

## A Tumorigenic Factor Interactome Connected through Tumor Suppressor MicroRNA-198 in Human Pancreatic Cancer

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### Abstract

**Purpose:** The majority of pancreatic cancers overexpress mesothelin (MSLN), which contributes to enhanced proliferation, invasion, and migration. However, the MSLN regulatory network is still unclear. Here, we investigated the regulation of a panel of tumorigenic factors and explored the potential of MSLN-regulated miR-198 treatment *in vivo*.

**Experimental Design:** The expression and functional regulation of the tumorigenic factors MSLN, NF- $\kappa$ B, and the homeobox transcription factors (TF) POU2F2 (OCT-2), Pre-B-cell leukemia homeobox factor 1 (PBX-1), valosin-containing protein (VCP), and miR-198 were studied in pancreatic cancer cell lines, patient tumor samples, and xenograft pancreatic cancer mouse models.

**Results:** We found that miR-198 is downregulated in pancreatic cancer and is involved in an intricate reciprocal regulatory loop with MSLN, which represses miR-198 through NF- $\kappa$ B-mediated OCT-2 induction. Furthermore, miR-198 repression leads to overexpression of PBX-1 and VCP. The dysregulated PBX-1/VCP axis leads to increased tumorigenicity. Reconstitution of miR-198 in pancreatic cancer cells results in reduced tumor growth, metastasis, and increased survival through direct targeting MSLN, PBX-1, and VCP. Most interestingly, reduced levels of miR-198 in human tissue samples are associated with upregulation of these tumorigenic factors (MSLN, OCT-2, PBX-1, VCP) and predict poor survival. Reduced miR-198 expression links this tumor network signature and prognosticates poor patient outcome. High miR-198 disrupts the network and predicts better prognosis and increased survival.

**Conclusions:** miR-198 acts as a central tumor suppressor and modulates the molecular makeup of a critical interactome in pancreatic cancer, indicating a potential prognostic marker signature and the therapeutic potential of attacking this tumorigenic network through a central vantage point. *Clin Cancer Res*; 19(21); 5901–13. ©2013 AACR.

### Introduction

The complex biologic functions that give rise to cancer pathogenesis can rarely be attributed to individual molecules but rather arise from key interactions among heterogeneous components interacting in modular regulatory

networks. The result is a specific disease signature with far-reaching clinical effects (1). miRNAs are small, noncoding RNA molecules frequently dysregulated in cancers (2). miRNAs can individually regulate several links of a functional network; therefore, miRNA dysregulation can give rise to a complex disease phenotype (3). In this study, we have identified a novel network of heterogeneous prognostic factors for pancreatic cancer interconnected through modulation of a central, tumor-suppressive miRNA, miR-198. These include mesothelin (MSLN), NF- $\kappa$ B, and homeobox transcription factors POU2F2 (OCT-2), pre-B-cell leukemia homeobox factor 1 (PBX-1), and valosin-containing protein (VCP).

miR-198 was previously reported to be downregulated up to 5-fold in hepatic cancers compared to normal liver parenchyma (4) and was reported as a suppressor of hepatocellular carcinoma cell invasion through negative regulation of the HGF/c-MET pathway (5). Yet miR-198 was also found to be upregulated in retinoblastoma, indicating that it may not behave as a tumor suppressor in all cases (6). However, its role in pancreatic cancer pathogenesis has not been studied.

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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### Translational Relevance

We have identified a novel network of tumorigenic prognostic factors that plays a critical role in pancreatic cancer pathogenesis. This interactome is interconnected through a central tumor suppressive microRNA, miR-198, which is able to both directly and indirectly modulate expression of the various members of this network to alter the molecular makeup of pancreatic tumors, with important clinical implications. When this tumor signature network is intact, miR-198 expression is reduced and patient survival is dismal; patients with higher miR-198 present an altered tumor signature network, better prognosis, and increased survival. miR-198 replacement reverses tumorigenicity *in vitro* and *in vivo*, indicating the therapeutic potential of attacking a complex heterogeneous network of factors through a central vantage point.

MSLN is a cell surface glycoprotein overexpressed in about 90% of human pancreatic adenocarcinomas (7–10). We have reported that MSLN overexpression leads to increased pancreatic cancer cell proliferation, invasion, and migration *in vitro* and increased tumor growth *in vivo* (9) and constitutively activates NF- $\kappa$ B to promote cell survival (11, 12). Yet the mechanisms through which MSLN mediates pathogenesis remained largely unexplored. Here, we identify the MSLN/NF- $\kappa$ B axis as part of the interactive network regulating miR-198, along with a molecule whose expression had not been previously observed in pancreatic cancer, OCT-2. OCT-2 is a bifunctional TF that can exert both activating and repressing functions (13). Its expression was previously thought primarily restricted to B cells and tumor cells of the B-cell lineage, neuronal cells, and keratinocytes (14–17). In this study, we found that OCT-2 is expressed in pancreatic cancer cells, is induced by MSLN, and acts as a regulator of miR-198 activity, leading to dysregulation of downstream effectors PBX-1 and VCP. PBX-1 was initially identified as a participant in pre-B-cell acute lymphoblastic leukemia (18, 19) and has been associated with progression of melanoma, (20). Furthermore, PBX-1 is an inducer of the gene for VCP, a ubiquitously expressed protein involved in cell survival (21), with prior indications as a prognostic marker for pancreatic cancer metastasis (22–24). We found that the PBX-1/VCP axis plays a key role in MSLN-mediated pancreatic cancer pathogenesis.

With miR-198 as a central vantage point, we examined the molecular makeup of heterogeneous patient tumors. We identified a pattern of expression that correlates with decreased tumorigenesis in mice and has clinical relevance with patient prognosis and survival, with implications for both therapeutic intervention and diagnostic applications.

### Materials and Methods

#### Cell lines and cell culture

Human pancreatic cancer cell lines used in this study were purchased from the American Type Culture Collection and

were authenticated by DNA fingerprinting at the University of Texas MD Anderson Cancer Center Characterized Cell Line Core. Human pancreatic duct epithelial (HPDE) cells were provided as a gift from Dr. Ming-Sound Tsao (Ontario Cancer Institute, Ontario, Canada). All cells were cultured as previously described (8, 9, 25). MSLN-overexpressing stable cells (or empty vector controls) were selected in MIA PaCa-2 cells or HPDE cells (MIA-MSLN, HPDE-MSLN) or vector (MIA-V, HPDE-V) using retrovirus vectors expressing puromycin resistance (Origene) with 0.5  $\mu$ g/mL of puromycin added into the medium as previously described. Stable cells overexpressing miR-198 or vector control were generated in AsPC-1 (AsPC-1-miR-198, AsPC-1-miR-Ctrl), MIA-MSLN (MIA-MSLN-miR-198, MIA-MSLN-miR-Ctrl), MIA-V (MIA-V-miR-198, MIA-V-miR-Ctrl), and HPDE cells (HPDE-miR-198, HPDE-miR-Ctrl) using the Lenti-miR miRNA Precursor Clone Collection (SBI). The miRZip Lentivector collection from SBI was used to stably knock-down miR-198 in MIA-PaCa2 (MIA-V-Zip-198, MIA-V-Zip-Ctrl) and HPDE (HPDE-Zip-198, HPDE-Zip-Ctrl) cells.

#### Patient tissue collection and preparation

Human pancreatic adenocarcinoma specimens were collected from the patients of any age, gender, and race who underwent resectable surgery according to an approved human IRB protocol (H-16215) at BCM. A complete matching set of surrounding normal pancreas, plasma, serum, and blood cells from the same patient were also collected and stored at the Elkins Pancreas Center tissue bank. All patient samples used in this study were diagnosed pancreatic adenocarcinoma stages IIA or IIB between ages of 40 and 79 years. Tissue total RNA isolation was done by using Ambion "RNAqueous-4PCR" kit. Total miRNAs were extracted and purified by using mirVana miRNA isolation kit (Applied Biosystems/Ambion). For detailed method, please see the Supplementary Experimental Procedures.

