Published in final edited form as:

Cancer Lett. 2014 May 28; 347(1): 54–64. doi:10.1016/j.canlet.2014.01.020.

MicroRNA Let-7 Downregulates STAT3 Phosphorylation in Pancreatic Cancer Cells by Increasing SOCS3 Expression

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Abstract

Although dispensable for normal pancreatic function, STAT3 signaling is frequently activated in pancreatic cancers. Consistent downregulation of expression of microRNA let-7 is also characteristic of pancreatic ductal adenocarcinoma (PDAC) biopsy specimens. We demonstrate in this study that re-expression of let-7 in poorly-differentiated PDAC cell lines reduced phosphorylation/activation of STAT3 and its downstream signaling events and reduced the growth and migration of PDAC cells. Let-7 re-expression did not repress expression of STAT3 protein or its activator cytokine interleukin 6 (IL-6). However, let-7 re-expression enhanced cytoplasmic expression of suppressor of cytokine signaling 3 (SOCS3), which blocks STAT3 activation by JAK2. Our study thus identified a mechanism by which STAT3 signaling can be inhibited in pancreatic cancer cells by modifying let-7 expression.

Keywords

Pancreatic cancer; STAT3; let-7; SOCS3; EMT

1. Introduction

As the fourth leading cause of cancer-related death in United States and worldwide, pancreatic cancer continues to remain a devastating disease with a 5-year survival rate of less than 5% [1]. Because of asymptomatic early stages and the aggressive phenotype of the cancer cells, less than 20% patients present with resectable disease [2]. Gemcitabine alone or in combination with fluorouracil or other drugs produce only a modest benefit in prolonging survival [3–5]. However, non-gemcitabine containing combination chemotherapy (FOLFIRINOX) recently have reported improvements in overall survival by ~4.3 months, but only at the expense of severe side effects [6]. Genetic analysis of pancreatic cancers indicated that multiple mutations accumulate over time in these tumors with some of them being more frequent than others (such as KRAS [~90%], p16/CDKN2A [~75%], TP53 [~65%], SMAD4 [~50%]) [7,8]. However, blocking the activity of these frequently-mutated genes did not turn out to be a promising therapeutic strategy [9]. The Pancreatic Cancer

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Conflict of Interest Statement

The authors of this manuscript have no conflict of interest.

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Genome Project identified on average as many as 63 mutations per patient [10]. Identification of molecular mechanisms that are more directly associated with the aggressive and sustained proliferation of pancreatic cancer cells is therefore urgently needed.

Animal model studies on pancreatic cancer demonstrated that inflammatory responses play an important role in the progression of early pancreatic lesions into pancreatic ductal adenocarcinoma (PDAC) [11]. Indeed, activation of signal transducer and activator of transcription 3 (STAT3) signaling, an important mediator of the inflammatory response, has been observed in high percentage of PDAC cases [12,13]. Although STAT3 is dispensable for normal pancreatic development, a genetically-engineered mouse model of PDAC demonstrated that STAT3-deficient mice had a lower incidence of PDAC development. The STAT3 family of transcription factors is activated in response to inflammatory cytokines, growth factors and hormones. Binding of these factors to their cognate receptors at the cell surface induces receptor dimerization and activation of the tyrosine kinase Janus-activated kinase (JAK), which phosphorylates the cytoplasmic domain of the receptor and facilitates binding of non-phosphorylated STAT3 for subsequent phosphorylation [14,15]. Phosphorylated STAT3 dimerize via an SH2 domain, migrates to the nucleus and in a sequence-specific manner activate transcription of genes that play a role in cell proliferation, inhibition of apoptosis and inflammatory response. STAT3 protein can also be phosphorylated and activated by other kinases such as c-src [16]. Consistent with such pro-tumorigenic activities of STAT3, constitutive activation of STAT3 has been reported in multiple human malignancies such as prostate cancer, breast cancer, lung cancer, multiple myeloma, and lymphoma [17–21]. Multiple studies reported activation of STAT3 in pancreatic cancer as well [22,23].

In recent years it has been proposed that in addition to the accumulated mutations that favor cancerous growth, epigenetic events may also play important role in the development and maintenance of pancreatic cancer [24]. MicroRNAs are already known as master epigenetic regulators whose role in regulating diverse cellular processes including cell proliferation, development, differentiation, apoptosis and consequently oncogenesis has been established unambiguously. Deregulation of several specific miRNA has been already noted in PDAC [25–27]. Interestingly, the let-7 miRNA has been found to be consistently downregulated in aggressive pancreatic cancer cell lines [28,29]. Further, recent studies with needle aspirates from primary pancreatic tumors also reported downregulation of let-7 in the majority of tumors [30]. Let-7 was originally identified in *C. elegans* as a gene whose mutation was lethal to these organisms because of abnormal larval growth [31]. Bioinformatics analysis demonstrated that let-7 microRNA is highly conserved among diverse animal species suggesting further its critical role in early development [32–34]. Let-7 ensures correct timing of events that are associated with exit from cell cycle and terminal differentiation. It targets oncogenic protein KRAS, transcription factor high mobility group AT-hook 2 (HMGA2), cell cycle regulatory protein such as cyclinD1 and many others [35]. Let-7 maturation is controlled by a stem cell maintenance factor Lin28a/Lin28b via a negative feed-back mechanism [36]. Let-7 expression is barely detectable in human and mouse embryos although its expression increases significantly upon differentiation [37,38]. Consistent with these observations, low levels of let-7 expression have been reported in many cancers [39–41].

In this study we evaluated relationship between let-7 expression and the STAT3 signaling pathway in pancreatic cancer cell lines. We found that let-7 expression is lower in the poorly-differentiated pancreatic cancer cell lines Panc1 and MiaPaCa and is inversely related to STAT3 phosphorylation in them. Re-expression of let-7 in these lines reduced the phosphorylation of STAT3, which resulted in reduction of migration and growth of these cells. Let-7 did not directly reduce the expression of STAT3 or its activator IL-6, but did

increase significantly the expression of a protein suppressor of cytokine signaling 3 (SOCS3), which inhibits phosphorylation of STAT3. We therefore, provide strong evidence that let-7 expression dictates STAT3 activity in pancreatic cancer cells and that reactivation of let-7 expression in these cells may have a therapeutic application.

2. Materials and Methods

2.1. Cell lines and reagents

Human pancreatic cancer cell lines BxPC-3, Panc1, MiaPaCa-2 and ASPC1 were obtained from American Type Culture Collection (Manassas, VA, USA). BxPC-3 and ASPC1 cells were maintained in RPMI1640 medium containing 10% fetal calf serum (FCS) supplemented with 50 µg/mL streptomycin and 50 units/mL of penicillin. Panc1 and MiaPaCa-2 cells were maintained in DMEM containing 10% FCS supplemented with antibiotics as above. Packaging cell line Phoenix (a HEK293 derivative that constitutively express murine leukemia virus envelope glycoprotein), was provided by Gary Nolan's laboratory (Stanford University) and maintained in 10% FCS supplemented DMEM. All cells were grown at 37°C in humidified incubator containing 5% CO₂. Recombinant interleukin-6 was purchased from Cell Signaling (Danvers, MA).

2.2. Transfection

Plasmid DNA was transfected by lipofectamine 2000 using manufacturer's protocol (Invitrogen, Grand Island, NY). Transfection of siRNA or microRNA mimics was done by RNAiMax transfection reagent from Invitrogen according to their protocol. Optimal concentrations of siRNA or miRNA for transfection were determined empirically. ON-TARGETplus SMARTpool siRNA for STAT3 and non-target control siRNA were purchased from Dharmacon/Thermo Scientific (Pittsburg, PA). The let-7a and let-7f microRNA mimics and microRNA mimic negative controls (miRIDIAN microRNA mimics) were also purchased from Dharmacon.

