Pancreatic stellate cells form a niche for cancer stem cells
niche and promote their self-renewal and invasiveness

Enza Lonardo, Javier Frias-Aldeguer, Patrick C. Hermann, Christopher Heeschen

Stem Cells & Cancer Group, Clinical Research Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain

Correspondence: Dr. Christopher Heeschen, MD, PhD, Stem Cells & Cancer Group, Clinical Research Programme, Spanish National Cancer Research Centre (CNIO), Madrid, Spain, E-mail: christopher.heeschen@cnio.es

Short title: Pancreatic stellate cells promote cancer stem cells
Abbreviations: PSC, pancreatic stellate cells; CSC, cancer stem cells; GEM, gemcitabine; CM, conditioned media; SB, SB431542; Alk, activin-like kinase
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ABSTRACT

Chronic pancreatitis and pancreatic ductal adenocarcinoma (PDAC) are characterized by extensive fibrosis. Importantly, in PDAC this results in poor vascularization and impaired drug delivery to the cancer cells. Therefore, the combined targeting of pancreatic tumor stroma and chemotherapy should enhance response rates, but the negative outcome of a recent phase III clinical trial for the combination of chemotherapy and hedgehog pathway inhibition suggests that other means also need to be considered. Emerging data indicate that elimination of cancer stem cells as the root of the cancer is of pivotal importance for efficient treatment of pancreatic cancer. Recently, we demonstrated in a highly relevant preclinical mouse model for primary pancreatic cancers that the combination of cancer stem cell-targeting strategies in combination with a stroma-targeting agent such as a hedgehog pathway inhibitor and chemotherapy results in significantly enhanced long-term and progression-free survival. Mechanistically, in the present study we now demonstrate that Nodal-expressing pancreatic stellate cells as an important component of the tumor stroma create a paracrine niche for pancreatic cancer stem cells. Secretion of the embryonic morphogens Nodal/Activin by pancreatic stellate cells promoted in vitro sphere formation and invasiveness of pancreatic cancer stem cells in an Alk4-dependent manner. These data imply that the pancreatic cancer stem cell phenotype is promoted by paracrine Nodal/Activin signaling at the tumor-stroma-interface. Therefore, targeting the tumor microenvironment is not only able to improve drug delivery, but even more importantly destroys the cancer stem cell niche and therefore should be an integral part of cancer stem cell-based treatment strategies.
Pancreatic ductal adenocarcinoma (PDAC) remains one of the most devastating cancers, and is the fourth leading cause of cancer death with a survival rate of less than 5%. Extensive fibrosis represents a hallmark of PDAC and may play a crucial role in the aggressive behavior of these carcinomas. One of the driving cellular components of this desmoplastic response are pancreatic stellate cells (PSCs), which have been identified as the principal source of excessive extracellular matrix production during chronic pancreatitis and pancreatic adenocarcinoma. PSCs are tissue resident cells, which can also be found in normal pancreas and exert two different stages. In their quiescent (non-activated) state, the cells reveal abundant cytoplasmatic fat droplets containing vitamin A. Once activated in response to pancreatic injury, PSCs lose their vitamin A stores and acquire a myofibroblast-like phenotype characterized by expression of alpha-smooth muscle actin (α-SMA) and production of various extracellular matrix proteins.

Moreover, recent evidence from our and other laboratories suggests that PDAC also harbors a distinct subpopulation of so called cancer stem cells (CSCs) defined by their ability to self-renew and exclusively promote in vivo tumor formation as well as metastasis. Most importantly, CSCs have been identified as the major player in the resistance toward conventional chemotherapy and radiotherapy. Specifically, the standard chemotherapeutic agent gemcitabine was not capable of eliminating pancreatic CSCs, but rather lead to a relative increase in the number of CSCs, indicating a preferential targeting of more differentiated cancer cells.

