The Matricellular Protein CCN1/Cyr61 Is a Critical Regulator of Sonic Hedgehog in Pancreatic Carcinogenesis*§

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Background: CCN1 plays a vital role in pancreatic carcinogenesis with an unknown mechanism.

Results: CCN1 regulates Sonic-Hedgehog in pancreatic cancer cells via integrin-Notch-signaling pathway to promote in vitro motility and in vivo tumorigenic growth.

Conclusion: CCN1 is a critical regulator of Sonic-Hedgehog signaling in pancreatic cancer cells.

Significance: Studies suggest a mechanism whereby CCN1 regulates carcinogenic events in the pancreas.

CCN1 is a matricellular protein and a member of the CCN family of growth factors. CCN1 is associated with the development of various cancers including pancreatic ductal adenocarcinoma (PDAC). Our recent studies found that CCN1 plays a critical role in pancreatic carcinogenesis through the induction of EMT and stemness. CCN1 mRNA and protein were detected in the early precursor lesions, and their expression intensified with disease progression. However, biochemical activity and the molecular targets of CCN1 in pancreatic cancer cells are unknown. Here we show that CCN1 regulates the Sonic Hedgehog (SHh) signaling pathway, which is associated with the PDAC progression and poor prognosis. SHh regulation by CCN1 in pancreatic cancer cells is mediated through the active Notch-1. Notably, active Notch-1s recruited by CCN1 in these cells via the inhibition of proteasomal degradation results in stabilization of the receptor. We find that CCN1-induced activation of SHh signaling might be necessary for CCN1-dependent in vitro pancreatic cancer cell migration and tumorigenicity of the side population of pancreatic cancer cells (cancer stem cells) in a xenograft in nude mice. Moreover, the functional role of CCN1 could be mediated through the interaction with the αvβ3 integrin receptor. These extensive studies propose that targeting CCN1 can provide a new treatment option for patients with pancreatic cancer since blocking CCN1 simultaneously blocks two critical pathways (i.e. SHh and Notch1) associated with the development of the disease as well as drug resistance.

Pancreatic ductal adenocarcinoma (PDAC), with a prevalence of 2–3% of new cancer cases annually in the United States, is the fifth leading cause of cancer deaths in the United States and globally (1–4). Prognosis of PDAC is extremely dismal. Due to the impalpable nature of the disease, PDAC is hard to diagnose at an early stage and typically presents with metastasis at the time of diagnosis. Currently, there are no effective therapies for PDAC, and it exhibits a profound resistance to current chemotherapies (5). Therefore, new insights into the etiology of PDAC progression along with its precise mechanisms of drug resistance need to be discovered.

PDAC develops from pancreatic intraepithelial neoplastic (PanIN) precursor lesions through multiple histologic, genetic, and epigenetic changes (6, 7). Multiple studies have found that sonic hedgehog (SHh), a lipid-modified, secreted signaling protein, plays a critical role in PDAC's development from PanIN lesions to the invasive growth of the disease (8–12). Aberrant expression of SHh in pancreatic ductal epithelial cells, which is normally absent in the developing and mature pancreas (13), binds to the receptor patched (PTCH) to prevent an inhibitory impact of PTCH on the smoothened (Smo) receptor. The released and activated Smo then promotes translocation of GLI family of transcription factors from cytoplasm to the nucleus to induce the expression of SHh-targeted genes linked with carcinogenic events in the ducts of the pancreas (10, 11, 14, 15). The GLI-independent pathway may also be involved in SHh mediated pancreatic carcinogenesis (14). Despite the discrepancy in the literature, the previous studies have demonstrated that SHh is responsible for PDAC cell proliferation, epithelial-mesenchymal transition (EMT), maintenance of cancer stemness, migration, invasion, and metastatic growth in distant organs (5, 10, 11, 15–19). Moreover, SHh also plays a critical role in promoting desmoplasia and drug resistance in animal models (11, 20, 21). Notwithstanding knowledge of all these pathobiological impacts of SHh-signaling in PDAC, the mechanism(s) whereby SHh is activated in pancreatic cancer cells remains elusive. Insight into this regulation could provide a new rationale for improved therapy against this disease.