2.3. Construction of miRNA expression vector and retroviral transduction

Expression vector for individual let-7 microRNA members were constructed by cloning mature let-7 sequences in pSuper.Retro.Puro vector (Oligoengine, Seattle, WA). Individual positive strand oligo (68 to 70 bases) were designed according manufactures protocol. A complementary strand was then designed so that after annealing they would result in a dsDNA insert with *Bam*HI and *Xho*I restriction sites at the end. The annealed product was then cloned into a *Bgl*II-*Xho*I cut pSuper vector. The let-7 sequences used in cloning experiments were: let-7a, 5'-TGAGGTAGTAGGTTGTATAGTT-3'; let-7c, 5'-TGAGGTAGTAGGTTGTATGGTT-3'; let-7f, 5'-TGAGGTAGTAGATTGTATAGTT-3'; and let-7g, 5'-TGAGGTAGTAGTTTGTACAGTT-3'. Non-targeting Sh-RNA sequence used for cloning was 5'-TAAGGCTATGAAGAGATAC-3'. Authenticity of all recombinant clones was verified by sequencing of the entire insert. Individual clones were transfected in the Phoenix packaging cell line. Recombinant retrovirus particles (replication-defective) were harvested from the culture supernatant 48 hrs post-transfection, passed through a 0.45 micron membrane and concentrated 100-fold by ultracentrifugation at 100,000×g. To generate let-7 expressing stable lines, cells were infected with the concentrated virus stock and selected in presence of 3 µg/mL puromycin.

2.4. miRNA isolation and quantitation

MicroRNA-enriched total RNA from cells were isolated by miRNeasy kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Quantitation of individual microRNAs were done by real-time PCR based Taqman microRNA assay system using microRNA-

specific RT-primers (for let-7a, let-7c, let-7f and let-7g) and cognate PCR primers (Applied Biosystems, Grand Island, NY). RNU44 specific reagents were used as internal control.

2.5. Immunoblotting analysis

Whole cell lysates were prepared by disrupting cells in RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1 mM EDTA) containing 5 µl/mL of combined protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein samples (30µg) were separated in readymade 4–20% MiniProtein –TGX precast polyacrylamide gels (BioRad, Hercules, CA) and transferred onto BA-85 nitrocellulose membranes (Perkin-Elmer, Boston, MA). Rabbit primary antibodies STAT3 (79D7), phosphoSTAT3-Tyr 705 (D3A7), E-Cadherin (24E10), ZEB1 (D80D3), Vimentin, KRas (D2C1), HMGA2 (D1A7), HMGA1 (D6A4), PIAS3 (D5F9), and SOCS3 (#2923) were all from Cell Signaling Technology. Mouse monoclonal antibodies for VEGF (SC-7269) and Vimentin (SC-51720) was from Santa Cruz Biotechnology (Santa Cruz, CA), Fibronectin (MAB1918) was from R&D Systems, whereas β-actin (A1978) was from Sigma Chemicals. All primary antibodies were used at 1:1000 dilutions. Horseradish peroxidase-conjugated secondary antibodies (against rabbit or mouse IgG) obtained from Amersham/GE Healthcare Corporation were used at 1:2000 dilutions. Bands were detected by Western Lightning Plus-ECL chemiluminescence substrates (Perkin-Elmer) and photographed in Imagequant LAS4000 (GE Healthcare). Band intensities were quantitated using Image Studio Lite software from LI-COR Biosciences (Lincoln, Nebraska).

2.6. Cell growth and viability assay

To determine the viability of different pancreatic cancer cells following enforced expression of let-7 or transfection of STAT3 siRNA, 30,000 cells were seeded in individual well of 96-well plate and allowed to grow for 48 or 72 hrs. Cell growth were analyzed by MTS assay using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, Wisconsin) according to the manufacturer's protocol.

2.7. Immunofluorescence microscopy

Cells were grown in Nunc Lab-Tek II 8-well chamber slides (Fisher Scientific) for 48 hrs. In some experiments cells were transfected with siRNA directly in chamber slides. Cells were fixed in 4% paraformaldehyde for 15 min at room temperature before treating them with 0.1% sodium citrate solution (containing 0.1% Triton X100) for 45 min on the bench. One to two drops of ImageIT FX signal enhancer (Invitrogen) was then added to each well and incubated again for 30 min at room temperature. Non-specific binding of antibodies was blocked by treating the cells on the bench for 1 hr with 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Cells were treated with appropriately diluted antibody in 3% BSA overnight at 4°C. Next day, cells were treated with 1:400 dilution of Alexafluor 488-conjugated secondary antibody (Invitrogen) for 1 hr at room temperature. Cells were washed 3 times with PBS at the end of each incubation step. Cells were then mounted with Ultracruz mounting medium containing 4',6-diamidino-2-phenylindole (DAPI, Santa Cruz Biotechnology) and viewed under a Olympus IX51 fluorescence microscope.

2.8. Cell Migration assay

Effect of let-7 re-expression on the migration of PDAC cells was tested by wound-healing and chemotaxis assays. For wound healing assay stably-transfected Panc1 cells were allowed to grow to confluency for 24hr in 60mm culture dishes. The cell monolayer was scratched gently with a sterile pipet tip. Migration of the cells in the scratched area was then monitored over time by light microscopy. Chemotaxis assays were performed in 24-well plates containing transwell chambers with membranes containing 8µm size pores (Corning).

Panc1 cells were transfected with negative control mimic or let-7a mimic at a 20nM final concentration using RNAiMax transfection reagent as described in section 2.2. Three days later transfected cells were harvested by trypsinization and washing in serum-free DMEM. The upper chamber received 65,000 cells suspended in 300 μ L serum free DMEM growth medium. The lower chamber received 1.0 mL of conditioned media from actively growing PANC1 cells (after filtration through 0.45 μ M membrane. Membranes were stained 16 hrs later with Diff-Quik Stain (IMEB Inc. San Marcos, CA) and migrated cells were view under light microscope and photographed.

2.9. Reverse transcription-Real time polymerase chain reaction (qRT-PCR)

Total RNA from stably- or transiently-transfected cells was isolated by Trizol exaction according to manufacturer's protocol (Invitrogen). Reverse transcription of 1 μ g of total RNA was performed using Superscript III enzyme and random hexamer primer (Invitrogen). Expression of individual genes was then analyzed by semiquantitative qRT-PCR using SYBR green technology in ABI Prism Sequencer 7500 (Applied Biosystems). Relative quantification of gene expression was determined by the comparative threshold method (Δ CT), as described previously [42]. Expression level of the β -actin mRNA in each individual sample was used to normalize our results. Primer pairs used in qRT-PCR assays are listed in Table 1.

2.10. Statistical analysis

Cell culture experiments were repeated three times or more. Two-tailed Students t-test was used to ascertain statistical differences between groups, and a p-value of < 0.05 was considered significant.

3. Results

3.1. STAT3 phosphorylation levels are higher in aggressive pancreatic cancer cell lines

Persistent STAT3 signaling is believed to play a role in cancer progression, as its upregulation has been documented in several human cancers. Recent reports from animal model studies also suggested that STAT3 signaling not only plays a role in the development of pancreatic cancer but may also be required for cancer initiation [43]. To investigate the significance of STAT3 signaling in human pancreatic cancers we first investigated STAT3 phosphorylation in four pancreatic cancer cell lines that represent different stages of the disease. As shown in Figure 1A, STAT3 phosphorylation was evident all cell lines we tested. However, level of phosphorylation was much higher in lines whose differentiation level was moderate to poor (Panc1) or poor (MiaPaCa). Moderately-differentiated lines BxPC-3 and ASPC1 lines showed much lower levels of phosphorylation. We also compared the relationship of STAT3 phosphorylation of these lines with the expression of the angiogenic factor VEGF and epithelial-mesenchymal transition (EMT) markers. As shown in Figure 1A, expression of the epithelial marker e-cadherin was very low in Panc1 and undetectable in MiaPaCa but was abundant in BxPC-3 and ASPC1. Expression of ZEB1, which inhibits e-cadherin expression, was high in Panc1 and MiaPaCa but absent in other two lines. Similarly, VEGF expression was also high in Panc1 and MiaPaCa compared to BxPC3, although its level in ASPC1 was also relatively elevated. This data demonstrates that higher STAT3 phosphorylation level correlates with the markers of aggressiveness of pancreatic cancer cell lines.

