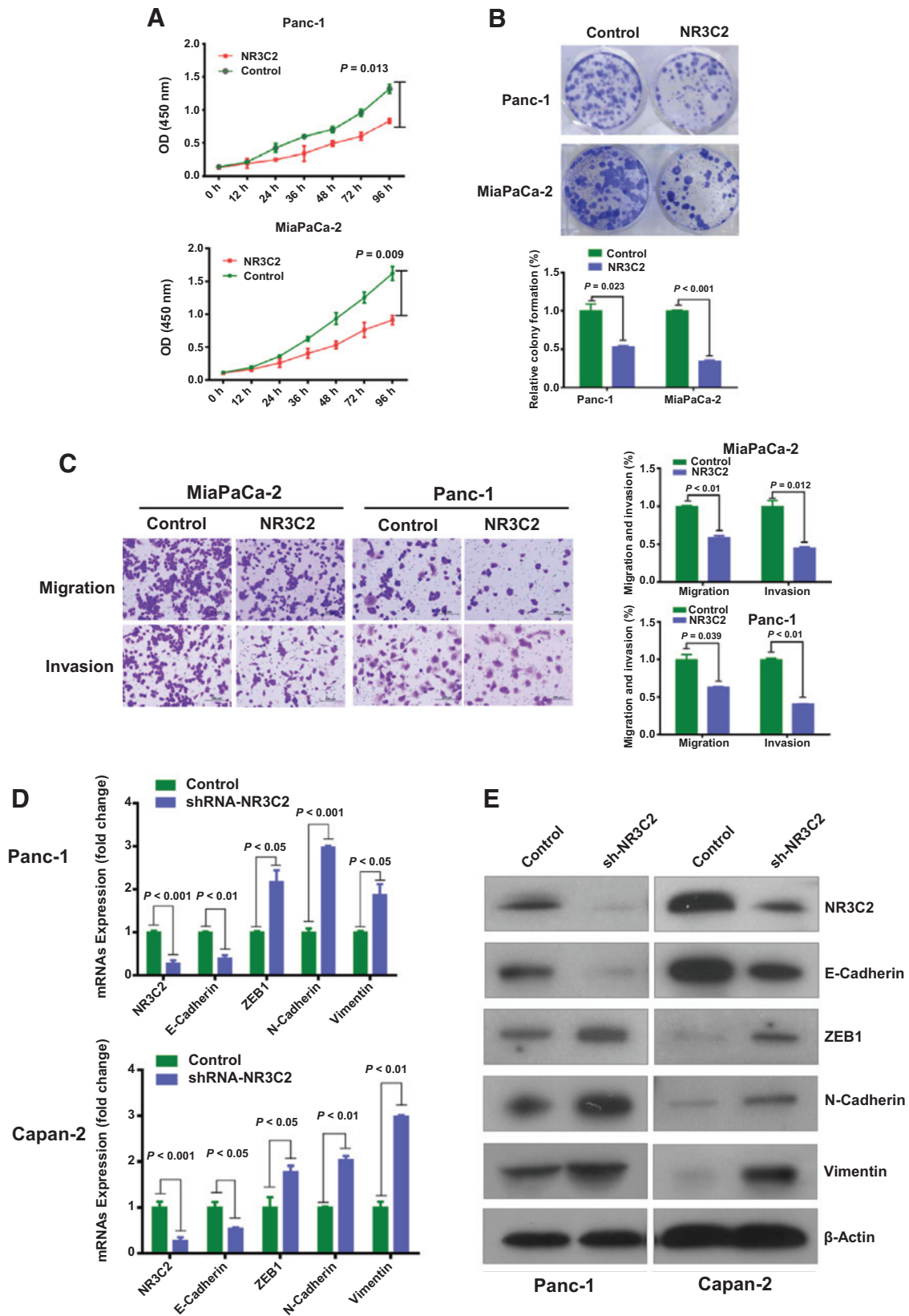


Figure 3. NR3C2 inhibits proliferation, colony formation, invasiveness, and EMT. A, NR3C2 overexpression suppresses the proliferation of Panc-1 and MiaPaCa-2 cells. B, NR3C2-overexpressing cells showed reduced colony formation as compared with control cells. C, NR3C2 significantly attenuated cell motility and invasiveness in Panc-1 and MiaPaCa-2 cell lines. D and E, NR3C2-knockdown resulted in a marked decrease in E-cadherin and increase in zeb-1, N-cadherin, and vimentin.