Members of the TGF-β family, such as TGF-β and Nodal/Activins, exert multiple, and sometimes opposing effects on a variety of cell types depending on the cellular context including the stage of the disease, the local environment, and the identity and the dosage of the ligand. Nodal and
Activins bind to the Activin-like type I and II receptors Alk4 and 7, while Cripto-1 constitutes an essential co-receptor for Nodal only. Nodal/Activins are secreted proteins, which are expressed during embryonic development and implicated in developmental processes like mesoderm formation and left-right axis specification. Moreover, Nodal and Activins are essential for maintaining the pluripotency of human embryonic stem cells.

Very recently, we have now demonstrated that Nodal/Activin signaling is also essential for the self-renewal capacity and stemness properties of pancreatic CSCs. Surprisingly, however, genetic targeting of Nodal in cancer stem cells reproducibly abrogated their self-renewal capacity \textit{in vitro}, but did not result in a similar abrogation of their \textit{in vivo} tumorigenicity. In contrast, genetic targeting of the Alk4 receptor consistently abrogated \textit{in vitro} sphere formation and \textit{in vivo} tumorigenicity of the CSCs. These data suggest that the stroma could function as a paracrine source of Nodal/Activin \textit{in vivo} bypassing the effect of Nodal knockdown in the CSCs itself. Therefore, a more thorough characterization of the interface between human PSCs as the driving component of pancreatic desmoplasia and CSCs should provide important insights into the pathophysiology of PDAC with potential important therapeutic implications.
RESULTS

Pancreatic stellate cells express components of the Nodal/Activin signaling cascade.

Immunohistochemistry for GFAP, an established marker for PSCs \(^{18}\) was utilized for the identification of PSCs in serial sections of pancreatic ductal adenocarcinoma. We found that GFAP was expressed in only a minor subset of stromal cells surrounding cancer cells and neoplastic ductal structures (Fig. 1A). Positive GFAP expression in stromal cells was found in all investigated tumors (n=4). Consistently, isolated PSCs also expressed GFAP as well as \(\alpha\)-SMA as a second, albeit unspecific marker of PSCs (Fig. 1B). Next, we demonstrated by qPCR that Nodal/Activin pathway-associated genes (Nodal, ActivinA, Gdf1, Smad2, Smad4, and Alk4) are expressed in PSCs (Fig. 2A). Western blot analysis confirmed that Nodal and ActivinA are indeed expressed in PSC at levels comparable to those observed in PDAC (Fig. 2B). Moreover, as determined by immunocytochemistry, we show that PSCs do not only express high levels of Nodal, but also express the co-receptor Cripto, which is essential for functional Nodal signaling. Interestingly the Nodal expression was restricted to intracellular vesicles, consistent with the ability of PSCs to secrete this protein and thus putatively interact with cancer stem cells in a paracrine fashion (Fig. 2C).

PSC-derived Nodal/Activin drive self-renewal and invasiveness of pancreatic cancer stem cells.

We have shown previously that primary pancreatic CSCs can be enriched \textit{in vitro} as anchorage-independent three-dimensional colonies, which are also termed spheres.\(^{8}\) These spheres are enriched for cells with stem cell-like properties including the ability to form secondary spheres as well as more differentiated progenies. Furthermore, we also reported the enrichment of pancreatic CSCs within the CD133\(^{+}\) expressing cell population as assessed by flow cytometry.\(^{8}\) Thus, in the present study, we used these two supplementary methods for functionally identifying pancreatic CSCs.
A total number of three human pancreatic adenocarcinoma xenografts were used with A6L and 185, being described earlier\textsuperscript{19,20} as well as 354, which was obtained using the same methodology. Importantly, for \textit{in vitro} experiments all cells were freshly isolated from early passage xenografts. Isolated cells from these xenografts were cultured in low passages as adherent cells (monolayer) or anchorage-independent spheres at low passages. Moreover, for some experiments, the established pancreatic cancer cell line L3.6pl was also used. Cells were phenotyped by flow cytometry for the expression of CD133, CD44, CXCR4, SSEA-4, and SSEA-1 (\textit{data not shown}). As previously reported, spheres are enriched in CD133+ cells and several other markers that have been associated with a CSC phenotype such as CXCR4, SSEA-4, and SSEA-1 as compared to adherent cells.\textsuperscript{17}