Further, to determine a direct relationship of STAT3 and growth of poorly-differentiated pancreatic cancer cells, we compared the viability of Panc1 and MiaPaCa cell lines following siRNA-mediated inhibition of STAT3 expression. As shown in Figure 1B and 1C, downregulation of STAT3 expression significantly reduced the growth rate of both Panc1

and MiaPaCa lines ($P < 0.05$). Together, our data provide evidence that activated STAT3 contributes to the high growth rate in these pancreatic cancer cell lines.

3.2. Let-7 downregulates STAT3 phosphorylation in pancreatic cancer cell lines

Let-7 has been implicated in carcinogenesis as its downregulation has been reported in many cancers [33]. Abundant expression of let-7 has been reported in differentiated tissues, although its level typically remains low to undetectable during embryogenesis, further supporting its role as a tumor suppressor [37,44–46]. Here we investigated whether STAT3 phosphorylation in pancreatic cancer cells is regulated by let-7 microRNA. First we confirmed that when compared with the moderately differentiated line BxPC-3, the expression of individual members of let-7 is indeed reduced in aggressive cell lines MiaPaCa and Panc1 (Figure 2A). We generated MiaPaCa and Panc1 cell lines that stably express individual members of let-7 and then analyzed how such reexpression affects STAT3 phosphorylation. Our results show that in MiaPaCa cells stably expressing let-7a (MP-7a), let-7f (MP-7f) or let-7g (MP-7g) phospho-STAT3 levels were significantly reduced compared to the levels in the paired cell line that expressed non-targeting Sh-RNA (MP-Sh) (Figure 2B, top left). Although minor variations were observed in repeat experiments, band intensity data from three independent experiments clearly demonstrated the downregulation of STAT3 phosphorylation by all let-7 members (Figure 2B, top right). Similarly, Panc1 cell lines stably-expressing let-7a, let-7c or let-7f (Pa-7a, Pa-7c or Pa-7f, respectively) had significant reduction of phospho-STAT3 levels compared to the paired cell line expressing non-targeting Sh-RNA (Pa-Sh) (Figure 2B, bottom left and right). Immunofluorescence analysis of phospho-STAT3 expression in Panc1 cells showed distinct nuclear labeling in Pa-Sh cells but not in Pa-7a cells (Figure 2C). Because phosphorylated STAT3 dimerizes and migrates to the nucleus and activates transcription of STAT3-dependent genes, our data further demonstrates an inverse relationship between let-7 expression and STAT3 activation.

Next, to determine the effect of let-7 reexpression on the growth of pancreatic cancer cells, we performed cell survival assays on MiaPaCa cells stably expressing let-7a, let-7f or let-7g. As demonstrated in Figure 2D, MP-7a, MP-7f and MP-7g lines showed significant reduction in cell growth compared to MP-Sh ($P < 0.01$). Taken together, our results clearly demonstrate that let-7 reexpression reduces growth of poorly-differentiated pancreatic cancer cell lines possibly by controlling STAT3 signaling activity.

3.3. Let-7 re-expression retards migration of pancreatic cancer cells

Tissue invasion and migration to distant locations that are attributed to the acquisition of EMT are hallmarks of cancer metastasis [47]. Several studies reported that aggressive pancreatic cancer cell lines express higher levels of mesenchymal markers (such as Zeb1, Vimentin, Slug, Snell, Twist) and lower levels of epithelial markers (such as E-cadherin, γ -cadherin) [48,49]. Further, acquisition of gemcitabine resistance in pancreatic cancer cells has been associated with EMT [50]. Because our data showed increased levels of mesenchymal markers in Panc1 and MiaPaCa cells and that the growth of these cells were reduced following let-7 re-expression, we investigated whether their EMT phenotype would be affected by let-7. We first compared E-cadherin expression of Panc1 cells expressing non-targeting Sh-RNA and let-7a or let-7f by immunofluorescence analysis. As shown in Figure 3A, although E-cadherin was barely visible in Pa-Sh cells, significant expression was detected in Pa-7a and Pa-7f cells, while only few E-cadherin expressing cells were visible in Pa-7c line. To examine the effect of re-expression of let-7 microRNA on the expression of other epithelial and mesenchymal markers, we transfected Panc1 cells with either non-targeting control microRNA mimic or let-7a mimic followed by immunostaining 4 days later. We found that let-7 re-expression decreased the expression of mesenchymal marker

vimentin and fibronectin. Let-7 re-expression also significantly increased the expression of cytoplasmic and membrane bound β -catenin, which is typically associated with epithelial phenotype. (Figure 3B). Next, we compared the migration characteristics of Pa-7a and Pa-7f cells compared to Pa-Sh cells in a wound-healing assay. These two lines were chosen because of their higher ability to express E-cadherin. Although no cell growth could be noted in any of the lines by 6 hr, there was significant migration of Pa-Sh cells by 24hr, but not of the Pa-7a or Pa-7f cells (Figure 3C). To further substantiate the effect of let-7 re-expression on cell migration, we also performed chemotaxis assay in transwell chambers on Panc1 cells that were transfected with either control mimic miRNA or let-7a mimic. As shown in Figure 3D and 3E, migration of let-7a mimic-transfected Panc1 cells through the membrane was severely retarded when compared to that of the same cells transfected with control mimic miRNA. Therefore, these results suggest that let-7 expression indeed retards EMT in pancreatic cancer cells.

3.4. Let-7 reexpression inhibits expression of STAT3-activated genes

We rationalized that if STAT3 is indeed a major effector in pancreatic tumorigenesis and let-7 is a critical regulator this process, changes in the expression of STAT3-regulated genes will accompany let-7 re-expression in pancreatic cancer cells. To test this possibility, we first examined the expression of genes that are known targets of let-7. In this experiment we transiently-transfected MiaPaCa or Panc1 cells with let-7a or let-7f mimic miRNAs and compared the results with those from cells transfected with non-targeting control mimic miRNA. Immunoblotting experiments confirmed markedly lower levels of phospho-STAT3 in let-7a-transfected MiaPaCa or Panc1 cells, compared to control mimic miRNA-transfected cells (Figure 4A). Substantial decreases in KRas (in both lines) and HMGA2 (in Panc1 only) proteins were also noted. The absence of HMGA2 protein expression in MiaPaCa cells has been noted in another study as well [51]. The HMGA1 protein level was lower in let-7a-transfected MiaPaCa cells. It is noteworthy that although a let-7 binding site has not been reported in HMGA1 mRNA, an inverse relationship of HMGA1 with let-7 has been reported in pancreatic neuroendocrine tumors [52]. Further, qRT-PCR analysis of total RNA isolated from the same let-7 mimic-transfected Panc1 cells demonstrated marked reduction in the expression of HMGA2, Lin28a and Lin28b transcripts (Figure 4B).

Next, we analyzed the expression of STAT3-regulated genes in the same RNA extracts (from control mimic- or let-7 mimic-transfected MiaPaCa or Panc1 cells). As shown in Figures 4C, VEGF, CycD1 and MMP9 genes were greatly reduced in MiaPaCa cells. Similarly, significant downregulation of VEGF and CycD1 were also noted in Panc1 cells following transfection with let-7 mimic (Figure 4D). These results suggest that let-7 re-expression in aggressive PDAC cell lines abrogates STAT3 signaling. It was possible that downregulation of genes by let-7 re-expression are STAT3-signaling independent. Interestingly, introduction of a constitutively-active STAT3 expression plasmid (STAT3C) [53] in MP-Sh cells induced CycD1 gene expression as expected, but such introduction failed to do so in MP-7a cells (data not shown). The STAT3C construct acts as constitutively-active form because of it higher binding affinity to the STAT3 target gene promoters but it still needs phosphorylation at Y705 position [54]. We believe that because in our experiment let-7 inhibits STAT3 phosphorylation, STAT3C also cannot be phosphorylated and as such cannot demonstrate its constitutively-active phenotype. Therefore inability of this construct to upregulate CycD1 gene expression in Pa-7a cells indirectly demonstrates that let-7-mediated effects on STAT3 downstream targets are indeed occurring through STAT3.