#### Subcutaneous and orthotopic pancreatic cancer mouse models

Animal procedures were conducted under guidelines approved by the Institutional Animal Care and Use Committee (IACUC). Tumor cells ( $3 \times 10^6$ ) were inoculated into the right flank (s.c) or into the pancreas (orthotopic) of 5- to 6-week-old male nude mice (NCI-Charles River) as previously described (25). For the s.c. model, tumor size was measured using digital calipers at different time points indicated throughout the study. Tumor volume was determined with the following formula: tumor volume ( $\text{mm}^3$ ) = [length (mm)]  $\times$  [width (mm)]<sup>2</sup>  $\times$  0.52. For the orthotopic model, tumor implantation and growth were monitored using a fluorescent filter to view GFP expression in the abdomen. Any mice experiencing leakage from the orthotopic injection were removed from the study. After 4 weeks, mice were euthanized and evaluated macroscopically for the presence of tumor/metastases in the abdominal cavity. Tumor nodules were explanted, weighed, and stored in RNALater solution (Ambion) in  $-80^\circ\text{C}$  for subsequent analysis.

### Statistical analysis

Statistical analysis was conducted using the Student *t* test (paired, 2-tailed). Statistical significance was defined as  $P < 0.05$  (denoted by \*). Survival data were analyzed using log-rank test and the data was plotted using the Kaplan–Meier method. Patients still living were censored from the results. In addition, expression values were log<sub>2</sub>-transformed, and Cox proportional hazards model was used to examine the association between the variables and the overall survival. *P* values and 95% confidence intervals are 2-sided. SAS 9.3 (SAS institute Inc.) was used for statistical analysis. Data are presented as mean ± SD. Linear regression analyses were conducted using Prism (GraphPad).

Additional experimental procedures including *in vitro* assays and confirmatory experiments can be found in the Supplementary Experimental Procedures.

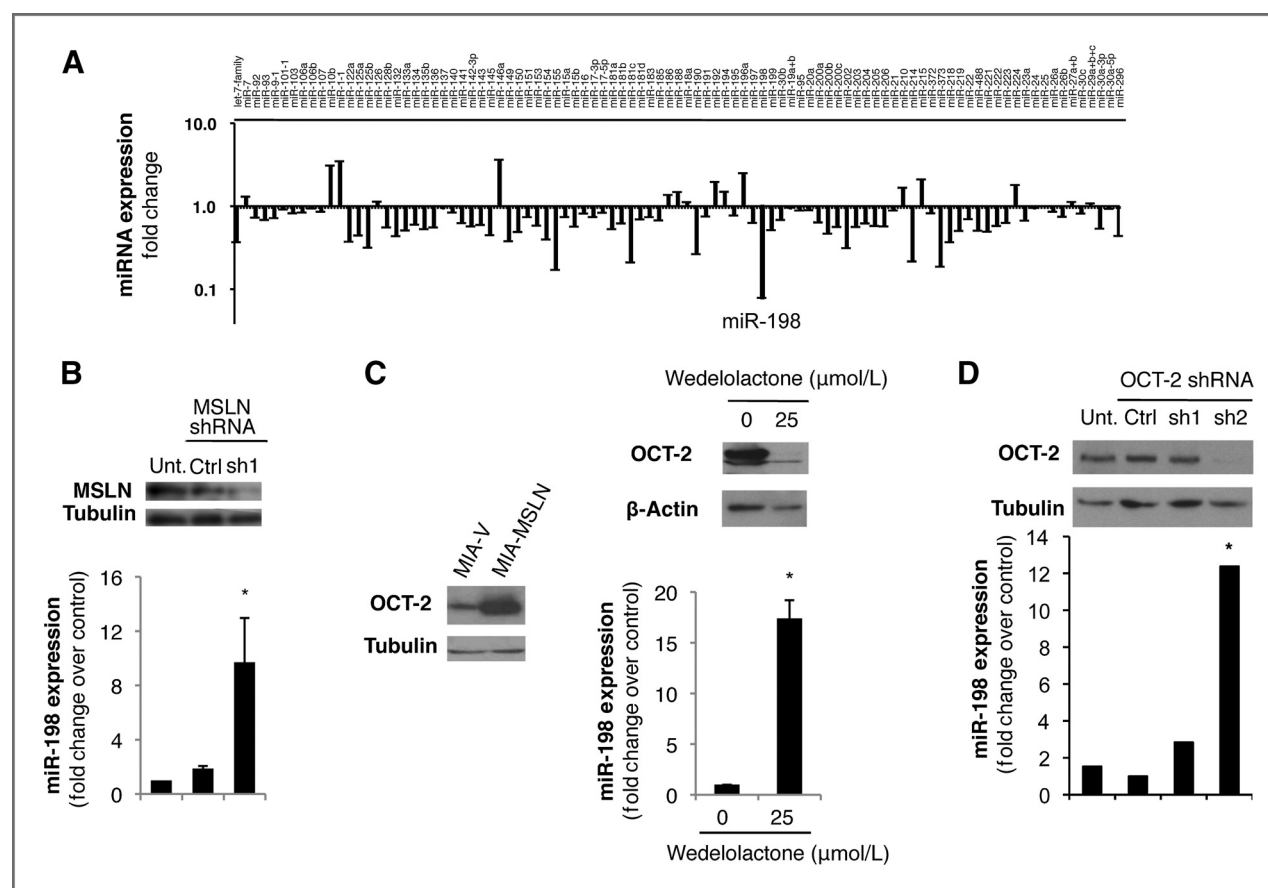
### Results

#### MSLN-mediated NF- $\kappa$ B activation represses the miR-198 promoter through OCT-2 induction

We have previously reported that MSLN overexpression leads to increased pancreatic cancer cell tumorigenesis (9).

To further examine the mechanisms of MSLN-mediated pathogenesis, we compared MIA-MSLN (overexpressed MSLN in MIA-PaCa2 cells which have low endogenous MSLN levels) and MIA-V (vector control) cells (8) in expression of 95 cancer-associated miRNAs (selected after a thorough review of the literature) by using real-time reverse transcription (RT) PCR. We found a global dysregulation of miRNA expression, with several miRNAs either upregulated (i.e., miR-10b, miR-196a) or downregulated (i.e., miR-198, miR-200c, miR-155) following MSLN overexpression (Fig. 1A, Supplementary Table S1). miR-198 was the most significantly downregulated (~15-fold downregulation). Conversely, transfection of MSLN-specific shRNAs in MSLN-overexpressing cells restored miR-198 expression ~12-fold ( $P < .05$ ; Fig. 1B), further indicating that MSLN was responsible for the observed miR-198 downregulation. We therefore selected miR-198 for further study and found a negative relationship between miR-198 and MSLN expression in a panel of pancreatic cancer cell lines (Supplementary Fig. S1A and S1B).

miR-198 is an intronic miRNA, located in the 3'-untranslated region (UTR) of the gene for human follistatin-related