To evaluate the paracrine effects of PSC-derived Nodal/Activin on cancer cells and vice versa, we cultured the respective cells using identical media to collect respective conditioned media on day 3. Then, we incubated the corresponding cells (PSC or cancer cells) with the conditioned media for 24h. By qPCR we demonstrate that conditioned media conditioned by cancer cells slightly increased Nodal expression, but strongly enhanced ActivinA expression in PSCs (\textbf{Fig. 3A&B}). In contrast, conditioned media derived from PSC was able to only slightly enhance the expression of ActivinA in cancer cells (\textbf{Fig. 3C}).

Functionally, however, conditioned medium derived from PSCs was capable of significantly enhancing self-renewal capacity and invasiveness of pancreatic cancer cells. In the sphere formation assay, single cells in suspension were grown in presence of conditioned media, and showed a significantly increased number of spheres after 7 days as compared to cell exposed to control medium (\textbf{Fig. 4A}). Differences in the response between individual tumors are likely related to differences in endogenous Nodal/Activin secretion. Indeed, A6L is a tumor with very
strong Nodal expression and did not significantly respond to conditioned medium. Next, we investigated the influence of the conditioned media on the invasive capacity of pancreatic CSC. Matrigel™-coated modified Boyden chambers were used to quantitatively evaluate cell invasion. As shown in figure 4B, the percentage of transmigrated cells significantly increased following stimulation with conditioned media. Inhibition of the common Nodal/Activin receptors Alk4/7 by SB431542 abrogated the enhanced invasiveness of pretreated CSCs. These data indicate that Nodal/Activin secreted from PSC drives invasiveness of CSCs. Consistently, in the migration-based wound-healing assay, we observed that cells exposed to conditioned medium responded by enhanced migratory activity, which again was abolished in presence of SB431542 (n=3) (Fig. 4C).

**The hedgehog signaling inhibition targets pancreatic stellate cells.** The hedgehog signaling inhibitor CUR199691 significantly inhibited population doubling of PSCs (Fig. 5A), while adherent cancer cells remained mostly unaffected (data not shown). Mechanistically, we were able to show that treatment with CUR199691 did not alter the expression profile of PSC with respect to components of the Nodal/Activin pathway (Fig. 5B), but resulted in a significantly enhanced rate of apoptotic PSC (Fig. 5C). Histological evaluation of the tumors explanted after single agent therapy (gemcitabine) and triple combination therapy (gemcitabine, SB431542, and CUR199691) showed a marked depletion of tumor stroma in the triple therapy group. Here we now extend these data by showing that GFAP+ pancreatic stellate cells were also strikingly reduced in the triple treatment group as compared to tumors harvested from mice treated with gemcitabine alone (Fig. 5D). These data confirm in vivo that PSCs can indeed be targeted by inhibition of the hedgehog pathway and that the treatment effect of the triple combination treatment is at least in part due to elimination of PSCs.
DISCUSSION

Here we provide evidence for the existence of a specific cancer stem cell niche in pancreatic ductal adenocarcinoma formed by pancreatic stellate cells, an important component of the stromal tumor microenvironment. Nodal/Activin are secreted by pancreatic stellate cells and strongly promote the self-renewal capacity and invasiveness of primary pancreatic cancer stem cells. Therefore, targeting the stroma compartment of PDAC including pancreatic stellate cells does not only enhance drug delivery to highly malignant pancreatic cancer cells as shown previously, but also destroys the supportive niche formed by pancreatic stellate cells. Therefore, a triple combination treatment including a hedgehog pathway inhibitor for targeting pancreatic stellate cells and the dense stroma, an inhibitor of the Nodal/Activin pathway for directly targeting the self-renewal capacity of cancer stem cells as well as chemotherapy for elimination of the more differentiated progenies of the cancer stem cells represents a promising novel strategy for patients with pancreatic ductal adenocarcinoma.