3.5. STAT3 or IL-6 expression is not affected by let-7 re-expression in PDAC cell lines

To understand how let-7 might be down-regulating phospho-STAT3 levels in pancreatic cancer cells, we evaluated whether let-7 was directly interfering with the expression of STAT3 mRNA. The inflammatory cytokine IL-6 is a major activator of STAT3 signaling. Recent studies demonstrated that both STAT3 and IL-6 are direct targets of let-7. In breast cancer cell lines let-7-mediated down-regulation of IL-6 expression has been documented [55]. Wang et. al. demonstrated that hepatitis C virus (HCV) down-regulates let-7 expression in HepG2 cells, which in turn prevents degradation of STAT3 mRNA and supports persistent STAT3 signaling [56]. Interestingly, our qRT-PCR experiments did not show any significant change in the expression of either STAT3 or IL-6 expression in MiaPaCa or Panc1 cells (Figure 5A). Curiously, treatment of Pa-7a, or Pa-Sh lines with IL-6 protein (20ng/ml, 20 min) increased phospho-STAT3 levels in both of them (Figure 5B). These results suggest that let-7 down-regulates STAT3 signaling in PDAC cells by an IL-6-independent mechanism, but is ineffective against any robust exogenous cytokine-mediated STAT3 activation.

3.6. SOCS3 expression is upregulated by let-7 in pancreatic cancer cells

To understand the mechanism of downregulation of STAT3 signaling by let-7, we investigated the activity of other important regulators of STAT3 signaling in MiaPaCa and Panc1 cells under the condition of let-7 re-expression. Because exogenous IL-6 induced STAT3 phosphorylation similarly in both Pa-Sh and Pa-7a cells, we reasoned that the activity of the upstream regulator JAKs are not grossly affected by let-7 re-expression. Therefore we investigated the activity of the two downstream regulators of STAT3 signaling, namely protein inhibitor of activated STAT3 (PIAS3) and SOCS3. PIAS3 inhibits STAT3 signaling by interacting with the DNA-binding domain of STAT3 and preventing its transcriptional activator function [57]. The other regulator SOCS3, whose expression is initially induced by STAT3 signaling, binds to the catalytic domain of JAK and prevents phosphorylation of STAT3 via negative feedback regulation [58]. As shown in Figure 6A, let-7 re-expression did not alter the level of PIAS3 protein in either MiaPaCa (compare MP-Sh and MP-7a) or Panc1 cells (compare Pa-Sh and Pa-7a). However, there was a significant increase in the level of SOCS3 protein in both MP-7a and Pa-7a cells compared to MP-Sh and Pa-Sh cells, respectively. Interestingly, when Pa-Sh and Pa-7a cells were stimulated with IL-6 (20 min or 4 hr), SOCS3 level increased in both cells by 20 min (Figure 6B). However, the SOCS3 level went down in Pa-Sh cells within 4 hrs, but still remained high in Pa-7a cells. To further verify this let-7-mediated upregulation of SOCS3, we transfected Panc1 cells with non-targeting control mimic or let-7a mimic siRNA and analyzed SOCS3 protein expression by immunoblotting and immunofluorescence. As shown in Figure 6C, let-7a mimic-transfected cells had relatively less phospho-STAT3, as expected. The SOCS3 protein level was upregulated by let-7a mimic transfection, just as found in the Pa-7a cells in Figure 6A. An immunofluorescence assay demonstrated that let-7a mimic-transfected Panc1 cells had a much higher level of cytoplasmic expression of SOCS3 compared to non-targeting control mimic-transfected cells (Figure 6D). A similar staining pattern was also noted in let-7 mimic-transfected MiaPaCa cells (Figure 6D, lower panel). These data demonstrate that let-7 upregulates SOCS3 expression in PDAC cells and suggests that such upregulation reduces STAT3 phosphorylation and downstream signaling events.

3.7. STAT3-signaling pathway regulates let-7 re-expression mediated effects

To investigate the role of STAT3-signaling pathway in mediating let-7 re-expression related activities we wished to knockdown one STAT3-signaling pathway intermediate and analyze let-7-induced activities under such condition. We transfected Panc1 cells with let-7a mimic, SOCS3 siRNA or combination of both let-7a mimic and SOCS3 siRNA and analyzed expression of epithelial and mesenchymal markers by immunofluorescence staining.

Analysis of SOCS3 mRNA by qRT-PCR analysis in these cells demonstrate upregulation of SOCS3 expression by let-7a compared to that in control siRNA transfected cells as expected (Figure 7A). SOCS3 siRNA greatly reduced SOCS3 mRNA expression well below baseline level and combination of SOCS3 siRNA and let-7a mimic increased SOCS3 expression moderately but not to the level seen in control siRNA transfected cells. Analysis of phospho-STAT3 level in the whole cell lysates from similarly transfected cells demonstrated exactly opposite trend that is downregulation by let-7a, upregulation by SOCS3 siRNA and intermediate expression with the combination (slightly lower than that with SOCS3 alone) (Figure 7B). Immunofluorescence staining demonstrated that let-7a mimic induced expression of cytoplasmic and membrane-bound β -catenin (epithelial marker) as well as SOCS3 expression but reduced fibronectin expression (mesenchymal marker) compared to those in control siRNA transfected cells (Figure 7C). SOCS3 siRNA had no effect on β -catenin expression, increased fibronectin expression and reduced SOCS3 expression over those compared to control siRNA transfected cells. Interestingly, when let-7 mimic was added to SOCS3 siRNA during transfection, let-7 characteristic features started to appear again although not as strong as with let-7 mimic alone (right most panel in Figure 7C). β -catenin expression was more than that with SOCS3 siRNA alone, fibronectin expression was much lower than that with SOCS3 siRNA alone and SOCS3 expression was slightly higher compared to that with SOCS3 siRNA alone. These data clearly indicate that inhibition of a STAT3 signaling pathway intermediate significantly reduce let-7 re-expression-induced effects in PDAC cells. In other words these results suggest that STAT3 signaling pathway acts as a mediator of the effects seen with let-7 re-expression.

4. Discussion

Expression of let-7 microRNA is downregulated in many pancreatic cancer cell lines that are poorly-differentiated, although how such deregulation affect their growth is not well understood. The pro-proliferative transcriptional activator STAT3 remains active in most pancreatic cancer lines and in patient-derived tumors. In this study, we investigated whether let-7 has a role in modulating STAT3 signaling in pancreatic cancer cells. We demonstrated that STAT3 signaling is constitutively-active in poorly-differentiated pancreatic cancer cell lines and expression of let-7 microRNA is downregulated in them. Further, re-expression of let-7 in these cells decreased STAT3 phosphorylation and downstream signaling suggesting a direct link between let-7 expression and STAT3 signaling in PDAC cell lines.

Activating mutations of KRas and inactivating mutations of p16 or p53, which all favor cell proliferation, are very common in pancreatic cancer [7,8]. The most frequent KRas mutations found in pancreatic cancers, G12D and G12V, encode constitutively-active KRas, which persistently activates the Ras-Raf-MEK-ERK pathway and provides a strong growth stimulatory signal. STAT3 signaling is typically activated by cytokines and growth factors following their binding to the cell surface gp130 receptor. Interestingly, oncogenic KRas is also known to support STAT3 signaling [59] which suggests that KRas may have a role in regulating STAT3 signaling in pancreatic cancer cells. However, knocking down STAT3 expression alone in both MiaPaCa and Panc1 cell in our studies significantly reduced their growth (Figure 1B and IC), although both these cell lines bear an activated KRas (G12D) mutation. Our study thus suggests that STAT3 has a KRas-independent role in the growth of PDAC cells.