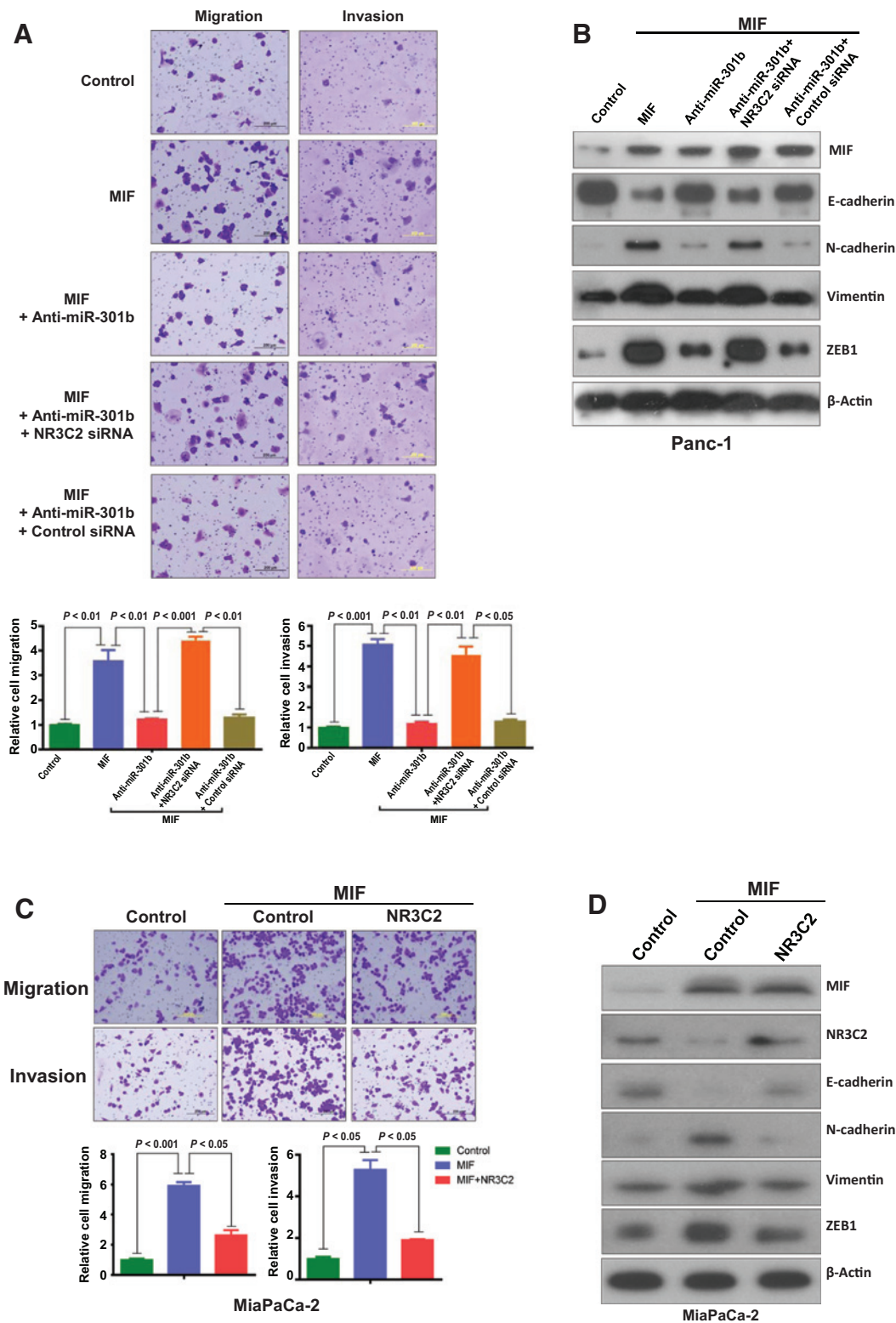


Figure 4.