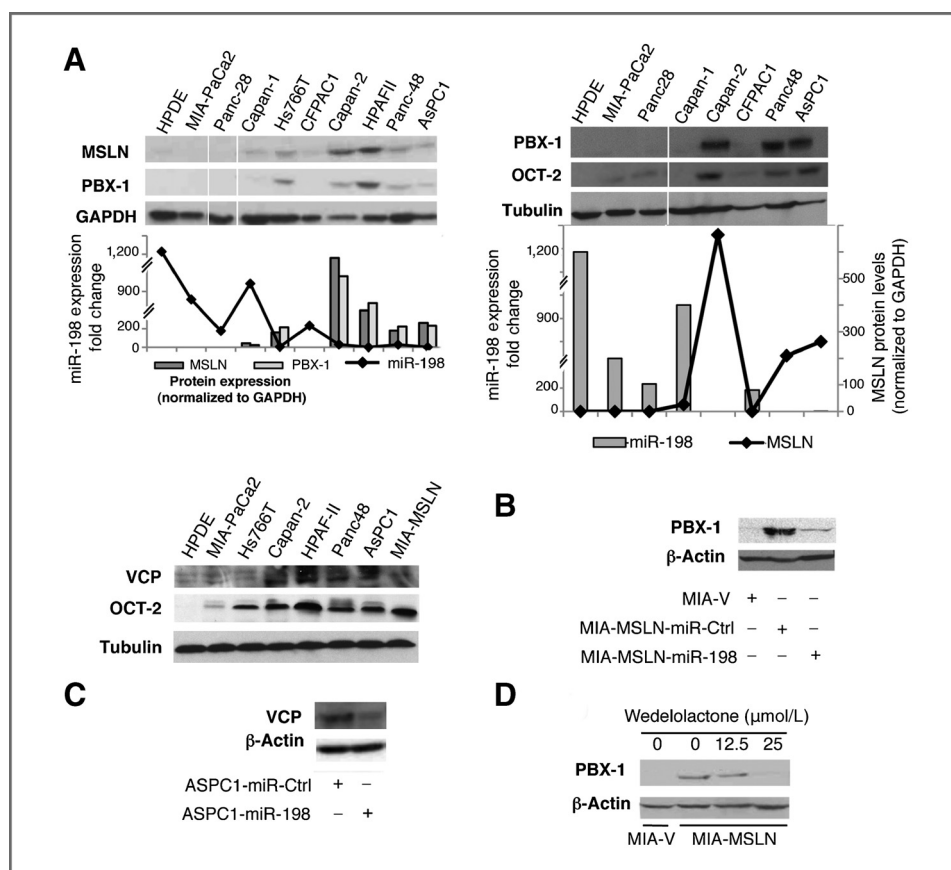


**Figure 1.** MSLN regulates miR-198 expression through NF- $\kappa$ B-mediated induction of OCT-2. A, profiling of 95 cancer-associated miRNAs in MIA-V versus MIA-MSLN cells. miR-198 is ~15-fold downregulated following MSLN overexpression. B, silencing MSLN restores miR-198 expression. Unt, untransfected cell control; Ctrl, scrambled shRNA control; sh1, one of the MSLN shRNA constructs. C, MSLN overexpression induces OCT-2. Wedelolactone treatment restores miR-198 expression and blocks OCT-2 induction in MIA-MSLN cells. D, shRNA-mediated silencing of OCT-2 rescues miR-198 expression in MIA-MSLN cells. \*,  $P < 0.05$ .

protein (*FSTL-1*) and as such was purported to be under control of the same promoter (26, 27). We established a link between miR-198 and *FSTL1* (Supplementary Fig. S1C and S1D) and conducted *in silico* analyses of the *FSTL-1/miR-198* promoter to determine potential binding sites for repressive transcription factors that could be downregulating miR-198 in tumors (Supplementary Table 2). We identified 3 binding sites for the NF- $\kappa$ B-inducible, repressive transcription factor OCT-2 (consensus sequence ATGCAAAT) in the *FSTL-1/miR-198* promoter. As one of the hallmarks of MSLN overexpression is that it constitutively activates NF- $\kappa$ B in pancreatic cancer cells (12), we examined whether OCT-2 may provide the link between MSLN and miR-198 repression. Forced MSLN expression in MIA-PaCa2 cells led to a strong induction in OCT-2 (Fig. 1C). We treated MIA-MSLN cells with NF- $\kappa$ B inhibitor Wedelolactone and observed a ~17-fold increase in miR-198 expression (Fig. 1C) as well as an almost complete block in OCT-2 expression (Fig. 1C). These results were also confirmed in endogenously high MSLN cell line AsPC1 (Supplementary Fig. S1E). Finally, OCT-2 knockdown with short hairpin RNAs (shRNA) resulted in a 12-fold upregulation of miR-198 expression after 72 hours, restoring miR-198 levels close to pre-MSLN expression levels (Fig. 1D). These results show that MSLN-mediated NF- $\kappa$ B activation induces OCT-2, which in turn represses miR-198. Blocking either NF- $\kappa$ B or OCT-2 in this pathway can effectively restore miR-198 expression.

### miR-198 is the central link between upstream regulatory factors MSLN and OCT-2 and downstream targets PBX-1 and VCP

We used miRNA target prediction software to find potential miR-198 targets. After screening a wide number of genes using real-time RT-PCR (data not shown), we identified a potential miR-198 binding site within the 3'UTR of PBX-1 (Supplementary Fig. S2A). This binding site is evolutionarily conserved in 22 of 23 species examined (Supplementary Fig. S2B). PBX-1 has direct effects on tumorigenicity and is also a regulator of VCP; increased expression of the PBX-1-VCP axis has been associated with cancer metastasis, increased cell survival, and poor prognosis (28). VCP also has a binding site for miR-198 within its 3'UTR (Supplementary Fig. S2C) that is conserved in a majority of species (Supplementary Fig. S2D). We used immunoblotting to show the link between upstream MSLN/OCT-2 and the downstream PBX-1/VCP axis (Fig. 2A). As shown in Fig. 2A, MSLN was co-expressed with PBX-1 ( $P < 0.05$ ) and both correlated inversely with miR-198 in a majority of pancreatic cancer cell lines. We also confirmed the same correlation at the mRNA level in Supplementary Fig. S2E and S2F. We then examined OCT-2 and PBX-1 protein expression in our pancreatic cancer cell line panel (Fig. 2A). HPDE cells had almost undetectable OCT-2 expression levels, in contrast to the majority of pancreatic cancer cell lines, which had robust OCT-2 expression that correlated closely with



**Figure 2.** miR-198 is the central link between upstream regulatory factors MSLN and OCT-2 and the closely correlated downstream PBX-1/VCP tumorigenic axis. A, MSLN protein expression correlates positively with PBX-1 expression and both correlated negatively with miR-198 ( $P < 0.005$ ), in a pancreatic cancer cell line panel. Increased OCT-2 protein expression is accompanied by an increase in PBX-1 expression. VCP expression along with downregulation in miR-198 expression. B, PBX-1 expression increases following MSLN overexpression in MIA-PaCa2 cells and is reduced after miR-198 overexpression. C, VCP expression in AsPC1 cells is downregulated ~50-fold following miR-198 overexpression. D, Wedelolactone blocks PBX-1 expression.

both MSLN and PBX-1 ( $P < 0.05$ ). A similar correlation was observed between OCT-2 and VCP (Fig. 2A), tying together the last of the factors in our interactome in the pancreatic cancer cell line panel.