Although the specific mechanism that determines the highly malignant growth and dissemination pattern as a defining feature of PDAC still remains heavily under-investigated territory, the aggressive behavior of pancreatic cancer cells has always been assumed to be linked to the tumor microenvironment. Pancreatic cancer cells including the distinct subpopulation of cancer stem cell are embedded in fibroblast-like cells as well as inflammatory cells and a non-cellular component consisting of dense extracellular matrix. This pancreatic fibrogenesis occurs in response to (chronic) injury in general and during carcinogenesis in particular. The desmoplastic reaction is at least in part responsible for the poor vascularization of PDACs, as its high density impairs vessel ingrowth, thus producing a hypoxic environment. Indeed, so far anti-angiogenic therapies have essentially failed in PDAC. Since hypoxia has been shown to play an important role in the resistance to chemotherapy and promotion of stemness, the hypoxic environment
of PDACs may promote cancer stem cells directly, but also indirectly by hypoxia- as well as injury-induced activation of the stroma.26

Importantly, the most crucial functional component of the tumor microenvironment is represented by the pancreatic stellate cells. Pancreatic stellate cells are localized around the ducts of the normal pancreas when PSC are mostly maintained in a quiescent/non-secreting state. But these cells become highly activated in response to pancreatic injury and/or in response to the secretion of paracrine factors such as TGF-b, fibrinogen,27 or sonic hedgehog28. PSC have clearly been demonstrated to play a key role in PDAC-associated fibrogenesis by secreting components of the extracellular matrix4,29 and formation of the tumor-associated stroma. Importantly, soluble factors secreted by activated PSCs also promote proliferation and migration of cancer cell.30-32

Previously, we have shown that the Nodal/Activin pathway is essential for the self-renewal capacity and stemness of pancreatic cancer stem cells and therefore represents a valid and novel therapeutic target. Subsequent studies in mice transplanted with primary human pancreatic cancer cells demonstrated that the inherited cancer stem cell compartment is severely altered by inhibition of this pathway resulting in chemo-sensitization of the cancer stem cells, which then can be eliminated by standard chemotherapy resulting in significantly increased progression-free survival. However, as the two hallmarks of this disease are also poor vascularization and massive stroma content, drug delivery has always been a major challenge in PDAC. Indeed, the stroma represents a key physical barrier that separates the cancer (stem) cells from the non-tumor tissue and therefore has been described as a protective embedding of the cancer cells resulting in treatment resistance.33 Importantly, clinically relevant stroma formation is regularly absent or at least of only minor extend in these mouse models generated by xenografting of isolated cancer cells. In contrast, xenografting of primary human tumour tissue pieces represents a much better preclinical model of human pancreatic cancer based on abundant stroma content.34 Consistent
with this notion, subsequent \textit{in vivo} testing of this new treatment modality targeting the Nodal/Activin pathway in mice with xenografted primary cancer tissue did not result in similar treatment responses as compared to data derived from models with xenografted singularized cells.\textsuperscript{14}

It was then demonstrated that the concentrations of the Nodal/Activin pathway inhibitor in the transplanted primary tumor tissue was indeed insufficient for effectively blocking this pathway. Intriguingly, this hurdle could be overcome by simultaneous targeting of the sonic hedgehog pathway as a crucial signaling component for pancreatic stellate cells and other stroma cells. The resulting triple therapy containing a Nodal/Activin inhibitor, a hedgehog pathway inhibitor, and a chemotherapeutic agent resulted in long-term survival of all mice, while mice receiving only the chemotherapeutic agent had to be sacrificed within a few weeks due to rapid tumor progression.