MIF enhances tumor cell invasiveness by targeting NR3C2 through the upregulation of miR-301b. A, MIF-induced increase in tumor cell migration and invasion is suppressed by the inhibition of miR-301b. However, the suppression of migration and invasion following miR-301b inhibition was abrogated by knocking down NR3C2. B, MIF overexpression decreases E-cadherin and increases N-cadherin, vimentin, and zeb1 protein levels in pancreatic cancer cells. Although, miR-301b inhibition in the MIF overexpressing cells is associated with an increase in E-cadherin, and a decrease in N-cadherin, vimentin, and zeb1, these effects were abolished when NR3C2 was inhibited as well. C, MIF-induced increase in tumor cell invasiveness is suppressed by overexpressing NR3C2. D, the effect of MIF on the expression of EMT-associated markers was attenuated by NR3C2.

complimentary approach of overexpressing NR3C2 in pancreatic cancer cells with stable high expression of MIF. NR3C2 overexpression led to a decrease in MIF-induced migration and invasion (Fig. 4C). Furthermore, NR3C2 overexpression resulted in an increase in E-cadherin and decrease in N-cadherin, vimentin, and zeb1 (Fig. 4D). Taken together, these findings showed that MIF-induced tumor cell invasiveness and EMT involve the upregulation of miR-301b and subsequent targeting and downregulation of NR3C2.

MIF-mediated regulation of miR-301b and NR3C2 involves PI3K/Akt pathway

MIF activates PI3K/Akt and MEK/MAPK signaling pathways, which plays a role in tumorigenesis (10, 21, 22). We investigated whether the MIF-induced upregulation of miR-301b and subsequent targeting and downregulation of NR3C2 involve the activation of PI3K/Akt and MEK/MAPK signaling. Treatment of MIF-overexpressing pancreatic cancer cells with PI3K/Akt inhibitor LY294002 resulted in the significant decline in miR-301b and enhancement of NR3C2 expression (Fig. 5A). However, treatment with AZD6244, an inhibitor of MEK, did not have any effect on miR-301b or NR3C2 expression levels (Supplementary Fig. S10). Consistently, MIF overexpression showed an increase in the level of phosphorylated AKT (p-AKT^{ser473}) and a decrease in NR3C2 protein level determined by Western blot analysis (Fig. 5B). We then confirmed whether the treatment with LY294002 inhibits phosphorylation of AKT, and affects the expression of miR-301b and NR3C2. LY294002-treatment of MIF-overexpressing cells markedly reduced p-AKT^{ser473}, which resulted in a decline in miR-301b expression and increase in mRNA and protein level of NR3C2 (Fig. 5C-F). These *in vitro* findings show that MIF-induced upregulation of miR-301b and subsequent targeting and downregulation of NR3C2 are accomplished by activation of PI3K/Akt signaling. To validate these findings *in vivo*, we examined the orthotopic pancreatic tumors that were earlier generated from MIF-overexpressing or control human pancreatic cancer cells (7). Examination of MIF-overexpressing orthotopic tumors showed an elevated miR-301b and a marked decline in NR3C2 expressions as compared with control (Fig. 5G and H). Furthermore, an elevated expression of p-AKT^{ser473} in MIF-overexpressing tumors confirmed the activation of PI3K/Akt signaling pathway in these tumors (Fig. 5H).

Genetic ablation of MIF disrupted MIF-miR-301b-NR3C2 axis, enhanced survival, and reduced metastasis in KPC mouse model of pancreatic cancer

To examine whether MIF signaling is a potential therapeutic target in PDAC, we used a genetic strategy to target MIF in KPC mice, a genetically engineered mouse model of PDAC. Kaplan-Meier analysis showed that MIF-deficient KPC (MKPC) mice survived significantly longer than that of KPC mice with wild-type MIF ($P < 0.0001$; Fig. 6A). In addition, MKPC mice showed a significant reduction in metastatic burden ($P < 0.01$; Fig. 6B). A majority of these metastasis occurred in liver of both KPC and MKPC mice. Furthermore, primary tumor cells isolated from MKPC mice showed an enhanced expression of E-cadherin and reduced expression of vimentin, zeb1, and N-cadherin at both the protein and mRNA levels, as compared with primary tumor cells from KPC mice, suggesting an attenuation of EMT in MKPC tumors (Fig. 6C-E). Next, we examined whether MIF-deficiency affected MIF-miR-301b-NR3C2 signaling pathway in this pre-

clinical model of PDAC. Primary tumor cells from MKPC mice showed a reduced expression of miR-301b and an enhanced expression of NR3C2 as compared with KPC mice (Fig. 6E and F). In addition, IHC analysis of pancreatic tumors showed a significantly higher expression of NR3C2 in MKPC mice as compared with KPC mice (Fig. 6G). Taken together, these findings provide *in vivo* evidence that targeting MIF-miR-301b-NR3C2 signaling pathway reduce metastasis and enhance survival in KPC mouse model of pancreatic cancer.