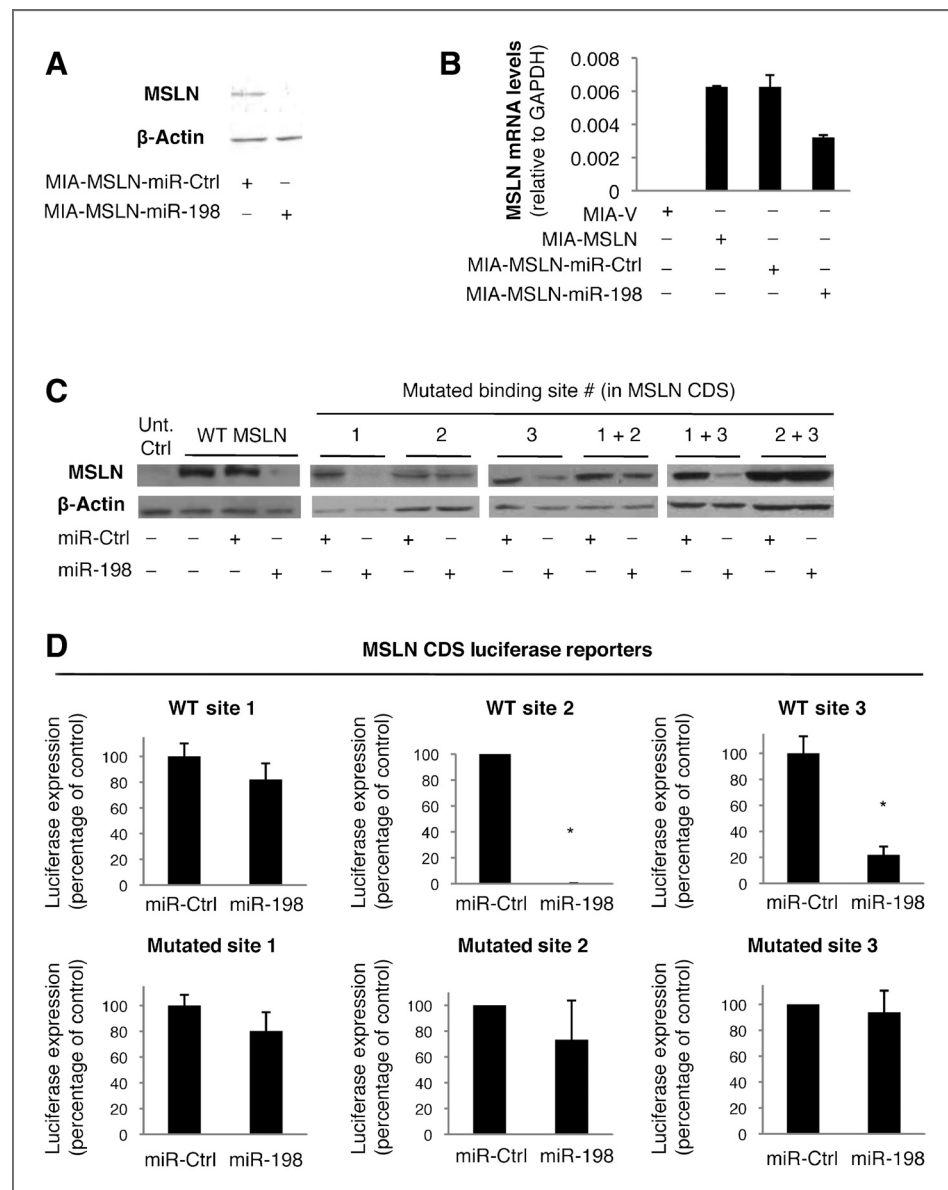
To establish PBX-1 and VCP as targets of miR-198, we stably overexpressed miR-198 in MIA-MSLN cells and AsPC1 cells (miR-198 expression in various engineered cell lines is shown in Supplementary Fig. S3), resulting in a decrease in PBX-1 mRNA (Supplementary Fig. S4A and S4B) and protein expression (Fig. 2B), making them almost undetectable. A similar result was observed for VCP following miR-198 reconstitution (Fig. 2C and Supplementary Fig. S4C and S4D), although a basal level of expression was still detectable. We tied these results to the rest of our interactome using either Wedelolactone inhibition of NF- $\kappa$ B (Fig. 2D) or shRNAs against OCT-2 (not shown), both of

which led to the downregulation of PBX-1 expression. Finally, we used luciferase reporters and site-directed mutagenesis to confirm direct targeting of miR-198 to both VCP and PBX-1, as discussed in the supplemental results (Supplementary Fig. S4E–S4H).

### MiR-198 reciprocally regulates MSLN expression by binding to target sites within the MSLN coding region (CDS)

Interestingly, we observed that miR-198 overexpression in MIA-MSLN cells led to marked reduction of MSLN protein expression (Fig. 3A), with a partial decrease in the mRNA levels (Fig. 3B), indicating a mechanism of post-transcriptional regulation. This suggested a reciprocal regulatory loop between MSLN and miR-198, yet we found no putative miR-198 targets in the 3'UTR of MSLN using target

**Figure 3.** miR-198 reciprocally regulates MSLN expression by targeting its coding region. A, miR-198 overexpression blocks MSLN at the protein level. B, MSLN mRNA levels are partially downregulated following miR-198 overexpression. C, site-directed mutagenesis of each of the 3 miR-198-binding sites within the MSLN coding region separately or in combination leads to differential restoration of MSLN expression in the presence of miR-198. D, miR-198 decreases luciferase expression in WT MSLN CDS constructs; mutating the miR-198 seed region restores luciferase expression. Expressed as firefly/*Renilla* ratio. \*,  $P < 0.05$ .



prediction software. However, recent evidence suggested that miRNAs can also act within the CDS of genes (29). We therefore used RNA22 to search for potential miR-198 binding sites within the MSLN CDS and found 3 potential miR-198 binding sites (Supplementary Fig. S5A). We used site-directed mutagenesis to alter the nucleotide sequence of the 3 sites without altering the amino acid composition of the MSLN protein (Supplementary Fig. S5B) and co-transfected WT or mutant MSLN plasmids along with miR-198 or control precursors into COS-7 cells, which were chosen because they have undetectable endogenous levels of both MSLN and miR-198 (Supplementary Fig. S5C). miR-198 transfection abolished expression of the WT MSLN protein (Fig. 3C). Mutating the 3 binding sites resulted in differential miR-198-mediated regulation of MSLN. When site 1 was mutated, there was no noticeable recovery of MSLN expression. However, mutating site 2 resulted in an almost complete recovery of protein expression, with the expression of the site 3 mutant falling in between the 2. Double mutants of sites 1 + 2 or 1 + 3 resulted in no noticeable improvement in expression than their single counterparts. However, the site 2 + 3 double mutant resulted in complete restoration of MSLN expression (Fig. 3C). This indicates that miR-198 binding sites 2 + 3 cooperate to block MSLN protein expression.

We further confirmed miR-198 targeting of the MSLN CDS regions by constructing luciferase reporter constructs containing portions of the MSLN CDS with WT or mutated seed regions (Supplementary Fig. S5D). Firefly luciferase expression was significantly decreased in 2 of the WT constructs in the presence of miR-198 ( $P < 0.05$ ) and was restored in the mutant constructs (Fig. 3D).

#### **miR-198 is an antagonist of MSLN-mediated autocrine pancreatic cancer cell survival and resistance to TNF- $\alpha$ -induced apoptosis**

We have previously reported that MSLN overexpression confers resistance to TNF- $\alpha$ -induced apoptosis (12). We conducted a TUNEL assay in MSLN-high cells to determine whether miR-198 expression could reverse this acquired resistance. After TNF- $\alpha$  treatment, only about 11% of control MIA-MSLN and about 1.2% of AsPC1 cells are undergoing apoptosis as measured by TUNEL. On the other hand, MIA-MSLN-miR-198 and AsPC1-miR-198 cells exposed to the same conditions show a dramatic increase in the number of cells undergoing apoptosis, of >60% and >90% over controls, respectively (Fig. 4A). Conversely, blocking miR-198 expression results in a significant and dramatic decrease in the number of apoptotic cells, down to ~19% in MIA-V-Zip-198 cells (Fig. 4B). Further overexpressing miR-198 in MIA-V cells had a smaller effect in further increasing apoptosis from ~68% to ~75%, consistent with the effects seen on proliferation. We confirmed apoptosis induction through a Western blotting for caspase-3 activation. The levels of uncleaved caspase-3 decrease in miR-198-high cells (Fig. 4C). These results indicate that miR-198 can antagonize the cell survival effects conferred by MSLN overexpression and its subsequent modulation of downstream targets.

#### **miR-198 overexpression reduces tumor growth and metastatic spread *in vivo***

We examined the functional relevance of miR-198-mediated targeting of our interactive network in a series of *in vitro* experiments shown in the Supplementary Data. MiR-198 replacement *in vitro* reduced proliferation, migration, and invasion of pancreatic cancer cell lines, as well as sensitized resistant MSLN-overexpressing cells to the chemotherapeutic effects of TNF- $\alpha$  (Supplementary Fig. S6 details in Supplementary Results).