CCN1, which is also known as Cyr61 (cysteine-rich 61), is a member of the CCN family of matricellular proteins which consists of CTGF, NOV, WISP-1, WISP-2, and WISP-3 (22–26).
Role of CCN1 in Regulation of SHh

CCN1 is a secretory, multifunctional protein, growth factor inducible, and an immediate early response gene (27). CCN1 is either localized intracellularly or associated with the cell surface and extracellular matrix, and it is involved in the adhesion, proliferation, migration, differentiation and angiogenesis during normal and patho-physiological processes (23, 24). The histopathological and immunohistochemical studies indicate that, except in lung cancers (28) and leiomyomas (29), CCN1 expression is markedly increased in different human cancers including PDAC (7, 30, 31). Our recent studies show that CCN1, when overexpressed in PDAC and its precursor lesions, promotes proliferation, EMT, and migration of pancreatic cancer cells and, possibly, regulates stemness of these cells through the regulation stemness regulatory genes and microRNAs (7). However, it remains unknown how the CCN1 system becomes rewired at the molecular and cellular levels to promote PDAC growth.

Given that SHh and CCN1 signaling are associated with the genesis of human PDAC, one could speculate that these signaling molecules walk hand-over-hand or their regulation is mutually dependent for the development of PDAC. These studies support the hypothesis and show that CCN1 is an upstream regulator of SHh in pancreatic carcinogenesis. Our data also provide evidence that integrin αvβ3-Notch1 signaling is critical in CCN1 induced SHh expression in pancreatic cancer cells. Collectively, these studies illustrate that CCN1 could be an ideal target in pancreatic cancer cells to prevent the action of two critical signaling cascades.

MATERIALS AND METHODS

Cell Lines and Cell Culture—Human pancreatic cancer cell lines i.e. BxPC-3, Capan-1, AsPC-1, HS766T, Panc-1 and MIA-PaCa-2 were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma), supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (Sigma) in a 37 °C incubator in the presence of 5% CO2. CCN1-silenced Panc-1 and MIA-PaCa-2 cell lines were prepared and maintained by our laboratory (7). Amphot-e pak 293 packaging cell line was purchased from Clontech and was maintained in high glucose DMEM containing 10% FBS. Cells were used for the experiment between four and six passages.

Reagents and Antibodies—Human polyclonal anti-rabbit CCN1 antibody, rabbit polyclonal anti-human Notch-1, mouse monoclonal anti-human CD24, human polyclonal anti-galectin-1, polyclonal goat anti-rabbit IgG-HRP, human polyclonal anti-rabbit Notch-1 and monoclonal goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-mouse GAPDH antibody was purchased from Applied Biosystems (Foster City, CA). Human monoclonal anti-rabbit SHh antibody, Mouse monoclonal Collagen 1, and human polyclonal anti-rabbit Ptc1 antibody were obtained from Abcam (Cambridge, MA). Human rabbit polyclonal Gli1 was purchased from Cell Signaling (Boston, MA). Cyclin D1 and Bcl-2 antibodies were purchased from BD Biosciences (San Jose, CA) and Calbiochem respectively. pSilencer™ 5.1-U6 retroviral vector and siPORT™ XP-1 transfection agent were obtained from Applied Biosystems (Foster City, CA). All other chemicals were obtained either from Sigma or Fisher Scientific (Houston, TX). Cysteinyl recombinant protein was purchased from Fisher Scientific (St. Louis, MO). Notch-1 inhibitor DAPT [N-(N-(3, 5-difluorophenacetyl)-l-alanyl)-S-phenylglycine t-butyler ester] was purchased from Sigma. Matrigel was purchased from BD Biosciences (San Jose, CA). Cyclomamine was obtained from Sigma.

Mouse Xenograft Experiments—The animal studies were conducted according to the approved Guidelines of the Animals Care and Use Committee of Kansas City VA Medical Center. For subcutaneous nude mice xenograft studies, side population (SP), and non-side population (NSP) of Panc-1 cells were isolated by a BD FACS Aria SORP flow cytometer (BD Biosciences) using ~405 nm excitation and 440 nm emission as described previously by Haque et al. (7). Sorted cells (i.e. SP and Non-SP) were briefly cultured in DMEM with 10% FCS in 5% CO2 at 37 °C, and then cells (5 × 104 cells suspended in Matrigel to a final volume of 100 µl) were injected s.c. into the right rear flank of 6–8-week-old male athymic nude mice (6 mice per group) and tumor growth was monitored starting after the 2nd day of injection. This was continued for up to 45 days or more using our previous methods (32, 33). Male athymic nude mice (nu/nu genotype) were obtained from Charles Rivers (Wilmington, MA) and acclimated to our facility for 1 week before starting the experiments.