Clinical validation of MIF-miR-301b-NR3C2 axis in human pancreatic cancer

Next, we examined the relevance of MIF-miR-301b-NR3C2 axis in the disease aggressiveness in human pancreatic cancer using multiple independent cohorts of PDAC. MIF-high tumors showed a reduced expression of NR3C2 in test cohort ($N = 69$; Fig. 7A, panel 1). Furthermore, MIF expression in the tumors was positively correlated with miR-301b and negatively correlated with NR3C2 expression (Fig. 7A, panels 2 and 3). In addition, a negative correlation was found between miR-301b and NR3C2 expression in these tumors (Fig. 7A, panel 4). Moreover, a lower expression of NR3C2 in tumors was associated with poorer survival of patients in this cohort (Fig. 7, panel 5). We then validated these findings in our validation cohort-2 ($n = 64$; Supplementary Table S3). Adding to our confidence, all the findings in validation cohort were consistent with the test cohort (Fig. 7B). Furthermore, a reduced NR3C2 expression was found in tumors as compared with adjacent nontumor pancreas in both of these cohorts (Supplementary Fig. S11A and S11B). Next, IHC analysis of TMA in a second independent validation cohort-3 of PDAC ($n = 173$) further validated that a lower expression of NR3C2 is associated with poorer patient survival (Fig. 7C and D; Supplementary Table S5). In addition, a third validation could be achieved by examining the gene expression profile of PDAC patient cohorts in "The Cancer Genome Atlas" (TCGA; www.cancergenome.nih.gov). Tumors from nonmetastatic PDAC cases showed an elevated level of NR3C2 as compared with metastatic cases in TCGA cohort (Supplementary Fig. S12A). Furthermore, a lower expression of NR3C2 in tumors correlated with poorer survival of patients in this cohort as well (Supplementary Fig. S12B). Finally, a lower expression of NR3C2 was validated in metastatic tumors as compared with primary tumors in a recently published large gene expression dataset (GSE71729, Supplementary Fig. S12C; ref. 23). A lower expression of NR3C2 in these tumors also predicted poorer survival (Supplementary Fig. S12D). Taken together these findings and their multiple validations demonstrate the clinical relevance of MIF-miR-301b-NR3C2 signaling pathway in disease aggressiveness and survival in patients with PDAC.

Discussion

Inflammatory mediators are critical components of pancreatic tumor biology, and their paracrine and autocrine functions significantly contribute to tumor progression and disease aggressiveness in patients with PDAC (20, 24). MIF was primarily described as a proinflammatory cytokine; however, recent evidence of a high level of MIF expression in many tumor types and its association with prognosis implicates it in the development and progression of human cancer. Pancreatic tumors with an