We recapitulated these results using 2 separate xenograft mouse models with 2 different pancreatic cancer cell lines, the AsPC1-derived AsPC1-miR-198 and the MIA-PaCa2-derived MIA-MSLN-miR-198 (both cell lines properties were characterized in Supplementary Figs. S3 and S6). In a subcutaneous model using AsPC1-miR-198 or corresponding vector control cells, 8 of 8 mice injected with AsPC1-miR-Ctrl cells developed tumors by 3 days postinjection (d.p.i.), which reached an average volume of 1,500 mm<sup>3</sup> by 24 d.p.i. None of the mice injected with AsPC1-miR-198 cells (0 of 8) developed tumor by 35 d.p.i. ( $P \leq 0.0001$ ; Supplementary Fig. S6P). These results were confirmed in an s.c. model using MIA-MSLN-miR-198 and vector control cells. In this model, 9 of 9 mice injected with MIA-MSLN-miR-Ctrl cells developed large tumors with rapid onset (measurable at 7 d.p.i.), with an average tumor volume of ~2,000 mm<sup>3</sup> by 25 d.p.i. In contrast, only 3 of 9 mice injected with miR-198 overexpressing cells presented small tumors, which were not measurable until 35 d.p.i.; by day 45, they had grown to an average volume of only ~37 mm<sup>3</sup>, a highly significant difference compared to the controls ( $P < 0.0005$ ). The rest of the mice (6 of 9) remained completely tumor free (Supplementary Fig. S6Q and S6R).

Mice injected orthotopically with 2 different cell lines showed a similar phenotype as shown in the subcutaneous mouse model. Nude mice were orthotopically injected with AsPC1-miR-Ctrl cells and AsPC1-miR-198 cells, respectively, for 4 weeks ( $n = 6$  per group). The tumor weight was measured at time of sacrifice. The average weight of primary tumor derived from AsPC1-miR-Ctrl cells was 0.568 g; whereas the weight of tumors derived from AsPC1-miR-198 cells was 0.047 g, which is more than 10-fold difference ( $P < 0.0001$ ; Fig. 5A). Similar results were obtained from the orthotopic model with MIA PaCa2-derived cell lines. Mice ( $n = 8$ ) received MIA-MSLN-miR-Ctrl cells developed primary tumors with an average weight of 0.6 g, ~10-fold greater than primary tumors in mice injected with MIA-MSLN-miR-198 cells (~0.06 g;  $P < 0.0005$ ; Fig. 5B). Control mice all had observable jaundice, weight loss, and abdominal ascitic fluid, whereas none of the mice injected with high miR-198 cells had these symptoms. GFP expression cassettes in our stable cells allowed us to visualize tumor spread after sacrificing the mice (Fig. 5C). Control mice all presented with liver, spleen, and intestinal metastasis, and tumor spread throughout the body cavity. The MIA-MSLN-miR-198-injected mice, on the other hand, had few metastases in addition to decreased primary tumor growth. Five of 8 mice had no metastases; one did not develop any tumor;

the remaining 3 mice showed limited tumor spread. We confirmed the expression levels of the factors in our proposed network in mouse tumor tissues and found that control tumors had high levels of MSLN, OCT-2, PBX-1, and VCP and low levels of miR-198. In contrast, the smaller, less aggressive MIA-MSLN-miR-198 tumors were characterized by a significant decrease in expression of all factors in the network, indicating the potential of miR-198 to directly target PBX-1, VCP, and MSLN and indirectly modulate expression of all by OCT-2 ( $P < 0.05$ ; Fig. 5D).

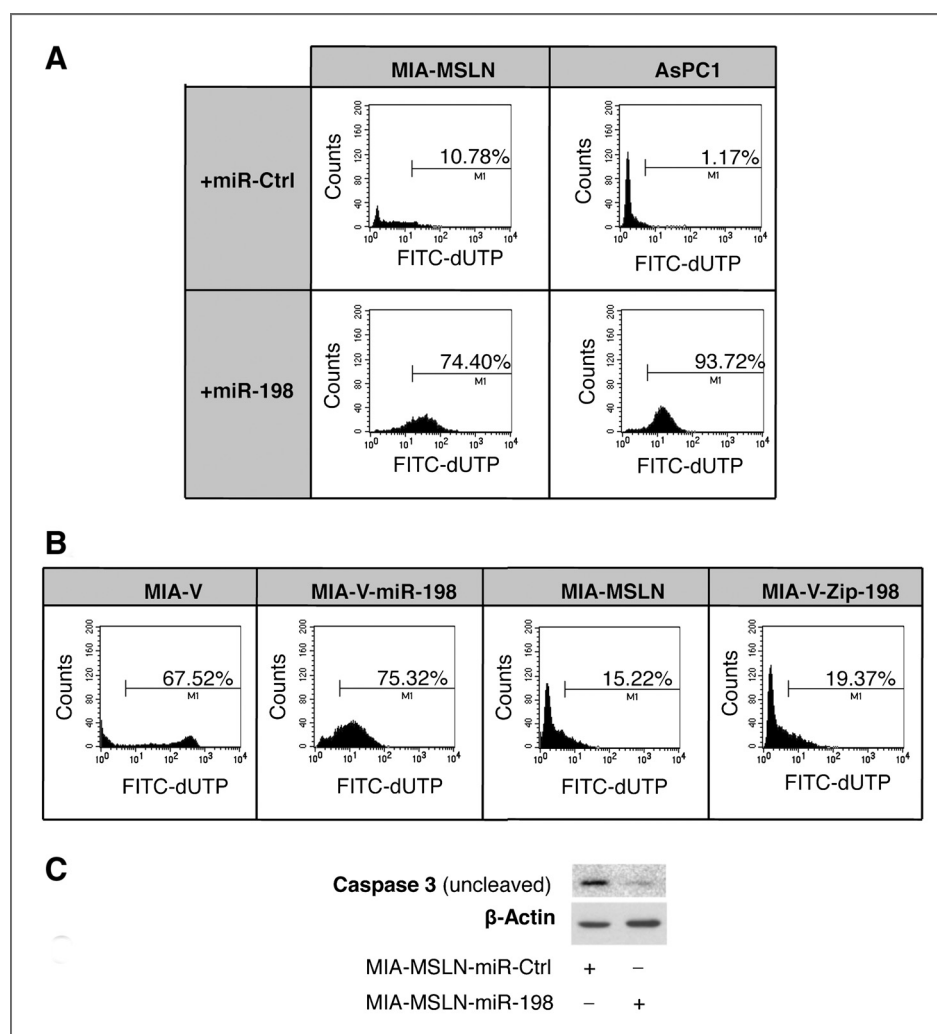
### An interactome of tumorigenic factors interconnected through miR-198 serves as a prognostic indicator of pancreatic cancer

To provide a glimpse into the potential clinical relevance of our *in vivo* results, we examined expression of network factors in 37 patients with pancreatic cancer who underwent resectable surgery at Elkins Pancreas Center. We found that miR-198 was downregulated in ~78% of tumors compared to adjacent normal tissues (Fig. 6A), with downregulation ranging from 2- to 142-fold (Supplementary Table S3). We further found that patients could be classified into 2 groups

based on overall tumor miR-198 levels: patients with miR-198 levels of 0.01 or greater (relative to U6 control) were included in the miR-198-high group and patients with a miR-198 level of 0.001 or lower were included in the miR-198-low group. The average relative miR-198 levels were 0.08 and 0.0004, respectively. Therefore, the miR-198-high group had a minimum 10-fold higher level of miR-198 than the miR-198-low group, with the majority of samples having a greater than 100-fold difference between the groups (Fig. 6B).