Retroviral Production and Transduction of Cells—CCN1-knock-out or scrambled pancreatic cancer cell lines (MIA-PaCa-2 CCN1KO and Panc-1 CCN1KO) were generated using pSilencer™ 5.1-U6 Retro-viral system (Ambion, Grand Island, NY) as per the protocol described earlier (7). Briefly, cloned human CCN1-shRNA/scrambled vector were transduced into an Amphpak™293 packaging cell line using siPORT™ XP-1 transfection agent. After transfection, the culture medium was changed and cells were incubated 48 more hours prior to collection of viral particles. Approximately, 60% of cells were infected with CCN1-shRNA containing viral supernatant or scrambled control and incubated for 72 h. Stable transfected clones were selected by puromycin treatment until the uninfected cells died. Stable cells were then cultured in regular DMEM with 10% FBS and harvested for Western or Northern blot analysis to check the transfection efficiency.

Western Blot Analysis—Cell lysates prepared from pancreatic cancer cell lines and tumor xenografts containing 30–50 µg proteins were analyzed by Western blot using the appropriate antibodies according to the method described previously (34). Signals were detected with SuperSignal Ultra Chemiluminescent substrate (Pierce) using ID Image Analysis software Version 3.6 (Eastman Kodak Company, Rochester, NY).

Immunohistochemistry—Immunohistochemistry was performed on 4% formalin-fixed, paraffin-embedded tissue sections according to our previous method (7, 35). Briefly, tissue sections were de-paraffinized in Xylene, rehydrated in different grades of alcohol, washed with PBS, and blocked with tissue blocker (Zymed Laboratories Inc.) for 10 min, and then immunostained by specific antibodies overnight in a moist chamber. The immunoreactivity was detected by conjugated streptavidin, and the sections were counterstained with hematoxylin.
The sections were imaged with a Leica photomicroscope. All samples were used according to VA Medical Center and University guidelines after receiving Institutional Review Board approval.

**Immunofluorescence**—The immunofluorescence assay was carried out as described earlier (32, 36). Cells were plated in chambered slides, fixed in methanol and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. After using a blocking solution, the cells were incubated with monoclonal mouse anti-SHh overnight at 4 °C and incubated with a goat anti-mouse FITC-conjugated Alexa Fluor 488 secondary antibody. Cells were washed with 1 × PBS and mounted in PBS glycerin. Immunofluorescent-stained cells were visualized using a Nikon Eclipse TE-300 microscope, and images were analyzed by software. Cells incubated without the primary antibody were treated as negative controls.

**Scratch Wound Healing Assay**—The motility behavior of the cells of different experimental conditions was examined by the scratch wound healing assay. Briefly, different cells were seeded in the chamber slides and allowed to reach 70–80% confluence as a monolayer and then scratched with a pipette tip diagonally. After scratching, chambers were gently washed with fresh media to remove detached cells. Migration into this area was documented and measured after 24 h. Each analysis was repeated three times.

**Proteasome Activity Assay**—To determine the chymotrypsin-like proteasome activity, 20 S proteasome activity assay kit (EMD Millipore, Billerica, MA) was used according to the manufacturer’s instructions. Briefly, harvested cells were incubated on ice for 30 min in lysis buffer containing 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 150 mM NaCl, 1% Nonidet P-40, and a protease inhibitor mixture including 1 μg/ml aprotinin, 1 μg of leupeptin, and 1.0 mM PMSF. Cellular debris was removed by centrifugation (18,000 × g, 1 h, 4 °C), and the supernatant was collected for the assay. For each reaction, 50 μg of sample proteins were used in assay buffer (50 mM Tris-HCl (pH 7.5), 25 mM KCl, 10 mM NaCl, 1 mM MgCl₂) where the chymotrypsin fluorogenic substrate N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC) was added to each well at a concentration of 50 μM, and the plates were incubated for 1 h in the dark at 37 °C. The fluorescence of AMC due to proteasome-mediated cleavage of the fluorogenic substrate was measured by excitation at 360 nm and emission at 460 nm in a SynergyTM HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT).

**Statistical Analysis**—All experiments were performed in triplicate for each observation. Each of the data represent the mean ± S.E. from the three separate experiments. Statistical analysis was performed between the two groups of data by an unpaired Student’s t test GraphPad Prism 4 software. Values were considered statistically significant at p < 0.05.