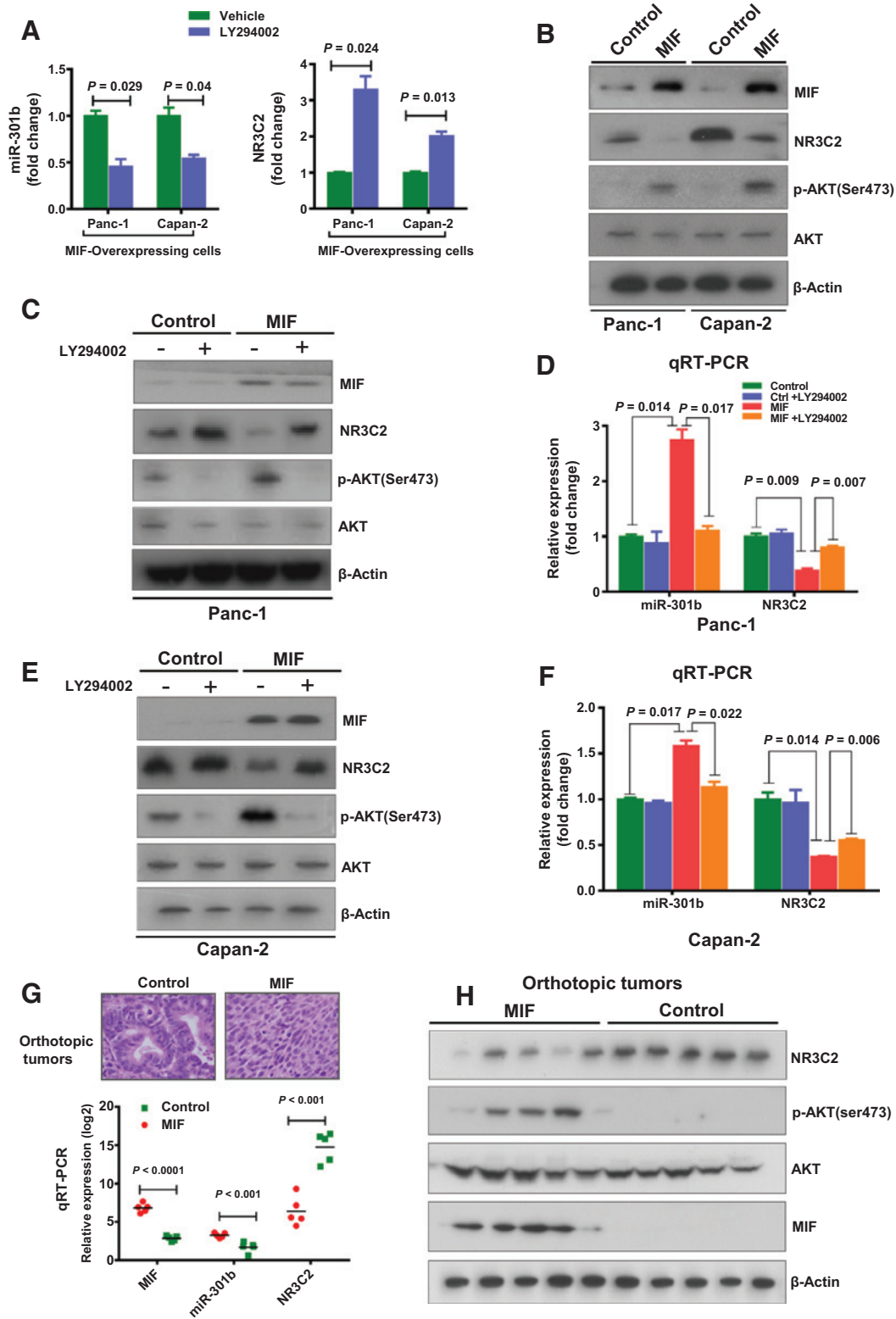
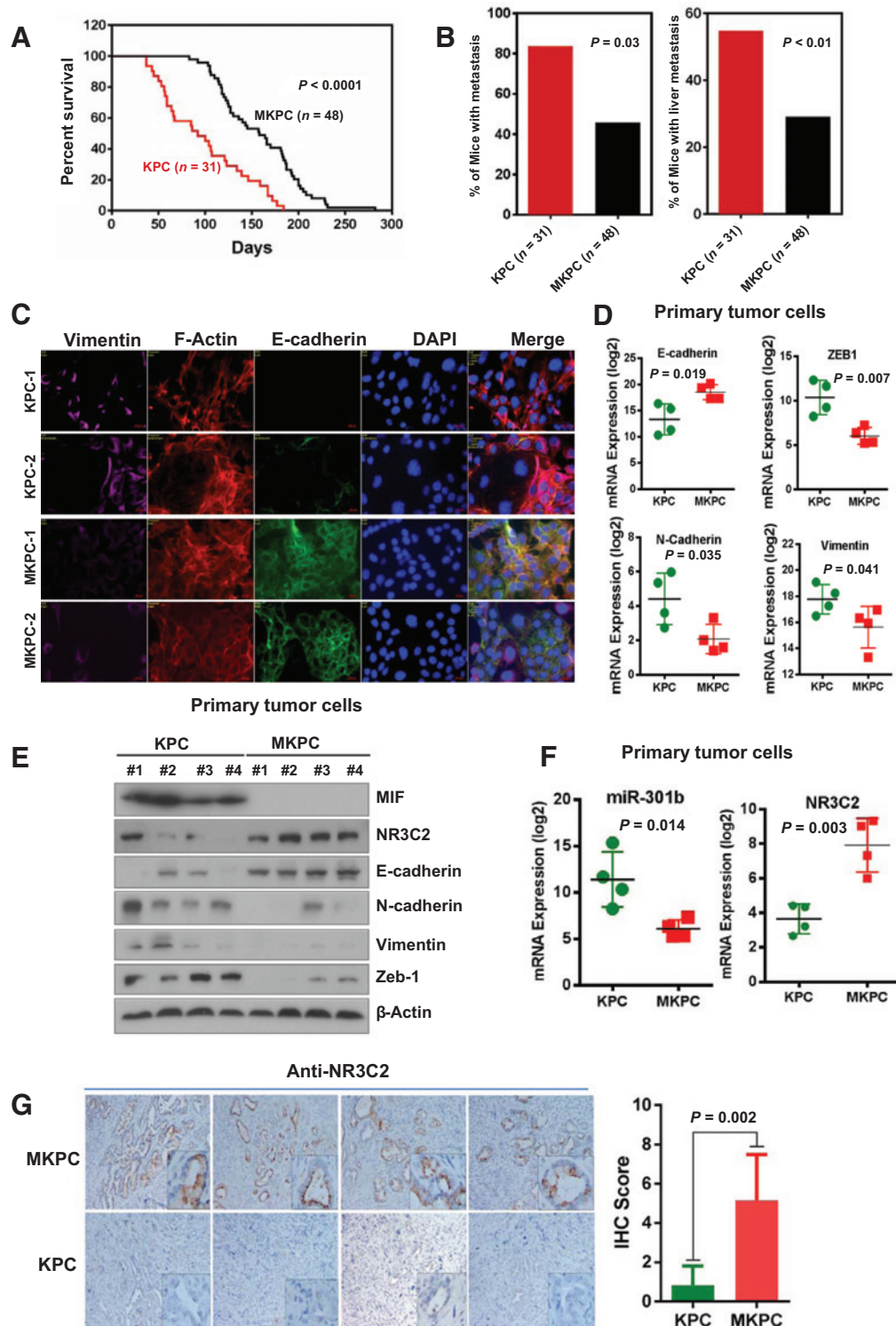