Genes that are regulated by let-7 typically have a binding site for the mature form of the microRNA on their 3'UTR, although indirect regulation via interaction with a secondary target is not uncommon. A recent report demonstrated that cisplatin-induced phosphorylation of STAT3 in esophageal squamous cell carcinoma was slightly reduced when let-7c was transfected into them [60]. Such reduction was attributed to the reduction in

the level of IL-6 that was induced following cisplatin treatment. In a previous study, let-7a was found to down-regulate IL-6 expression in a breast cancer cell line, which in turn also down-regulated STAT3 transcript expression as measured by real time PCR experiments [55]. The presence of a let-7 target site on 3'UTR of IL-6 mRNA was suggested as the reason for this let-7-mediated effect. In a study of hepatitis B virus x protein (HBx)-induced microRNA regulation in hepatocellular carcinoma cell line HepG2, Wang et. al. suggested that STAT3 is a direct target of let-7, as a tentative binding site of let-7 was identified in the 3'UTR of STAT3 mRNA [56]. To our knowledge however, any relationship between let-7 expression and STAT3 signaling in PDAC cells has not been investigated. Interestingly, in our studies with poorly-differentiated pancreatic cancer cell lines we did not find downregulation of either IL-6 or STAT3 transcription following let-7 expression, although suppression of STAT3 phosphorylation was apparent in all of our experiments (Figure 5A and Figure 4A). As large excess of exogenous IL-6 reversed the let-7-mediated downregulation of STAT3 signaling, there is likely no defect in JAK activity in the PDAC cells in our experiments. It is noteworthy that although KRas is a direct target of let-7 and we did find downregulation of KRas protein in pancreatic cancer cell lines, Corcoran et. al. reported that shRNA-mediated knockdown of KRas did not reduce STAT3 phosphorylation in PDAC cell lines [43]. Our data also show that even in the presence of activating mutation of KRas in MiaPaCa and Panc1 cells, STAT3 knockdown alone reduced growth of these cells. Taken together, our data strongly suggests that let-7-mediated downregulation of STAT3 activity in PDAC cells does not involve IL6-IL6R-JAK portion of STAT3 signaling.

As STAT3 is a direct activator of SOCS3 expression, JAK-STAT signaling initially induces SOCS3 transcription. SOCS3 subsequently binds to JAK preventing its catalytic activity and thereby blocking JAK's ability to phosphorylate STAT3, which ultimately reduces SOCS3 production in a negative feedback manner [61,62]. SOCS3 also induces ubiquitination and proteasomal degradation of JAK and the gp130 IL-6 receptor. However, SOCS3 levels quickly fall after phosphorylation and nuclear translocation of STAT3 is inhibited. In our experiment we found increased expression of SOCS3 following let-7 re-expression. Interestingly, treatment with exogenous IL-6 although induced SOCS3 expression in both Pa-Sh and Pa-7a cells, only Pa-7a cells could maintain this induction for an extended period of time (Figure 6B). Clearly let-7 plays a role in stabilizing SOCS3 levels in PDAC cells. Further, we demonstrated that knockdown of SOCS3 expression led to reduced let-7-mediated effects in PDAC cells (Figure 7). As SOCS3 is a known inhibitor of STAT3 signaling, our data highlights the role of STAT3 signaling in the tumor suppressive activity of let-7. However, our study does not address how let-7 induces or stabilizes SOCS3 level and how those activities ultimately modulate STAT3 signaling in PDAC cells. Interestingly, Wei et. al. recently reported that SOCS3 is a direct target of miR-203 in keratinocytes [63] which could imply that STAT3 upregulation in pancreatic cancer cells may be the result of a similar activity. It was therefore plausible that let-7 down-regulated miR-203 in PDAC cells and thereby effected SOCS3 upregulation and STAT3 downregulation. However, we did not find any such relationship between let-7 and miR-203 expression in our studies (data not shown). It may be noted that a separate study on mouse keratinocytes by a different laboratory also could not identify any relationship between SOCS3 and miR203 [64]. Regardless, let-7 re-expression upregulates SOCS3 expression in PDAC cells (Figure 6D), and we are currently investigating the mechanism whereby let-7 increases SOCS3 expression in these cells.

An association of EMT with cancer cell invasion and metastasis has been established for many cancers including that of the pancreas [65]. Higher level expression of the factors that are associated with EMT, such as Snail, Slug and Twist has been reported in poorly-differentiated pancreatic cancer cells as well as in PDAC tissue samples [66]. Further, a recent study of clinically-resected pancreatic tumors in Nagoya University Hospital in Japan

demonstrated higher expression of the EMT phenotype as a poor prognostic marker for PDAC patients [67]. Our study demonstrated that let-7 re-expression not only downregulated STAT3 phosphorylation in PDAC cell lines but also increased the epithelial marker E-cadherin, and decreased the mesenchymal markers vimentin and MMP9, reversing the EMT phenotype. Let-7 re-expression in PDAC cells therefore could be highly beneficial clinically in preventing progression and metastasis.

A direct relationship between let-7 expression and cellular differentiation has been reported in number of studies. On the other hand, lack of their expression has been associated with variety of aggressive cancers [33,35]. Let-7 exerts these effects mostly through downregulating transcription factors or other proteins, such as HMGA2, KRAS, cyclinD1 and others that are positively associated with cell growth. Our study identifies another pathway whereby let-7 prevents activation of STAT3 signaling pathway that is linked with cellular response to external stimuli such as growth factors, interferons and cytokines and associated pro-proliferative responses. Although multiple studies have stated that inhibition of STAT3 signaling would be beneficial in treating variety of cancers, a consensus about how it should be done has not been reached. A complete shutdown of this pathway (as one would expect from the use of chemical inhibitors of STAT3) may interfere with multiple biological processes. Our work demonstrated that let-7, in contrast, regulates STAT3 signaling in a subtle manner through a physiological regulator. In our preliminary work, we found that certain histone deacetylase (HDAC) inhibitors actually increase the expression of let-7 in PDAC cells (data not shown). As some highly-active HDAC inhibitors are already being used in pre-clinical studies and clinical trials [42,68], a let-7 re-expression through the use of low doses of HDAC inhibitors or by gene therapy could be an useful therapeutic strategy against pancreatic cancer.

Acknowledgments

We thank Dr. Anurag Singh for his generous donation of the pancreatic cancer cell line ASPC1, Dr. Etty Benveniste for the STAT3C expression plasmid and Dr. Remco Spanjaard for numerous comments and suggestions. This study was supported by the Boston University Department of Medicine pilot grant and Genome Science Institute Seed grant (to S.K.G.), the National Cancer Institute grant (CA164245 to D.V.F.) and the Karin Grunebaum Foundation for Cancer Research (to D.V.F.). Kripa Patel and Anita Kollory received Boston University Undergraduate Research Opportunity Program Scholarships for their work under the mentorship of S.K.G.

References

1. Jemal A, Siegel R, Xu J, Ward E. Cancer statistics, 2010. *CA Cancer J Clin.* 2010; 60:277–300. [PubMed: 20610543]
2. Kleeff J, Michalski CW, Friess H, Buchler MW. Surgical treatment of pancreatic cancer: the role of adjuvant and multimodal therapies. *Eur J Surg Oncol.* 2007; 33:817–823. [PubMed: 17331695]
3. Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol.* 1997; 15:2403–2413. [PubMed: 9196156]
4. Poplin E, Feng Y, Berlin J, Rothenberg ML, Hochster H, Mitchell E, Alberts S, O'Dwyer P, Haller D, Catalano P, Cella D, Benson AB 3rd. Phase III, randomized study of gemcitabine and oxaliplatin versus gemcitabine (fixed-dose rate infusion) compared with gemcitabine (30-minute infusion) in patients with pancreatic carcinoma E6201: a trial of the Eastern Cooperative Oncology Group. *J Clin Oncol.* 2009; 27:3778–3785. [PubMed: 19581537]
5. Heinemann V, Quietzsch D, Gieseler F, Gonnermann M, Schonekas H, Rost A, Neuhaus H, Haag C, Clemens M, Heinrich B, Vehling-Kaiser U, Fuchs M, Fleckenstein D, Gesierich W, Uthgenannt D, Einsele H, Holstege A, Hinke A, Schalthorn A, Wilkowski R. Randomized phase III trial of

- gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer. *J Clin Oncol*. 2006; 24:3946–3952. [PubMed: 16921047]
6. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, Adenis A, Raoul JL, Gourgou-Bourgade S, de la Fouchardiere C, Bennouna J, Bachet JB, Khemissa-Akouz F, Pere-Verge D, Delbaldo C, Assenat E, Chauffert B, Michel P, Montoto-Grillot C, Ducreux M. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011; 364:1817–1825. [PubMed: 21561347]
 7. Schneider G, Schmid RM. Genetic alterations in pancreatic carcinoma. *Mol Cancer*. 2003; 2:15. [PubMed: 12605716]
 8. Hezel AF, Kimmelman AC, Stanger BZ, Bardeesy N, Depinho RA. Genetics and biology of pancreatic ductal adenocarcinoma. *Genes Dev*. 2006; 20:1218–1249. [PubMed: 16702400]
 9. Chiu J, Yau T. Metastatic pancreatic cancer: are we making progress in treatment? *Gastroenterol Res Pract*. 2012; 2012:898931.
 10. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science*. 2008; 321:1801–1806. [PubMed: 18772397]
 11. Perez-Mancera PA, Guerra C, Barbacid M, Tuveson DA. What we have learned about pancreatic cancer from mouse models. *Gastroenterol*. 2012; 142:1079–1092.
 12. Scholz A, Heinze S, Detjen KM, Peters M, Welzel M, Hauff P, Schirner M, Wiedenmann B, Rosewicz S. Activated signal transducer and activator of transcription 3 (STAT3) supports the malignant phenotype of human pancreatic cancer. *Gastroenterol*. 2003; 125:891–905.
 13. Toyonaga T, Nakano K, Nagano M, Zhao G, Yamaguchi K, Kuroki S, Eguchi T, Chijiwa K, Tsuneyoshi M, Tanaka M. Blockade of constitutively activated Janus kinase/signal transducer and activator of transcription-3 pathway inhibits growth of human pancreatic cancer. *Cancer Lett*. 2003; 201:107–116. [PubMed: 14580692]
 14. Ihle JN. STATs: signal transducers and activators of transcription. *Cell*. 1996; 84:331–334. [PubMed: 8608586]
 15. Zhong Z, Wen Z, Darnell JE Jr. Stat3: a STAT family member activated by tyrosine phosphorylation in response to epidermal growth factor and interleukin-6. *Science*. 1994; 264:95–98. [PubMed: 8140422]
 16. Cao X, Tay A, Guy GR, Tan YH. Activation and association of Stat3 with Src in v-Src-transformed cell lines. *Mol Cell Biol*. 1996; 16:1595–1603. [PubMed: 8657134]
 17. Abdulghani J, Gu L, Dagvadorj A, Lutz J, Leiby B, Bonuccelli G, Lisanti MP, Zellweger T, Alanen K, Mirtti T, Visakorpi T, Bubendorf L, Nevalainen MT. Stat3 promotes metastatic progression of prostate cancer. *Am J Pathol*. 2008; 172:1717–1728. [PubMed: 18483213]
 18. Berishaj M, Gao SP, Ahmed S, Leslie K, Al-Ahmadie H, Gerald WL, Bornmann W, Bromberg JF. Stat3 is tyrosine-phosphorylated through the interleukin-6/glycoprotein 130/Janus kinase pathway in breast cancer. *Breast Cancer Res*. 2007; 9:R32. [PubMed: 17531096]
 19. Bharti AC, Donato N, Aggarwal BB. Curcumin (diferuloylmethane) inhibits constitutive and IL-6-inducible STAT3 phosphorylation in human multiple myeloma cells. *J Immunol*. 2003; 171:3863–3871. [PubMed: 14500688]
 20. Yu H, Jove R. The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer*. 2004; 4:97–105. [PubMed: 14964307]
 21. Gao H, Ward PA. STAT3 and suppressor of cytokine signaling 3: potential targets in lung inflammatory responses. *Expert Opin Ther Targets*. 2007; 11:869–880. [PubMed: 17614756]
 22. Glienke W, Hausmann E, Bergmann L. Targeting STAT3 signaling in pancreatic cancer promotes antiapoptotic gene expression. *Pancreas*. 2011; 40:323–324. [PubMed: 21311318]
 23. Nagaraj NS, Washington MK, Merchant NB. Combined blockade of Src kinase and epidermal growth factor receptor with gemcitabine overcomes STAT3-mediated resistance of inhibition of pancreatic tumor growth. *Clin Cancer Res*. 2011; 17:483–493. [PubMed: 21266529]

24. McCleary-Wheeler AL, Lomberk GA, Weiss FU, Schneider G, Fabbri M, Poshusta TL, Dusetti NJ, Baumgart S, Iovanna JL, Ellenrieder V, Urrutia R, Fernandez-Zapico ME. Insights into the epigenetic mechanisms controlling pancreatic carcinogenesis. *Cancer Lett.* 2013; 328:212–221. [PubMed: 23073473]
25. Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, Liu CG, Bhatt D, Taccioli C, Croce CM. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA.* 2007; 297:1901–1908. [PubMed: 17473300]
26. Szafranska AE, Davison TS, John J, Cannon T, Sipos B, Maghnouj A, Labourier E, Hahn SA. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene.* 2007; 26:4442–4452. [PubMed: 17237814]
27. Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, Morgan DL, Postier RG, Brackett DJ, Schmittgen TD. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer.* 2007; 120:1046–1054. [PubMed: 17149698]
28. Ali S, Almhanna K, Chen W, Philip PA, Sarkar FH. Differentially expressed miRNAs in the plasma may provide a molecular signature for aggressive pancreatic cancer. *Am J Transl Res.* 2011; 3:28–47. [PubMed: 21139804]
29. Torrisani J, Bournet B, du Rieu MC, Bouisson M, Souque A, Escourrou J, Buscail L, Cordelier P. let-7 MicroRNA transfer in pancreatic cancer-derived cells inhibits in vitro cell proliferation but fails to alter tumor progression. *Hum Gene Ther.* 2009; 20:831–844. [PubMed: 19323605]
30. Ali S, Saleh H, Sethi S, Sarkar FH, Philip PA. MicroRNA profiling of diagnostic needle aspirates from patients with pancreatic cancer. *Br J Cancer.* 2012; 107:1354–1360. [PubMed: 22929886]
31. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature.* 2000; 403:901–906. [PubMed: 10706289]
32. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature.* 2000; 408:86–89. [PubMed: 11081512]
33. Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME. The role of let-7 in cell differentiation and cancer. *Endocr Relat Cancer.* 2010; 17:F19–36. [PubMed: 19779035]
34. Hertel J, Bartschat S, Wintsche A, Otto C, Stadler PF. Evolution of the let-7 microRNA family. *RNA Biol.* 2012; 9:231–241. [PubMed: 22617875]
35. Wang Y, Hu X, Greshock J, Shen L, Yang X, Shao Z, Liang S, Tanyi JL, Sood AK, Zhang L. Genomic DNA copy-number alterations of the let-7 family in human cancers. *PLoS One.* 2012; 7:e44399. [PubMed: 22970210]
36. Thornton JE, Gregory RI. How does Lin28 let-7 control development and disease? *Trends Cell Biol.* 2012; 22:474–482. [PubMed: 22784697]
37. Thomson JM, Parker J, Perou CM, Hammond SM. A custom microarray platform for analysis of microRNA gene expression. *Nat Methods.* 2004; 1:47–53. [PubMed: 15782152]
38. Bussing I, Slack FJ, Grosshans H. let-7 microRNAs in development, stem cells and cancer. *Trends Mol Med.* 2008; 14:400–409. [PubMed: 18674967]
39. Akao Y, Nakagawa Y, Naoe T. Let-7 microRNA functions as a potential growth suppressor in human colon cancer cells. *Biol Pharm Bull.* 2006; 29:903–906. [PubMed: 16651716]
40. Takamizawa J, Konishi H, Yanagisawa K, Tomida S, Osada H, Endoh H, Harano T, Yatabe Y, Nagino M, Nimura Y, Mitsudomi T, Takahashi T. Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res.* 2004; 64:3753–3756. [PubMed: 15172979]
41. Yu F, Yao H, Zhu P, Zhang X, Pan Q, Gong C, Huang Y, Hu X, Su F, Lieberman J, Song E. Let-7 regulates self renewal and tumorigenicity of breast cancer cells. *Cell.* 2007; 131:1109–1123. [PubMed: 18083101]
42. Ghosh SK, Perrine SP, Williams RM, Faller DV. Histone deacetylase inhibitors are potent inducers of gene expression in latent EBV and sensitize lymphoma cells to nucleoside antiviral agents. *Blood.* 2012; 119:1008–1017. [PubMed: 22160379]