Still, a puzzling finding of these comprehensive studies remained that knockdown of Nodal in the cancer stem cell compartment alone did only translate into reduced sphere formation in vitro, but \textit{in vivo} tumorigenicity remained mostly unaffected. Since only the knockdown of the common receptor Alk4 resulted in strong reduction of \textit{in vivo} tumorigenicity, these data actually suggest that \textit{in vivo} CSCs are most likely stimulated both in an autocrine and a paracrine fashion by the stromal compartment. In fact, here we now provide strong evidence that Nodal/Activin is not only produced and secreted by pancreatic cancer stem cells in an autocrine fashion, but also by pancreatic stellate cells, which are abundantly present in the stroma surrounding the pancreatic cancer stem cells. Our data suggest that the stroma does not only protect the embedded cancer cells from systemically administered anti-cancer drugs, but that it also contains functionally active components such as pancreatic stellate cells, which are capable of promoting the self-renewal capacity and the invasiveness of cancer stem cells. Therefore, our data implicate that
pancreatic stellate cells serve as a supportive niche for cancer stem cells protecting and supporting these highly tumorigenic cells.

These combined studies led us to conclude that targeting of the Nodal/Activin pathway does indeed represent a previously unknown and highly efficient modality for stalling progression and metastasis in pancreatic cancer. If combined with efficient depletion of the stroma to destroy the tumor microenvironment including the pancreatic stellate cells and to allow better accessibility of the cancer (stem) cells for the administered drugs, Nodal/Activin inhibitors are capable of dramatically improving the outcome in clinically most relevant human tissue xenograft models. Future studies now will have to validate these findings and should determine the general applicability of this treatment regimen using a large set of primary tumors. This will also help to address the question of which patients will most likely respond to this treatment modality and how best to identify them.
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MATERIALS AND METHODS

**Primary human pancreatic cancer cells.** Human pancreatic tumors were obtained with written informed consent from all patients. For in vitro studies, tissue fragments were minced, enzymatically digested with collagenase (Stem Cell Technologies, Vancouver, BC) for 90 min at 37°C and after centrifugation for 5 min at 1200 rpm the pellets were resuspended and cultured in RPMI, 10% FBS and 50 units/ml penicillin/streptomycin.

Pancreatic cancer spheres were generated and expanded in DMEM-F12 (Invitrogen, Karlsruhe, Germany) supplemented with B-27 (Gibco, Karlsruhe, Germany) and bFGF (PeproTech EC, London, UK). 10,000 cells/ml were seeded in ultra-low attachment plates (Corning B.V., Schiphol-Rijk, Netherlands) as described previously. After 7 days of incubation, spheres were typically > 75μm large with ~97% CD133 high. For serial passaging, 7-day-old spheres were harvested using 40 μm cell strainers, dissociated to single cells with trypsin, and then re-grown for 7 days. Cultures were kept no longer than 4 weeks after recovery from frozen stocks (passage 3-4).

**Human pancreatic cancer cell line.** The human pancreatic cancer cell lines L3.6pl was maintained as previously described.

**Human pancreatic stellate cell and conditioned media.** RLT-PSCs were maintained using RPMI or DMEM/F12 media supplemented with 1 or 2% FBS and 50 units/ml penicillin/streptomycin and fungizone. PSC conditioned media was collected three days after incubation with PSCs, centrifuged and filtered prior to incubation with primary cancer cells.

**AnnexinV assay.** PSCs were cultured in 10μM CUR199691 containing media for 48h and 72h. Attached and floating cells were collected, resuspended and stained with Annexin V (550474; BD Bioscience) after incubation with Annexin V binding buffer (556454, BD Pharmingen). Cells were then incubated with DAPI. Samples were analyzed by flow cytometry using a FACS Canto II (BD) and data were analyzed using FlowJo 9.2 Software (Ashland, OR).

**In vivo treatment of established pancreatic cancers.** Two mm³ pieces of primary, in vivo expanded pancreatic cancer tissue were implanted subcutaneously or orthotopically into the pancreas of female nude mice (Harlan Europe), and mice were randomized to the respective treatment groups. Size and weight of the
pancreatic tumors were monitored. Gemcitabine was administered twice a week (125 mg/kg i.p.).
SB431542 was used at 25 mg/kg and CUR199691 at 100 mg/kg, both by oral gavages twice daily for three weeks.