We then examined the correlations between our interactome members in the patient tumors, where miR-198 is shown to negatively correlate with all interactome factors except for the positive correlation with FSTL1 ( $P < 0.05$  for all; Supplementary Fig. S7, further discussion in Supplementary Results). The relationships between these tumorigenic factors and miR-198 were represented using the 5-order Venn diagrams depicted in Fig. 6C. Fold changes between tumor and adjacent normal tissues were determined for each factor, calculating the percentage of times each individual factor was upregulated and how often the upregulation in each was accompanied by an upregulation

**Figure 4.** miR-198 is an antagonist of MSLN-mediated autocrine pancreatic cancer cell survival and resistance to TNF- $\alpha$ -induced apoptosis. A, TUNEL assay shows a significant increase in apoptosis after TNF- $\alpha$  treatment in 2 high MSLN cells following overexpression of miR-198. B, TUNEL assay shows a significant decrease in apoptosis in MIA-V cells (high miR-198 cells) down to MIA-MSLN cell levels following blocking of miR-198 (MIA-V-Zip-198). C, overexpression of miR-198 in MIA-MSLN cells results in caspase-3 cleavage.



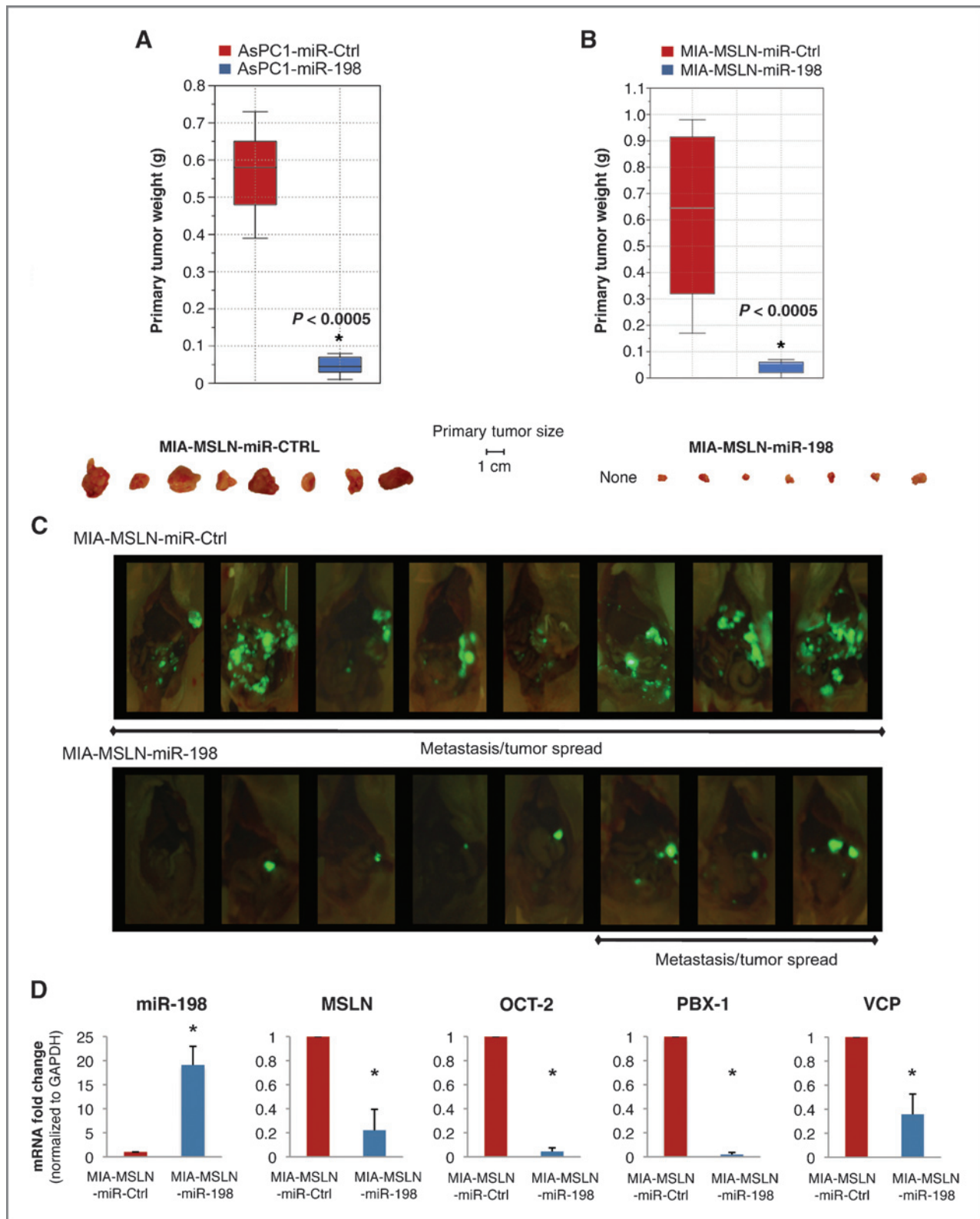


Figure 5. miR-198 overexpression modulates expression of regulatory network and reduces tumor growth and metastatic spread *in vivo*. A, nude mice injected orthotopically with AsPC1-miR-Ctrl cells develop tumors with an average weight of 0.568 g and only 0.047 g in AsPC1-miR-198 cells which is more than 10-fold difference ( $P < 0.0001$ ). B, nude mice injected orthotopically with MIA-MSLN-miR-Ctrl cells developed primary tumors at approximately 10-fold larger (by weight) than primary tumors in mice injected with MIA-MSLN-miR-198 cells after 4 weeks of injection. Resected primary orthotopic tumors (8 per group). C, GFP expression in the tumor cells allows for visualization of tumor spread in the orthotopic pancreatic cancer mouse model at 4 weeks after injection. D, real-time RT-PCR confirms RNA levels of all the factors in our proposed regulatory network. Mean  $\pm$  SD,  $n = 4$ . \*,  $P < 0.05$ .

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in the other factors and/or downregulation of miR-198. To reduce complexity, FSTL1 was not included in this graphical representation, as its close correlation with miR-198 makes them interchangeable in this analysis. For the total number of tissues ( $n = 37$ ), downregulation in miR-198 was accompanied by a simultaneous upregulation in all other network members (MSLN, OCT-2, PBX-1, and VCP) in 71% of tumors. When we conducted the same analysis for either the miR-198-low or miR-198-high groups separately, we observed a very clear change in the molecular makeup of this interactome: a simultaneous upregulation of MSLN, OCT-2, PBX-1, and VCP and downregulation of miR-198 occurs in 91.7% of the patients in the miR-198-low group. This percentage is the same for all the other possible permutations of interactions between the different factors, indicating a very close association between their expression changes in this subset of patients. On the other hand, in the miR-198-high group, none (0%) of the patients had a simultaneous modulation of the 5 factors in our interactome. This also extends to most of the other potential combinations in expression changes. These correlations are also depicted in a more traditional manner in Fig. 6C, where miR-198 is shown to correlate positively with FSTL1 and negatively with all other interactome factors (additional details in Supplementary Results). To determine whether this unique molecular signature correlated with a better patient prognosis, we examined patient survival in the 2 groups. As measured by log-rank test, patient survival was significantly increased in the miR-198-high group compared to the low group ( $P < 0.0005$ ,  $\chi^2 = 14.12$  df = 1), with an HR of 5.815 (95% CI, 2.321–14.57; Fig. 6D). The median survival of the miR-198-low patients was ~15.5 months, with only 11% of patients still alive after ~40 months. Conversely, miR-198-high patients who had died had a median survival time of 35.75 months, with 80% of miR-198-high patients were still alive at 40 months and 64% still alive after ~60 months. Cox proportional hazards model was used to examine the association between the various factors and the overall survival while adjusting for age. Univariate analysis shows a significant association between each one of the individual factors and survival except for PBX-1 (Supplementary Table S4), whereas a multivariate approach adjusting for both age and primary tumor size at the time of resection shows that miR-198 is significantly associated with overall survival [ $P = 0.0014$ ; 95% CI, 0.11(0.03–0.42); Supplementary Table S4]. These results indicate that this interactive tumor signature network is very tightly correlated in patients with pancreatic cancer with the worst prognosis, whereas those with the best prognosis have a global disruption in the network expression pattern.