**RESULTS**

**Differential Expression of SHh and CCN1 in Different Pancreatic Cell Lines and PDAC Samples**—To determine the status of SHh and CCN1 protein in different pancreatic cancer cell lines, we evaluated the level of SHh and CCN1 in different pancreatic cell lines (i.e. BxPC-3, Capan-1 (less aggressive), AsPC-1, Hs766T, Panc-1, and MIA-Paca-2 (highly aggressive) (7, 37)) by Western blot analysis using specific antibodies. We found that SHh and CCN1 are highly expressed in all cell lines except BxPC-3 cells where expressions of SHh and Cyr61/CCN1 were minimal or undetected (Fig. 1A). The highest levels of expressions of both proteins were detected in the Panc-1 cell line.

Next, we determined the expression profiles of CCN1 and SHh immunohistochemically in PDAC tissue arrays. Consistent with previous work (7, 14), both CCN1 and SHh were confined to the cytoplasm and their expressions were first detected in histologically defined precursor lesions (PanINs; PanIN-1A-PanIN-3) (data not shown), and the expression was markedly increased in the advanced stages of the disease (Fig. 1B).

**CCN1 Regulates SHh Signaling in Pancreatic Cancer Cells**—Recent studies have shown that SHh is an upstream regulator of CCN1 in breast cancer cells (39). Therefore, we sought to corroborate the reduced production of CCN1 protein in functionally deficient SHh pancreatic cancer cells. To do so, Panc-1 cells were exposed to a SHh receptor SMO inhibitor (39), Cyclosporine, with different doses (1–5 μg/ml) for 48 h,
and CCN1 and the downstream target of the SHh molecule cyclin D1 expression was measured using Western blotting. Unexpectedly, in contrast to the previous studies (39), we found that Cyclopamine was unable to block CCN1 expression in Panc-1 cells; however, it blocked the expression of Cyclin D1 in these cells (Fig. 2A). Furthermore, Panc-1 cells treated with SHh neutralizing antibody showed no effect on CCN1 expression in these cells (data not shown), demonstrating that the SHh signaling is not an upstream regulator of CCN1, at least not in Panc-1 cells. Next, we examined the status of SHh in CCN1-deficient Panc-1 and MIA-PaCa-2 cells. Thus, we generated stable CCN1 knock-out Panc-1 and MIA-PaCa-2 cell lines (designated as Panc-1CCN1KO and MIA-PaCa-2CCN1KO) by silencing CCN1 with CCN1-shRNA in these cells retrovirally (7) and examined the status of CCN1 and SHh using Western blot analysis. We found significantly reduced levels of CCN1 and SHh in the total cell lysates of Panc-1CCN1KO and MIA-PaCa-2CCN1KO cells as compared with mismatched-shRNA-transfected cells (Fig. 2B).

To exert biological and pathobiological functions, SHh binds with its receptor 12-span-transmembrane protein Patched (Ptc1) in a paracrine manner and relieves another 7-span-transmembrane protein receptor, Smoothened (Smo), which in turn induces the signal transduction pathway by activating the nuclear translocation of transcription factor Gli1 protein (11, 19). Therefore, in this study, we next tested whether CCN1 deficiency alters the basal level of Ptc1, Smo, and/or Gli1 in pancreatic cancer cells as a consequence of inhibiting SHh. We found that Ptc1 is up-regulated while levels of Smo and Gli1 were reduced markedly in the cell lysates of CCN1-deficient Panc-1 and MIA-PaCa-2 cells (Fig. 2C, left panel and supplemental Fig. S1).

The SHh-Ptc1-Smo-Gli1 signaling pathway promotes pancreatic ductal epithelial cell proliferation through transcription regulation of cyclin D1 and other cell cycle regulatory genes. In addition, the SHh-Ptc1-Smo-Gli1 signaling pathway protects pancreatic ductal epithelial cells from apoptotic cell death through the activation of molecules associated with Bcl-2 family for carcinogenic development (10, 19). Given that cyclin D1 and Bcl-2 are important SHh-target genes, we sought to determine whether CCN1 silencing reduces the expression of cyclin D1 and Bcl-2 in Panc-1 and MIA-PaCa-2 pancreatic cancer cells. To do so, the levels of cyclin D1, Bcl-2, and Bax were determined in Panc-1CCN1KO and MIA-PaCa-2CCN1KO cells using immune-Western blotting. We found both cyclin D1 and Bcl-2 levels were markedly reduced in CCN1-deficient cells (Fig. 2C, right panel and supplemental Fig. S1), while Bax expression is elevated in CCN1-silenced cells as compared with mismatched cell lines.