**Immunofluorescence and Immunohistochemistry.** Pancreatic stellate cells were seeded in 96-well dishes (Corning, NY) and incubated at 37°C for 3 hr. Cells were washed with cold PBS and then fixed with pre-chilled 4% PFA for 20 min at room temperature. After blocking with 1% bovine serum albumin in PBS cells were incubated with primary antibodies: Cripto (ab19917; Abcam, Inc.), Nodal (ab55676; Abcam, Inc.), α-Smooth Muscle Actin (A2547, Sigma-Aldrich), anti-GFAP (Z 0334; Dako) overnight at 4°C in the dark. Cells were then washed three times with PBS-Tween 0.1% and incubated with Alexa-Fluor-conjugated antibodies against mouse or rabbit (Invitrogen) at room temperature in the dark. Cells were mounted using Prolong Gold containing DAPI (Invitrogen) and analyzed using an SP5 confocal microscope (Leica, Heidelberg, Germany). Tumor xenografts were fixed in paraffin blocks. Tissue sections were analyzed for the presence of PSC using rabbit antibody anti-GFAP.

Western Blot Analysis. PVDF membranes containing electrophoretically separated proteins from human pancreatic stellate cells were probed with mouse antibodies against Nodal (ab55676; Abcam, Inc.) and ActivinA (ab89307; Abcam, Inc.) treated with peroxidase-conjugated sheep anti-mouse Ig secondary antibody (GE Healthcare) and then visualized by enhanced chemoluminiscence (Amersham).

**RNA preparation and RT-PCR.** Total RNAs from human primary pancreatic cancer cells and spheres were extracted with TRIzol kit (Life Technologies Inc.) according to the manufacturer’s instructions. One microgram of total RNA was used for cDNA synthesis with SuperScript II reverse transcriptase (Life Technologies Inc.) and random hexamers. Quantitative real-time PCR was performed using SYBR Green PCR master mix (QIAGEN), according to the manufacturer’s instructions. The list of utilized primers is depicted in Table 1.

**Invasion assays.** Invasion assays were performed using modified Boyden chambers filled with MatrigelTM (BioCoat, BD Biosciences, Heidelberg, Germany). Adherent and sphere culture derived cells were used for this experiment. Cells were added to the MatrigelTM-coated inserts and 750 µL of serum-free or conditioned media was added to the lower chamber. The assay chambers were incubated for 24h at 37°C.
Invasive cells were fixed in 4% PFA and stained with DAPI using Prolong Gold (Invitrogen). The number of invaded cells was compared to control.

**Wound healing assay.** Confluent cultures of primary cancer cells were scratched using a 200μl pipette tip. Cells were then incubated at 37°C with or without conditioned media in the presence or absence of 20μM of SB431542. The extent of migration was evaluated 24 hours later.

Proliferation assay. 10^4 cancer cells per well were plated in 24-multiwell plates in 10% FBS RPMI. 24h later, 3 days PSC-conditioned media was collected centrifuged, filtered and added to cultured cancer cells. Cell populations were measured at 0, 24, 48 and 72h.

**Sonic Hedgehog inhibition.** 5x10^3 cells per well were plated in 24-multiwell plates and incubated with 10% FBS RPMI. 24h after plating the cells, media was changed to 10% FBS RPMI containing 10μM CUR199691 dissolved in DMSO or DMSO only. Cell populations were quantified at 0, 24, 48 and 72h.

Statistical analyses. Results for continuous variables are presented as means ± standard deviation (SD) unless stated otherwise. Treatment groups were compared with the independent samples t test. Pair-wise multiple comparisons were performed with the one-way ANOVA (two-sided) with Bonferroni adjustment. P values < 0.05 were considered statistically significant. All analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, Illinois).
FIGURE LEGENDS

Figure 1: Pancreatic stellate cells are presents in pancreatic ductal adenocarcinoma. Formalin-fixed, paraffin-embedded human pancreatic cancer xenografts were stained for GFAP (brown) (A). Immunofluorescence for GFAP (green), α-SMA (red), and DAPI nuclear stain (blue) in PSC freshly isolated from pancreatic tumors (B) and immortalized and expanded PSC (C).