Figure 5. MIF-mediated regulation of miR-301b and NR3C2 involves AKT pathway. A, treatment of MIF-expressing stable Panc-1 and Capan-2 cells with Akt inhibitor, LY294002, inhibited miR-301b and enhanced the expression of NR3C2. Cells were treated with LY294002 for 48 hours. B, stable MIF-overexpressing cells expressed a higher level of phosphorylated Akt (p-Akt^{Ser473}) and a lower level of NR3C2. C–F, inhibition of Akt by LY294002 increased NR3C2 and decreased miR-301b level in MIF-overexpressing cells as compared with control cells. G, MIF-expressing orthotopic tumors showed an increase in miR-301b and decrease in NR3C2 expression as compared with control tumors. H, MIF activated AKT pathway and decreased NR3C2 level in MIF-expressing orthotopic tumors. MIF-expressing orthotopic tumors showed an enhanced level of p-Akt^{Ser473} and reduced level of NR3C2 as compared with control tumors.

**Figure 6.**

MIF deficiency increased NR3C2, enhanced survival, and reduced metastasis in KPC mouse model of pancreatic cancer. A, Kaplan-Meier analysis showing a significantly longer survival of MIF-deficient KPC mice (MKPC, $N = 48$) as compared with KPC mice ($N = 31$; $P < 0.0001$, log-rank test). B, a significantly lower percentage of MKPC mice showed metastasis as compared with KPC mice. C, immunofluorescence assay showing an enhanced expression of E-cadherin and reduced expression of vimentin in the primary tumor cells isolated from MKPC mice as compared with the KPC mice. D and E, MIF deficiency resulted in a marked increase in E-cadherin and decrease in zeb-1, N-cadherin, and vimentin at the mRNA and protein level in primary tumor cells. The reduction in the level of EMT markers was associated with an increase in NR3C2 expression in primary tumor cells from MKPC mice as compared with KPC mice. F, MIF deficiency resulted in an increased NR3C2 and a decrease in miR-301b expression in primary tumor cells from MKPC mice as compared with KPC mice. G, immunohistochemical staining of tumors showing a higher NR3C2 expression in MKPC as compared with KPC mice (Student t test, $P = 0.002$).