The Panc1 side population (SP), which is also considered to be cancer initiating cells/cancer stem cells, produced an s.c. tumor with overexpressed CCN1 in nude mice within a brief
FIGURE 3. SP of Panc-1 cell line exhibits higher tumour growth potential along with the overexpression of Shh signalling cascades than the NSP of Panc-1 cell line in vivo. A, SP and NSP cells were separated and propagated according to our previous method, which has been described in the “Materials and Methods.” Semiconfluent cells were injected s.c. into the right rear flank of athymic nude mice and tumor growth was monitored. The bar graph represents the size of the tumors after 45 days of injection of SP and NSP Panc-1 cells (n = 6 mice/exp). Error bars indicate ± S.D. *, p < 0.001 versus SP. B, photomicrographs represent the status of CCN1, Shh, and Shh signaling cascades in SP- and NSP-tumor xenografts. T1 and T2 represent tumor no. 1 and 2, respectively.

Role of CCN1 in Regulation of Shh

FIGURE 4. CCN1 knockdown suppresses active Notch-1 expression while Notch/γ-secretase inhibitor (DAPT) blocks Shh expression without affecting CCN1 expression in Panc-1 cells. A, Panc-1 cells were transfected with nontargeting shRNA (mismatched) or shRNA targeting CCN1. After 48 h, cells were harvested, and cell lysates were analyzed for Notch-1 (ICD) and β-actin protein expression by Western blotting using specific antibodies. B, Panc-1 cells were treated with DAPT (5 μM) for 24 and 48 h or left untreated. Cells were harvested at indicated times for Western blot with CCN1, Shh, GAPDH, and β-actin antibodies. The results of CCN1 and Shh are normalized by the intensities of GAPDH and β-actin respectively. Error bars indicate ± S.D. of three independent experiments. *, p < 0.001 versus controls.

time period as compared with the non-side population (NSP). Targeting CCN1 by shRNA most effectively reduced this feature of SP cells (7). In this study, we investigated whether Shh signaling pathways are active in SP- and NSP-xenografts. To do so, SP- and NSP-xenografts were established by injecting cells subcutaneously into the flanks of athymic nude mice. When SP-tumors reached ~200 mm³, they were compared for volume with NSP-tumors. Significantly, SP-tumors exhibited 3-fold more growth than NSP-tumors (Fig. 3A). On day 45, the tumors were excised, and total proteins were extracted for Western blot analysis using CCN1, Shh, PTCH-1, Gli-1, and Smo specific antibodies. Like the corresponding in vitro studies, levels of Shh and Notch-1 signaling proteins were elevated significantly in CCN1-overexpressed SP-tumors as compared with NSP-tumors where CCN1 is minimally expressed (Fig. 3B).

CCN1 Modulates Shh Expression through Notch-1 in Pancreatic Cancer Cells—The objective of the present work was to dissect the mechanism by which CCN1 regulates Shh expression in pancreatic cancer cells. Previously, we established that CCN1 activates Notch-1 (the released form of an intracellular domain (ICD) of Notch-1 into the cytoplasm) in pancreatic cancer cells and their CCN1-positive SP (7). Two independent studies have shown that Notch-1 activates Shh to reinforce the cell-fate switch in Xenopus (40), and that Shh regulates Notch-1-targeted genes in vascular smooth muscle cells (41). These two studies, although not related to pancreatic cancer, caused us to speculate that Notch-1 may be a requisite downstream mediator of CCN1-induced overexpression of Shh in pancreatic cancer cells. To test the hypothesis, first we determined the status of active Notch-1 (ICD) in CCN1-deficient Panc-1 (CCN1KO) cells by Western blot analysis using an ICD-specific antibody. Consistent with previous studies (7), our present finding demonstrating that CCN1 enhances the expression of active Notch-1 (ICD) in Panc-1 cells since shRNA-mediated silencing of CCN1 resulted in a near complete loss of active Notch-1 (ICD) protein expression in Panc-1 cells (Fig. 4A), without affecting the transcription of Notch-1 as demonstrated by qPCR analysis (data not shown). Next, we tested if active Notch-1 is requisite for CCN1-mediated activation of Shh in pancreatic cancer cells. To do so, Panc-1 cells were treated with a pharmacological inhibitor of ICD-releasing proteolytic enzyme γ-secretase (DAPT, 5 μM) (42) or vehicle for different times (24, 48, and 72 h), and the levels of CCN1 and Shh were determined in the supernatants of tissue extracts using Western blotting. Studies showed that the expression of Shh was markedly diminished by DAPT at 48 h of treatment (Fig. 4B). We found, however, that treatment of DAPT has no effect on CCN1 expression in Panc-1 cells (Fig. 4B). Therefore, we can conclude that Notch-1 is an intermediate molecule of CCN1 and Shh and may play a vital role in CCN1-mediated activation of Shh-signaling.
Role of CCN1 in Regulation of SHh