43. Corcoran RB, Contino G, Deshpande V, Tzatsos A, Conrad C, Benes CH, Levy DE, Settleman J, Engelman JA, Bardeesy N. STAT3 plays a critical role in KRAS-induced pancreatic tumorigenesis. *Cancer Res.* 2011; 71:5020–5029. [PubMed: 21586612]
44. Wulczyn FG, Smirnova L, Rybak A, Brandt C, Kwidzinski E, Ninnemann O, Strehle M, Seiler A, Schumacher S, Nitsch R. Post-transcriptional regulation of the let-7 microRNA during neural cell specification. *FASEB J.* 2007; 21:415–426. [PubMed: 17167072]
45. Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V. Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol.* 2004; 5:R13. [PubMed: 15003116]
46. Schulman BR, Esquela-Kerscher A, Slack FJ. Reciprocal expression of lin-41 and the microRNAs let-7 and mir-125 during mouse embryogenesis. *Dev Dyn.* 2005; 234:1046–1054. [PubMed: 16247770]
47. Lee JM, Dedhar S, Kalluri R, Thompson EW. The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol.* 2006; 172:973–981. [PubMed: 16567498]
48. Arumugam T, Ramachandran V, Fournier KF, Wang H, Marquis L, Abbruzzese JL, Gallick GE, Logsdon CD, McConkey DJ, Choi W. Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer. *Cancer Res.* 2009; 69:5820–5828. [PubMed: 19584296]
49. Hotz B, Arndt M, Dullat S, Bhargava S, Buhr HJ, Hotz HG. Epithelial to mesenchymal transition: expression of the regulators snail, slug, and twist in pancreatic cancer. *Clin Cancer Res.* 2007; 13:4769–4776. [PubMed: 17699854]
50. Shah AN, Summy JM, Zhang J, Park SI, Parikh NU, Gallick GE. Development and characterization of gemcitabine-resistant pancreatic tumor cells. *Ann Surg Oncol.* 2007; 14:3629–3637. [PubMed: 17909916]
51. Watanabe M, Sheriff S, Lewis KB, Tinch SL, Cho J, Balasubramaniam A, Kennedy MA. HMGA-targeted phosphorothioate DNA aptamers increase sensitivity to gemcitabine chemotherapy in human pancreatic cancer cell lines. *Cancer Lett.* 2012; 315:18–27. [PubMed: 22036895]
52. Rahman MM, Qian ZR, Wang EL, Sultana R, Kudo E, Nakasono M, Hayashi T, Kakiuchi S, Sano T. Frequent overexpression of HMGA1 and 2 in gastroenteropancreatic neuroendocrine tumours and its relationship to let-7 downregulation. *Br J Cancer.* 2009; 100:501–510. [PubMed: 19156147]
53. Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. Stat3 as an oncogene. *Cell.* 1999; 98:295–303. [PubMed: 10458605]
54. Li L, Shaw PE. Elevated activity of STAT3C due to higher DNA binding affinity of phosphotyrosine dimer rather than covalent dimer formation. *J Biol Chem.* 2006; 281:33172–33181. [PubMed: 16956893]
55. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. *Cell.* 2009; 139:693–706. [PubMed: 19878981]
56. Wang Y, Lu Y, Toh ST, Sung WK, Tan P, Chow P, Chung AY, Jooi LL, Lee CG. Lethal-7 is down-regulated by the hepatitis B virus x protein and targets signal transducer and activator of transcription 3. *J Hepatol.* 2010; 53:57–66. [PubMed: 20447714]
57. Yagil Z, Nechushtan H, Kay G, Yang CM, Kemeny DM, Razin E. The enigma of the role of protein inhibitor of activated STAT3 (PIAS3) in the immune response. *Trends Immunol.* 2010; 31:199–204. [PubMed: 20181527]
58. Croker BA, Krebs DL, Zhang JG, Wormald S, Willson TA, Stanley EG, Robb L, Greenhalgh CJ, Forster I, Clausen BE, Nicola NA, Metcalf D, Hilton DJ, Roberts AW, Alexander WS. SOCS3 negatively regulates IL-6 signaling in vivo. *Nat Immunol.* 2003; 4:540–545. [PubMed: 12754505]
59. Gough DJ, Corlett A, Schlessinger K, Wegrzyn J, Larner AC, Levy DE. Mitochondrial STAT3 supports Ras-dependent oncogenic transformation. *Science.* 2009; 324:1713–1716. [PubMed: 19556508]
60. Sugimura K, Miyata H, Tanaka K, Hamano R, Takahashi T, Kurokawa Y, Yamasaki M, Nakajima K, Takiguchi S, Mori M, Doki Y. Let-7 expression is a significant determinant of response to chemotherapy through the regulation of IL-6/STAT3 pathway in esophageal squamous cell carcinoma. *Clin Cancer Res.* 2012; 18:5144–5153. [PubMed: 22847808]

61. Babon JJ, Nicola NA. The biology and mechanism of action of suppressor of cytokine signaling 3. *Growth Factors*. 2012; 30:207–219. [PubMed: 22574771]
62. Silver JS, Hunter CA. gp130 at the nexus of inflammation, autoimmunity, and cancer. *J Leukoc Biol*. 2010; 88:1145–1156. [PubMed: 20610800]
63. Wei T, Orfanidis K, Xu N, Janson P, Stahle M, Pivarcsi A, Sonkoly E. The expression of microRNA-203 during human skin morphogenesis. *Exp Dermatol*. 2010; 19:854–856. [PubMed: 20698882]
64. Lena AM, Shalom-Feuerstein R, Rivetti di Val Cervo P, Aberdam D, Knight RA, Melino G, Candi E. miR-203 represses 'stemness' by repressing DeltaNp63. *Cell Death Differ*. 2008; 15:1187–1195. [PubMed: 18483491]
65. Tiwari N, Gheldof A, Tatari M, Christofori G. EMT as the ultimate survival mechanism of cancer cells. *Semin Cancer Biol*. 2012; 22:194–207. [PubMed: 22406545]
66. Karamitopoulou E. Role of Epithelial-Mesenchymal Transition in Pancreatic Ductal Adenocarcinoma: Is Tumor Budding the Missing Link? *Front. Oncol*. 2013; 3:221.
67. Yamada S, Fuchs BC, Fujii T, Shimoyama Y, Sugimoto H, Nomoto S, Takeda S, Tanabe KK, Kodera Y, Nakao A. Epithelial-to-mesenchymal transition predicts prognosis of pancreatic cancer. *Surgery*. 2013; 154:946–954. [PubMed: 24075276]
68. Qiu T, Zhou L, Zhu W, Wang T, Wang J, Shu Y, Liu P. Effects of treatment with histone deacetylase inhibitors in solid tumors: a review based on 30 clinical trials. *Future Oncol*. 2013; 9:255–269. [PubMed: 23414475]

Figure 1.

STAT3 phosphorylation facilitates the growth of pancreatic cancer cell lines. (A) Immunoblot analysis of whole cell lysates from actively-growing BxPC-3, Panc1, MiaPaCa and ASPC-1 cells. Thirty micrograms of protein from each sample was separated in 4–20% gradient SDS-PAGE. Similar results were obtained from lysates prepared from two other independent experiments. (B) Three thousand STAT3 siRNA-transfected (20nM) Panc1 cells were seeded per well in quadruplicate in 96-well plate and allowed to grow up to 6 days post transfection. Cell viability was measured by MTS assay. (C) Parallel experiments as in panel B, but using MiaPaCa cells. Error bars represent standard error of the mean (SEM) within three experiments. Statistical significance of <0.05 are indicated by *. Experiments in B and C were done at least three times with similar results.

Figure 2.