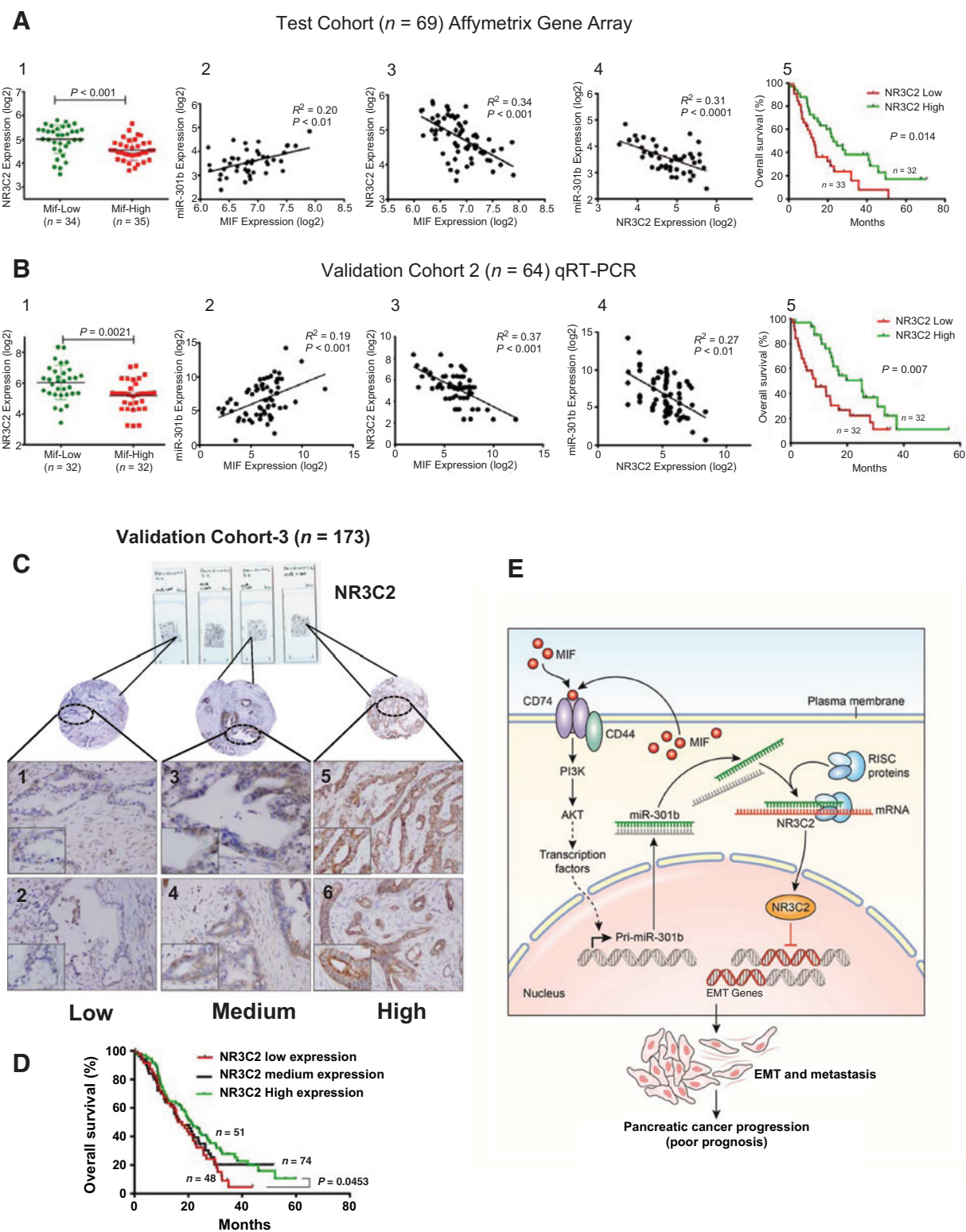


Figure 7. Clinical validation of MIF-miR-301b-NR3C2 axis in human pancreatic cancer. A, MIF-high tumors expressed a lower level of NR3C2 as compared with MIF-low tumors in PDAC cases ($N = 69$). Dot plots represent the normalized \log_2 -transformed NR3C2 expression values obtained by Affymetrix gene expression microarray analysis (panel 1); Pearson correlation analysis between MIF, miR-301b, and NR3C2 expression level in the same cohort (panel 2-4); and Kaplan-Meier analysis showing poor survival in patients with lower NR3C2 expression in tumors (panel 5). B, validation of MIF/miR-301b/NR3C2 axis in an independent cohort of PDAC cases ($N = 64$). C, tissue microarray-based immunohistochemical analysis of NR3C2 in an independent validation cohort-3 ($N = 173$). D, Kaplan-Meier analysis showing different survival in groups of PDAC patients with low and high NR3C2 expression levels in tumors. E, schematic representation of the regulation of NR3C2 by MIF in pancreatic cancer progression and disease aggressiveness.

aberrant MIF expression are highly aggressive and a higher MIF expression is associated with poorer survival in patients with PDAC (7). Recently, exosomal-MIF is linked with the formation of premetastatic niche in liver and described as a potential biomarker of liver metastasis in PDAC (25). In this study, we performed molecular characterization of MIF-high and MIF-low pancreatic tumors to examine the underlying mechanism of the highly aggressive behavior of pancreatic tumors with an aberrantly higher MIF expression in patients with PDAC, by applying an integrative analysis of coding and noncoding gene expression profiles. Our findings demonstrated that a novel MIF-miR-301b-NR3C2 signaling pathway is a key driver of disease aggressiveness and may be therapeutically targeted to improve survival in PDAC.