Regulation of Notch-1(ICD) by CCN1 in Pancancer Cells—To dissect the functional role of CCN1 in regulation of Notch-1, we asked whether CCN1 enhances the stability of active Notch-1(ICD) by blocking the proteasomal degradation via the ubiquitin-proteasome system that have been implicated in the regulation of the half-life of Notch-1 (43–45). To this aim, first, we determined the expression of CCN1 on 20 S proteasome, a catalytic core of proteasome complex. To do so, Panc-1CCN1KO cells under different treatment conditions was determined by the fluorogenic substrate Suc-LLVY-AMC using 20 S proteasome activity assay kit. Proteasomal activity is expressed in arbitrary fluorescence units (AFU). Error bars indicate Suc-LLVY-AMC using 20 S proteasome activity assay kit. Proteasomal activity is expressed in arbitrary fluorescence units (AFU). Error bars indicate 

* p < 0.005 versus mismatched shRNA; **, p < 0.001 versus CCN1 shRNA. B, representative Western blots show expression CCN1 (left panel) and Notch-1 (ICD) (right panel) in Panc-1 cells under different treatment conditions, histograms in the lower left panel and lower right panel show the CCN1 to β-actin ratio and Notch-1 to β-actin ratio, respectively. Error bars indicate ± S.D. of three independent experiments. * p < 0.005 versus mismatched shRNA; **, p < 0.001 versus CCN1 shRNA. Note: Lactacystin has no impact on CCN1 expression (left panel, lane 3). Based on the results, the studies indicate that CCN1 enhances the stability of Notch-1(ICD) by preventing the proteasomal degradation events in the pancreatic cancer cells.

CCN1-induced Notch-1 Activation and SHh Expression Are Mediated through Integrin αvβ3 Receptor in Pancancer Cells—Multiple activities of CCN1 are mediated through heterodimeric cell surface integrin receptors (24). Interestingly, αv subunit of integrin is prime mediator of cellular activities of CCN1 in different cancer cells (24, 25). Given αv, we sought to determine if this unit is involved in CCN1-induced activation of Notch-1 and SHh expression in pancreatic cancer cells. To do so, we asked whether CCN1 recombinant protein could promote motility in different pancreatic cancer cell lines by Western blotting using specific antibodies. We found that all tested integrins, except α4 and α6, were expressed in Panc-1 cells, while these integrins were differentially expressed in ASPC-1 and Mia-PaCa-2 cell lines (Fig. 6A and supplemental Table S1). Next, to investigate the possible role of integrin αv in CCN1-mediated regulation of Notch-1 and/or SHh expression, functional blocking monoclonal antibody (mAb) against αv was used to study if CCN1 interacts with αv to activate Notch-1 followed by SHh expression in Panc-1. As expected, αv mAb markedly blocked CCN1-induced activation of Notch-1 and SHh expression in Panc-1 cells (Fig. 6B–D). Furthermore, an antibody of β3 (B3A) but not β1 subunits of integrins significantly abolished the activity of CCN1 on Notch-1 activation and SHh expression in Panc-1 cells (Fig. 6E, F and F). Together, these studies suggest that the integrin αvβ3 heterodimer may play critical role in CCN1-induced activation of SHh signaling.

CCN1-induced Pancancer Cancer Cell Motility Is Mediated through SHh—Previously, we demonstrated that CCN1 is one of the prime regulators of in vitro migration and invasion of pancreatic cancer cells (7). SHh-signaling also promotes motility and invasiveness of gastric and pancreatic cancer cells (46, 47). Therefore, we speculate that CCN1-induced pancreatic cancer cell motility is mediated through SHh-signaling. To test the hypothesis, we used Panc-1CCN1KO and Mia-PaCa-2CCN1KO cells as well as Panc-1 side population (SP, CCN1-positive cells) and Panc-1 non-side population (NSP, CCN1-negative cells) cells. First, using a scratch wound assay, we determined the motile behavior of above mentioned cell lines. We observed an enhanced motility in CCN1-positive cells compared to CCN1-negative cells which exhibited a markedly reduced rate of wound closure after 24 h of culture in an identical culture environment (Fig. 7A–C).