## Discussion

We have dissected the mechanisms by which miR-198 can alter the molecular makeup of pancreatic tumors and present a unique perspective on the interplay between

several factors in a functional regulatory network in pancreatic cancer. The potential clinical implications are underscored by the significant reversal in tumorigenic aggressiveness that accompanies alteration of this network following miR-198 reconstitution in pancreatic cancer cells and mirrors the signature seen in xenograft tumor models and patient tissues. Our results are summarized in Fig. 7. In addition to elucidating the molecular mechanisms through which MSLN promotes pathogenesis, our study identifies OCT-2 and the PBX-1/VCP axis as critical biomarkers and targets for pancreatic cancer treatment. By acting as the key regulator of this network, miR-198 replacement therapy has the potential to influence interactions between these molecules and reverts the most aggressive pancreatic cancer cells to a more manageable, less invasive phenotype.

The heterogenic nature of tumor cell lines makes it impossible to ascribe all aspects of a more aggressive or less aggressive phenotype to one molecule and that the MSLN/miR-198 interaction may not be solely responsible for the entirety of the aggressive phenotype. We have observed that while MIA-PaCa2 cells or Panc-1 cells are inherently more aggressive than other cell lines, forced overexpression of MSLN in these cell lines leads to an even more significantly aggressive phenotype. The reverse effect can be seen in cell lines with high endogenous MSLN expression, such as ASPC1, which, while they may be inherently less aggressive than others, become even less invasive and proliferate more slowly if MSLN expression is artificially reduced. Loss of miR-198 may be indicative to aggressiveness of pancreatic cancer based on our data obtained from several human pancreatic cancer cell lines, which are commonly used in the scientific community. However, it could be a limitation that we did not include more cell lines such as L3.6pl (30) and SUIT-2 (31) in the current study.

Within this concerted network, we have uncovered a reciprocal regulatory loop between MSLN and miR-198 that gives us novel insight into the mechanisms of MSLN-mediated pathogenesis. miR-198 can reciprocally target multiple sites in the MSLN coding region, with an additive effect that leads to an almost complete block of MSLN protein expression. We have previously reported that MSLN overexpression leads to constitutive activation of NF- $\kappa$ B, resulting in increased cell survival and proliferation of pancreatic cancer cells through NF- $\kappa$ B-mediated interleukin (IL)-6 induction (11, 12). Here, we extend these findings to show that MSLN-mediated NF- $\kappa$ B activation can modulate miR-198 through induction of OCT-2. We have identified OCT-2 as an important factor in pancreatic cancer for the first time. Our findings that OCT-2 is overexpressed in a majority of pancreatic cancer cell lines and is upregulated in more than 80% of patient tumor tissues present a novel role for this protein as a pancreatic cancer prognostic factor and functional target.

We also found that miR-198 targets the tumorigenic factors PBX-1 and VCP. PBX-1 dysregulation has been implicated in increased proliferation of cancer cells (20, 32); VCP overexpression correlates with increased

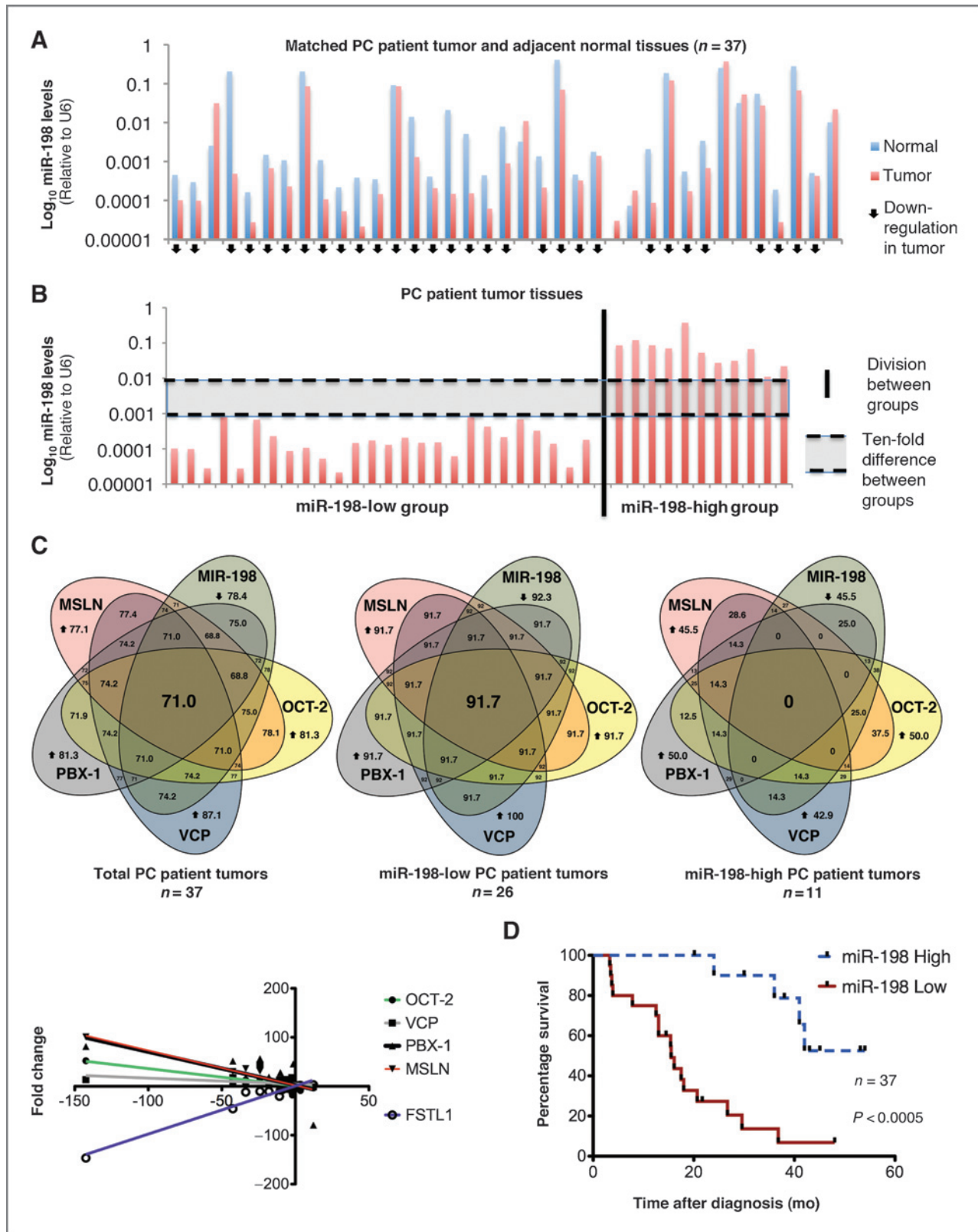


Figure 6. An interactome of tumorigenic factors interconnected through miR-198 serves as a prognostic indicator of pancreatic cancer. A, miR-198 is downregulated in 78.4% of pancreatic cancer tumors compared to normal adjacent tissue ( $n = 37$ ). Arrows point to the samples with miR-198 downregulated in tumor versus normal. B, patients were grouped into 2 cohorts based on miR-198 expression. (Continued on the following page.)