Several proinflammatory cytokines regulate miRNAs that are associated with the development and progression of human cancer, which may constitute one of the mechanisms underlying the role of cytokines in tumorigenesis (26). On the basis of this premise, we hypothesized that MIF regulates miRNAs, associated with tumor progression and disease aggressiveness in PDAC. The presence of an elevated level of miR-301b in MIF-high tumors as compared with MIF-low tumors in this study suggested a potential regulation of miR-301b by MIF in patients with PDAC, which was then confirmed through overexpression or depletion of MIF in genetically engineered pancreatic cancer cell lines and MIF-overexpressing orthotopic tumors, showing that MIF induces the upregulation of miR-301b and is mediated through the activation of PI3K/Akt pathway. MIF has been earlier shown to activate PI3K/Akt and ERK/MAPK signaling pathways. However, inhibition of ERK/MAPK signaling did not have any effect on MIF-induced increase in miR-301b (Supplementary Fig. S10) in pancreatic cancer cells. miR-301b overexpression enhances migration and invasion in pancreatic cancer cell lines (27). However, its implication in disease outcome in patients with PDAC is not described. An association between the enhanced miR-301b expression and poorer survival in multiple independent cohorts of PDAC patients analyzed in our study suggested that MIF-induced upregulation of miR-301b may contribute to enhanced disease aggressiveness.

Several miRNAs are implicated in the development and progression of pancreatic cancer (28). The aberrant expression of these miRNAs alters the expression of critical target genes responsible for maintaining cellular homeostasis. Our comprehensive analyses to identify the key target of MIF-induced miR-301b, which mechanistically enhances pancreatic tumor progression and disease aggressiveness, resulted in the discovery of NR3C2. NR3C2 is a mineralocorticoid receptor gene encoding mineralocorticoid receptor (MR), which functions as a transcription factor and plays a crucial role in regulating electrolyte balance (29). Interestingly, a lower expression of MR predicted poor prognosis in lung and colon cancer patients (18, 19). To our knowledge, the role of NR3C2 has not been described in pancreatic cancer. The discovery and validation of NR3C2 as a novel target of MIF signaling, and the poorer survival of patients with a lower expression of NR3C2 in tumors from multiple independent cohorts in our study, suggested its potential tumor-suppressive role in PDAC. The functional role of NR3C2 in tumorigenesis is not clear. An association between lower NR3C2 expression and increased angiogenesis has been described in colon cancer (19). EMT is a critical event in the progression of pancreatic cancer. In a recent

study, ataxia telangiectasia group D complementing gene (ATDC)-induced EMT critically led to the acquisition of invasive phenotype in KRAS-induced pancreatic tumorigenesis (30). The mechanistic and functional characterization of NR3C2 in our study revealed, for the first time, that it inhibits EMT and significantly reduced the metastatic potential of pancreatic cancer cells. These findings are consistent with a potential tumor-suppressive role of NR3C2 in pancreatic cancer. Consistently, we found that genetic deletion of MIF enhanced NR3C2 expression, reduced metastasis, and significantly prolonged survival in genetically engineered KPC mouse model of PDAC (Fig. 6). Aligned with these findings, a positive correlation between MIF and miR-301b, and negative correlations between MIF and NR3C2, and also NR3C2 and miR-301b expressions in human PDAC provided a clinical validation of MIF-miR-301b-NR3C2 axis, which enhances disease aggressiveness in these patients (Fig. 7E). Collectively, these data suggest that MIF-induced signaling pathway leading to the inhibition of NR3C2 is a key driver of disease aggressiveness in patients with PDAC.

Taken together, the findings in this study revealed a novel MIF-driven signaling pathway, which mechanistically links MIF-high tumors to enhanced disease aggressiveness and mortality, and also provide proof-of-principle that therapies targeting MIF-miR-301b-NR3C2 axis may improve disease outcome in patients with PDAC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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