To test if CCN1 recombinant protein could promote motility of CCN1-deficient cells through SHh signaling, we assayed the

somonal degradation in Panc-1CCN1KO cells. As shown in Fig. 5B, Notch-1(ICD) protein expression in Panc-1CCN1KO cells was expectedly reduced (right panel, lane 3) as compared with the mismatched shRNA stable transfected panc-1 cells (right panel, lane 1). However, the expression of Notch-1(ICD) in the Panc-1CCN1KO cells can be recovered by treating the cells with Lactacystin for 24 h. Lactacystin-treated cells exhibit slightly more expression of Notch-1 (right panel, lane 4) as compared with the mismatched shRNA stable transfected panc-1 cells (right panel, lane 1). Lactacystin has no impact on CCN1 expression (left panel, lane 3). Based on the results, the studies indicate that CCN1 enhances the stability of Notch-1(ICD) by preventing the proteasomal degradation events in the pancreatic cancer cells.

**FIGURE 5. CCN1 inhibits proteasomal activity and Notch1 degradation.** A, proteasomal activity (chymotrypsin-like peptidase activity) in Panc1 cells under different treatment conditions was determined by the fluorogenic substrate Suc-LLVY-AMC using 20 S proteasome activity assay kit. Proteasomal activity is expressed in arbitrary fluorescence units (AFU). Error bars indicate 

* p < 0.005 versus mismatched shRNA; **, p < 0.001 versus CCN1 shRNA. B, representative Western blots show expression CCN1 (left panel) and Notch-1 (ICD) (right panel) in Panc-1 cells under different treatment conditions, histograms in the lower left panel and lower right panel show the CCN1 to β-actin ratio and Notch-1 to β-actin ratio, respectively. Error bars indicate ± S.D. of three independent experiments. * p < 0.005 versus mismatched shRNA; **, p < 0.001 versus mismatched shRNA; **, p < 0.001 versus CCN1 shRNA.
motility of MIA-PaCa-2CCN1KO cells in the presence or absence of CCN1 recombinant protein with or without cyclopamine (an inhibitor of SHh signaling). As expected, the addition of CCN1 recombinant protein in the culture media helped in recovering the motile behavior of MIA-PaCa-2CCN1KO cells parallel with the induction of SHh expression, which was noticeably absent due to the shRNA-mediated silencing of CCN1 (Fig. 7, upper panel). The increased motility of MIA-PaCa-2CCN1KO cells by recombinant CCN1 protein can be abrogated by concomitant treatment with cyclopamine without altering the expression of SHh (Fig. 7D, lower panel). Collectively, these findings suggest that CCN1 induced motility of pancreatic cancer cells is SHh signaling dependent.

DISCUSSION

The importance of SHh signaling in the development of pancreatic cancer and chemoresistance has fueled intensive study on this signaling molecule (48). Ample evidence indicates that
SHh signaling is one of the “underpinning” signaling pathways which is aberrantly overexpressed in virtually all PDAC and is associated with tumor growth, metastasis and less survival in a genetically engineered mouse model of pancreatic cancer and an orthotropic xenograft model. Additionally, the viability of pancreatic cancer stem cells is also dependant on sustained expression of SHh signaling. Thus, blockading the SHh signaling pathway with an inhibitor reduces pancreatic cancer growth and improves outcomes (19, 48). Recently, we found that CCN1 functions in a similar pathobiological role in pancreatic carcinogenesis, indicating that CCN1 signaling is critical for epithelial-mesenchymal transition and stemness and is required to promote tumor growth by the side population (cancer stem cells) of pancreatic cancer cells in a xenograft model (7). In our present work, we sought to unravel whether and how these two signaling complexes interact or associate to orchestrate the dynamic events linked with the development of pancreatic cancer. Our studies reveal that CCN1 is an upstream regulator of SHh, modulating SHh expression through the integrin αβ3-Notch-1 signaling pathway (Fig. 8).