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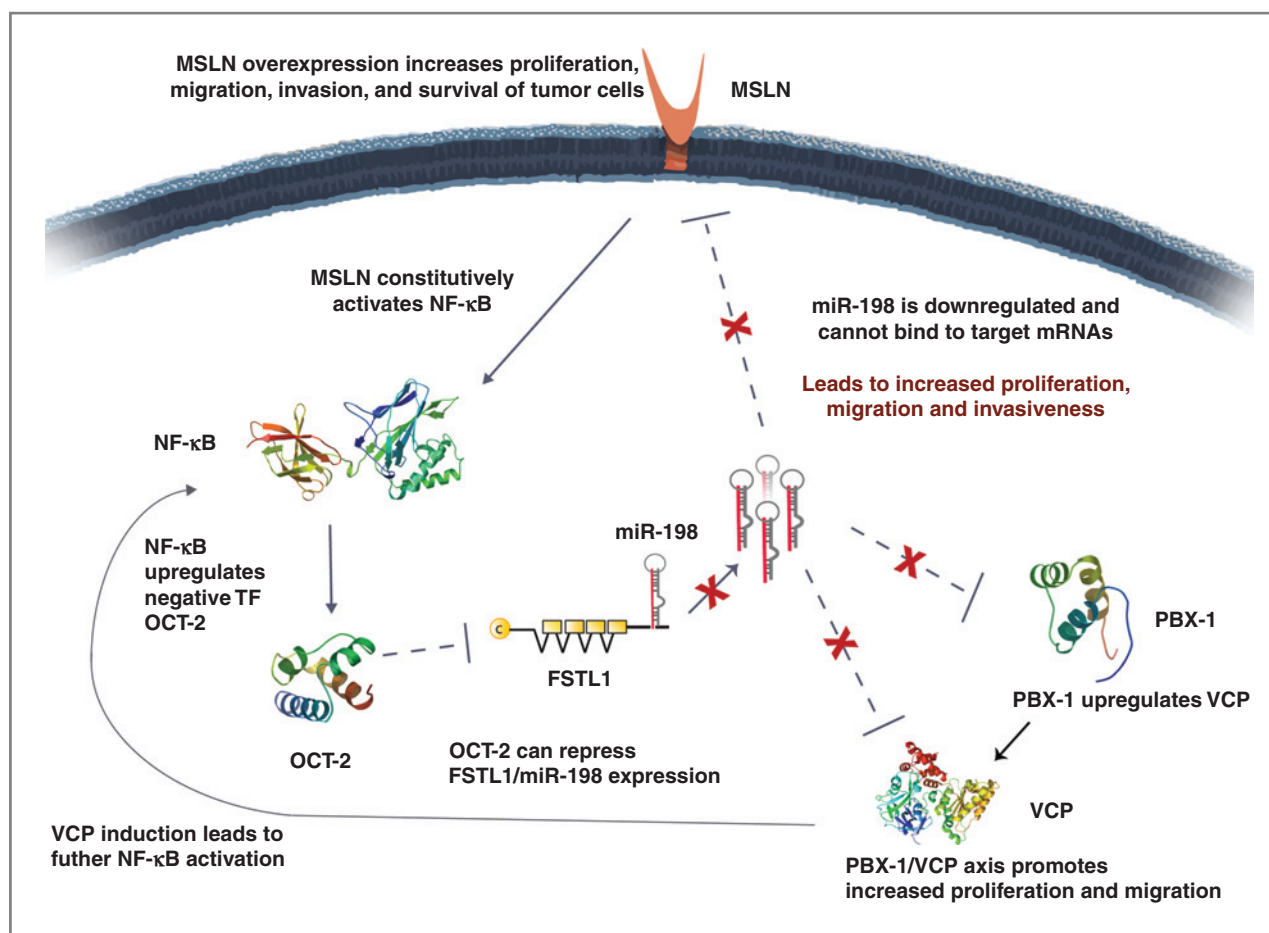


Figure 7. A novel network of heterogeneous prognostic factors for pancreatic cancer interconnected through modulation of tumor-suppressive miR-198. MSLN overexpression represses miR-198 through NF-κB-mediated OCT-2 induction, resulting in upregulation of miR-198 targets and increased tumorigenesis.

progression and metastatic potential of a variety of cancers (23, 33–36). We have shown for the first time that modulation of PBX-1 in pancreatic cancer cells contributes to MSLN-mediated proliferation. In addition, our results indicate a novel role for the PBX-1/VCP axis in pancreatic cancer cell migration and implicate PBX-1 as a key factor responsible for the metastatic potential of MSLN-overexpressing cells. Previous studies have linked the PBX-1/VCP axis to metastatic potential and survival through the VCP-mediated activation of NF-κB (22). Here, we have shown that NF-κB also reciprocally modulates PBX-1 and VCP expression through the regulation of miR-198 and the interconnectivity of our interactome. VCP can thereby feedback into the pathway, promoting

maintenance of NF-κB activation, OCT-2 induction, and subsequent miR-198 repression.

The PBX-1/VCP axis is important in cell survival under cytokine stress, as shown by the resistance of PBX-1/VCP-overexpressing cells in TNF- $\alpha$ -mediated induction of apoptosis (28). We have reported that MSLN-overexpressing cells are resistant to the apoptotic effects of TNF- $\alpha$  (12), and here we show that miR-198 reconstitution can act as an antagonist of these increased cell survival effects.

Two xenograft mouse models (subcutaneous and orthotopic) with human pancreatic cancer cell lines were used in the current study and show that miR-198 significantly inhibits tumor progression. In general, the orthotopic model and a genetically engineered mouse model that

(Continued.) The miR-198-high ( $n = 11$ ) group had a minimum 10-fold higher relative level of miR-198 than the miR-198-low group ( $n = 26$ ), as depicted by dashed area (0.01 and 0.001 miR-198 level relative to U6, respectively). C, five-order Venn diagram representing the complex interactome between the factors in our proposed network analyzed based on total patients with pancreatic cancer, patients with miR-198-low pancreatic cancer, or patients with miR-198-high pancreatic cancer. The numbers inside each colored oval represent the percent of patients in which the specific factors are upregulated (or downregulated in the case of miR-198) either individually (represented by up/down arrows in each oval) or for all possible combinations (represented by the numbers in the center of the diagram). Correlations between miR-198 and interactome members. D, patient survival after surgery was longer in the miR-198-high group than in the miR-198-low group ( $P = 0.0008$ ,  $\chi^2 = 14.12$ ,  $df = 1$ ), with an HR of 5.8 (95% CI, 2.321–14.57).

spontaneously develops pancreatic cancer (Kras<sup>LSL.G12D/+</sup>, p53<sup>R172H/+</sup>, PdxCretg<sup>+/+</sup>) (or KPC) (37) are more clinically relevant than subcutaneous model because of the differences in vascularity and microenvironment. However, as there is no mouse homolog of human miR-198, KPC mouse model was not chosen for the miR-198 study. In the current study, the cancer cells were labeled with GFP, and tumor size and metastasis status in the mouse models were directly visualized under the fluorescence apparatus when sacrificing mice for easy tracking of metastatic nodules. However, GFP is not an optimal imaging technology for tracking tumor progression in live animals. Luciferase, infrared, and mCherry are better than GFP for cancer imaging.

Taken together, our results show that miR-198-mediated modulation of our interactome has implications in preventing chemotherapeutic resistance of pancreatic cancer cells. Therefore, miR-198 may have clinical value from various perspectives. The identified interactome could act as a surrogate marker for extrapancreatic disease, as tumors identified with the low-miR-198 signature are more likely to metastasize or have metastasized. miR-198 replacement therapy may offer benefits as a deterrent to metastasis by reversing the metastatic and invasive potential of primary tumor cells; in addition, effective delivery of miR-198 may help to arrest proliferation of both the primary tumors and extrapancreatic disease by targeting the various interactome factors. Finally, miR-198 replacement may sensitize MSLN-overexpressing cells to chemotherapeutic agents such as TNF- $\alpha$  or gemcitabine, allowing for an improvement in treatment through a multimodal approach. We are currently exploring these possibilities in new studies.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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