Let-7 expression reduces STAT3 phosphorylation in pancreatic cancer cell lines. (A) Expression of let-7 members in microRNA preparations from three actively-growing pancreatic cancer cell lines was compared. All values were normalized against RNU44 expression and expression level of each individual let-7 microRNA was arbitrarily set to 1 in BxPC-3 cell line. Error bars represent SEM within three experiments. (B) Whole cell lysates from MiaPaCa or Panc1 cells stably-transfected with plasmid vectors expressing individual let-7 members (MP-7a, MP-7f, MP-7g or Pa-7a, Pa-7c, Pa-7f, respectively) or non-targeting Sh-RNA were analyzed for STAT3 and phosphoSTAT3 protein by immunoblotting. On the right of each panel is the band intensity quantitation chart that was generated after normalization for β-actin expression in each sample. STAT3 or phosphoSTAT3 levels in each sample were calculated against the level found in corresponding Sh-RNA expressing cell line. Error bars in these charts represent SEM from three independent experiments. (C) Immunofluorescence analysis of phosphoSTAT3 expression in Panc1 cells expressing either control Sh-RNA or let-7a. (D) Effect of let-7 overexpression on the growth of MiaPaCa cells was determined by seeding MP-7a, MP-7f, MP-7g or MP-Sh cells (3,000 cells/well) in a 96-well plate. Cell growth was evaluated 72 hrs after seeding by MTS assay as in Figure 1. This experiment was repeated at least three times and P values <0.001 are indicated by **.

Figure 3.

Let-7 reexpression reduces the EMT phenotype in pancreatic cancer cells. (A) Panc1 cells stably-expressing let-7a, let-7c, let-7f or non-targeting Sh-RNA (Pa-7a, Pa-7c, Pa-7f or Pa-Sh) were plated in chamber slides for 48hr before subjecting to immunofluorescence staining for E-cadherin (green). Counterstaining was done with DAPI (blue). Similar results were obtained in three independent experiments. (B) Expression of the mesenchymal markers vimentin and fibronectin and epithelial marker β -catenin in Panc1 cells transfected with either control miRNA mimic or let-7a mimic were analyzed by immunostaining as described in section 2.7. These experiments were repeated three times and a representative set is presented. (C) Pa-Sh, Pa-7a and Pa-7f cells were seeded in 60mm culture dishes to form confluent monolayers (for ~40hrs). Following infliction of a wound, migration of the cells was recorded at 0hr, 6hr and 24hr under a light microscope. These experiments were also repeated at least three times. A photograph of a representative experiment is presented. (D) Chemotaxis of Panc1 cells transfected with control miRNA mimic or let-7a mimic. Results from three independent wells for each set are presented. (E) Migrated cells from each individual experiments were counted and plotted graphically. Error bars represent standard deviations.

Figure 4.

Let-7 abrogates expression of STAT3-activated genes. (A) MiaPaCa and Panc1 cells were transfected with 20nM let-7a mimic or negative control mimic. Whole cell lysates from these cells were extracted 72 hrs after transfection and subjected to immunoblot analysis for let-7 targets as well as for total- and phospho-STAT3. Data presented is a representative of three independent experiments. (B) Effect of let-7 re-expression on let-7 targets in transfected Panc1 cells (with 20nM let-7a mimic, let-7f mimic or negative control mimic) were also tested by qRT-PCR experiments on 1 µg total RNA extracts from these cells. (C) Total RNA extracts from similarly transfected MiaPaCa cells were analyzed for expression of STAT3 target genes following qRT-PCR analysis on 1 µg of total RNA. (D) Parallel studies to panel C, but using RNA samples from transfected Panc1 cells. Results presented in (B), (C), (D) were all normalized against β-actin m-RNA levels in each individual sample. Error bars represent SEM within three experiments.

Figure 5.

Let-7 expression does not alter STAT3 or IL-6 expression in pancreatic cancer cells. (A) One microgram of total RNA from MiaPaCa and Panc1 cells transfected with 20nM let-7a mimic or negative control mimic (at 72 hrs post-transfection) was subjected to qRT-PCR analysis for STAT3 and IL-6 mRNA levels. All data were normalized against endogenous β -actin expression. SEM within three experiments are represented by error bars. P values were MiaPaCa =0.94, Panc1 =0.06 (for STAT3) and MiaPaCa =0.55, Panc1 = 0.93 (for IL-6). (B) Panc1 cells stably-transfected with let-7a or Sh-RNA were treated with 10ng/mL recombinant human IL-6 for 20 min before harvesting for whole cell lysate preparation and immunoblot analysis for total- and phospho-STAT3.

Figure 6.

Expression of let-7 induces expression of SOCS3, but not PIAS3, in PDAC cell lines. (A) Whole cell lysates from MP-Sh and MP-7a or Pa-Sh and Pa-7a cells were analyzed for PIAS3 and SOCS3 protein levels by immunoblot experiments. Band intensity quantitation chart generated after normalization for β-actin expression in each sample is presented in the lower panel (open bar is for PIAS3 and solid bar for SOCS3). All values are relative to the expression level in Sh-RNA expressing stable cell line. (B) Pa-Sh and Pa-7a cells were either untreated (0m) or treated with 10ng/mL recombinant IL-6 for 20m or 4hr before whole cell lysate preparation from them. Lysates were then evaluated for SOCS3 protein by immunoblot analysis. Band intensity chart presented in the lower panel represents the SOCS3 levels in the samples in comparison to the level seen in untreated Pa-Sh cells. These experiments were repeated three times with similar results. (C) Panc1 cells were transfected with let-7a mimic or negative control mimic (20nM) as in Figure 4B and the whole cell lysates were tested to confirm downregulation of STAT3 phosphorylation and upregulation of SOCS3 by let-7a mimic transfection before carrying out immunofluorescence analysis. (D) Panc1 and MiaPaCa cells were transfected with 20nM let-7a mimic or negative control mimic in chamber slides and processed for immunostaining on day 4 post-transfection. Bound antibodies were detected using Alexafluor 488-conjugated secondary antibodies

(green). These experiments were also repeated at least three times and a representative set is presented.

Figure 7.

Knockdown of SOCS3 abrogates let-7-mediated effects. Panc1 cells were transfected with control siRNA, let-7a mimic, ON-TARGETplus SMARTpool SOCS3 siRNA or a 1:1 mixture of let-7a mimic and SOCS3 siRNA (final concentration was 40 nM for all) using RNAiMax transfection reagent as described in section 2.2. (A) Total RNA extracts from transfected cells were reverse transcribed and SOCS3 mRNA expression in them was analyzed by qRT-PCR. SEM within three experiments are represented by error bars. (B) Whole cell lysates from Panc1 cells transfected as above were analyzed for total-STAT3 and phospho-STAT3 by western immunoblotting. Representative picture from three independent experiments are presented. (C) Panc1 cells transfected as above were subjected to Immunostaining with anti-β-catenin, anti-fibronectin and anti-SOCS3 antibodies. Bound antibodies were detected using Alexaflour 488-conjugated secondary antibodies (green). These experiments were repeated three times and a representative set is presented.

Table 1

Primers used in the study

Genes	Sequence (5' to 3')
Actin-F	TCCCTGGAGAAGAGCTACGA
Actin-R	AGCACTGTGTTGGCGTACAG
STAT3-F	CAGGAGGGCAGTTTGAGTCC
STAT3-R	CAAAGATAGCAGAAGTAGGAGA
IL6-F	CAGTTGCCTTCTCCCTGGG
IL6-R	ATGTTACTCTTGTTACATG
VEGF-F	GAATCATCACGAAGTGGTGA
VEGF-R	AACGCGAGTCTGTGTTTTTG
CycD1-F	CTGGCCATGAACTACCTGGA
CycD1-R	TCACACTTGATCACTCTCG
MMP9-F	GTCATCCAGTTTGGTGTCCG
MMP9-R	TGGTGCAGGCAGAGTAGGAG
Lin28a-F	AGAGTAAGCTGCACATGGAAGGGT
Lin28a-R	TATGGCTGATGCTCTGGCAGAAGT
Lin28b-F	CATGGTGGCAAAC TGCCACATAA
Lin28b-R	TTCGTGGAGGAAGCTTCTTGAGGT
HMGA2-F	GTGAGATGCAACAACCCCTGCTTT
HMGA2-R	TGTGGCCTTTGAAACTACCTCCCT