These studies have highlighted that the expression levels of CCN1 and SHh are markedly higher in aggressive pancreatic cell lines (i.e. Panc-1, Hs766T, and MIA-PaCa-2) as compared with less aggressive cells (Fig. 1). The immunohistochemical studies established that the expressions of CCN1 and SHh were first detected in the PanIN1 stage, a precursor lesion (8), and both proteins are persistently expressed at higher levels as lesions progress to more advanced stages. Furthermore, these studies show that CCN1 tightly controls SHh signaling in pancreatic cancer cells. We demonstrated that silencing of CCN1 inhibits the expressions of SHh and its downstream signaling proteins and effectors in pancreatic cancer cells, while SHh inhibition exhibits no effect on CCN1 expression but minimizes the migration of pancreatic cancer cells (Figs. 2 and 3). Collectively, these studies suggest that SHh lies downstream of
CCN1 and therefore, CCN1 can be considered a required signaling molecule for the initiation and progression of pancreatic carcinogenesis and drug resistance since SHh acts at multiple stages during pancreatic carcinogenesis and increases drug resistance (10, 11, 14, 38).

Different laboratories including ours have characterized the side population (SP) of pancreatic cancer cells (7, 49–51). Like other tumor cell lines, the SP of pancreatic cancer cell lines was enriched with cancer stem cells, exhibited an elevated migratory feature (Fig. 3) and formed tumors in the xenograft model (7). Given the pathobiological importance of the SP, we compared the tumorigenic potency of the SP and NSP in the mouse xenograft and determined the status of CCN1 and SHh in these xenografts. As expected, the SP cells formed larger tumors more quickly as compared with NSP when injected 1 x 10⁶ cells/mouse for 45 days. Further, the SP xenograft highly expressed CCN1 and SHh and downstream signaling partners as compared with the NSP xenograft (Fig. 3). Collectively, these studies strengthen the above perception and demonstrate a plausible connection between CCN1 and SHh signaling.

Given the activation of SHh by CCN1 in pancreatic cancer cells, how does CCN1 regulate SHh to orchestrate pathobiological functions such as cellular motility/migration? We envision at least one potential scenario: the participation of active Notch-1, a regulator of pancreatic tumorigenesis (40), as SHh is activated by Notch-1 or participates in Notch-1 signaling pathway in different biological contexts (41, 52) and CCN1 activates the Notch-1 in pancreatic cancer cells (Fig. 5) (7). Additionally, without interacting with CCN1, the “Notch activation process” inhibitor DAPT (γ-secretase inhibitor) blocks SHh expression in pancreatic cancer cells (Fig. 4), suggesting a unique signaling map in pancreatic carcinogenesis in which CCN1 regulates SHh through active Notch-1. However, it is still uncertain how CCN1 activates Notch-1 in this scenario. It could be mediated by transcriptional or posttranscriptional, or even both mechanisms. The transcriptional regulation can be ruled out because our preliminary qPCR analysis indicates that the Notch-1 mRNA expression is not significantly decreased in CCN1 lacking Panc-1 cells as compared with CCN1 expressing Panc-1 cells (data not included). Thus, it could be mediated by a post-transcriptional/post-translational mechanism. The post-transcriptional/post-translational regulation of Notch-1 is a complex multi-step process. These include maturation, activation, and finally proteasomal degradation after the transcriptional regulation of target genes (43–45, 53, 55, 56). Our studies indicate that CCN1 inhibits the proteasomal degradation process to keep Notch-1 (ICD) stable and active in pancreatic cancer cells (Fig. 5). Notch-1 activation, which is a process of releasing an intracellular domain of Notch (ICD) from the membrane into the cytoplasm, is primarily mediated by complex interactions of receptor Notch and ligands (i.e. DLL4, Jagged1 and Jagged2) at the surface of adjacent cells accompanied by a proteolytic process (54). Based on the preliminary results, which demonstrated shRNA-based CCN1 silencing inhibits Jagged1 expression in pancreatic cancer cells (supplemental Fig. S2), we cannot rule out the possibility that CCN1-induced activation of Notch-1 could be mediated through the induction of Jagged1 expression in these cells. The hypothesis is now under investigation in our laboratory.

CCN1 promotes cell adhesion, migration, proliferation, apoptosis, and angiogenesis under specific environmental conditions. It exerts its functions primarily through direct binding to integrin receptors with different combinations. For example, CCN1 promotes cell survival through integrin αvβ3, but apoptosis is induced through αvβ1 (24). These reports persuaded us to investigate whether CCN1, to promote an invasive phenotype such as cell motility (Fig. 7), modulates Notch-1 followed by SHh through the direct binding with distinct integrin
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