Figure 2: Nodal signaling components are expressed in pancreatic stellate cells. Expression profile of genes involved in the Nodal signaling in PSC and in pancreatic cancer cells (A). Western Blot analysis for Nodal and ActivinA protein in PSCs as compared to pancreatic tumors (185 and A6L) (B). Immunocytochemistry for Nodal (green) and its co-receptor Cripto (red) in PSCs (C).

Figure 3: Bidirectional communication between PSCs and pancreatic cancer cells. Schematic representation of conditioned media production by exposition to PSC (PSC-cond. medium) or cancer cells (CC-cond. medium) (A). qPCR analysis for Nodal and ActivinA mRNA expression in PSCs cultured in CC-conditioned medium as compared to control medium (B). qPCR analysis for Nodal and ActivinA mRNA expression in pancreatic cancer cells cultured in PSC-conditioned medium as compared to control medium (C).

Figure 4: Paracrine Nodal signals from pancreatic stellate cells promote cancer stem cell function. Sphere formation capacity of cancer stem cells isolated from different tumors where incubated with PSC-conditioned medium as compared to control medium (A). Invasion of sphere derived from A6L, 185, 354 and L3.6pl cancer stem cells in control medium or PSC-conditioned medium in the presence or absence of the Alk4/7 inhibitor SB431542 (B). Representative images
of wound healing assays showing migrated primary pancreatic cancer cells exposed to the indicated media in the presence or absence of SB431542 (C; upper panel). Quantification of the migratory activity (lower panel).

**Figure 5:** Inhibition of the sonic hedgehog pathway targets pancreatic stellate cells. Proliferation of PSC following exposure to 10μM CUR199691 as compared to vehicle (A). Changes in mRNA expression of Noda/Activin pathway components (B). Cytometry analysis for apoptotic cells by double-staining for Annexin V and DAPI (C). Detection of PSCs in pancreatic tumors treated with gemcitabine (Gem) alone or triple combination therapy (SB, SB431542; CUR, CUR199691) as evidenced by immunohistochemistry for GFAP as a marker for PSC (D).

**Figure 6:** Integrative concept of Nodal/Activin in pancreatic cancer stem cells. Autocrine Nodal/Activin signaling can be blocked in cancer stem cells by the Alk4/7 inhibitor SB431542 *in vitro*. In the presence of pancreatic stellate cells as a crucial component of the tumor microenvironment, it is required to simultaneously target these cells for better efficiency as these cells produce high amounts of Nodal / Activin and create a supportive niche for cancer stem cells. Importantly, in return cancer stem cells also promote PSC by secreting sonic hedgehog. This paracrine stimulation of pancreatic stellate cells can be inhibited by hedgehog pathway inhibitors such as the smoothened inhibitor CUR199691. For irreversible ablation of cancer stem cells it is still mandatory to additionally treat the cells with a cytotoxic drug such as Gemcitabine leading to in a multimodal triple combination therapy.
REFERENCES


Figure 1 – Lonardo et al.

A

A6L tumor

185 tumor

C+  C-

B

15 µm  15 µm  15 µm

α-SMA  GFAP  DAPI

C

GFAP  Hematoxylin

GFAP  DAPI

α-SMA  DAPI
Figure 2 – Lonardo et al.

A

- Graph showing protein expression levels
  - x-axis: Nodal, Activin, Gdf1, Smad2, Smad4, Alk4
  - y-axis: %PC (10^3)

B

- Table showing protein bands
  - PSC
  - PDAC
  - Proteins: Activin A, Nodal, GAPDH
  - Molecular weights: 56Kd, 40Kd, 37Kd

C

- Images of cellular staining
  - Nodal DAPI
  - Cripto DAPI
  - Scale bars: 25 µm
Figure 3 – Lonardo et al.

A

Conditioned medium
Respective cells

B

\[ 2 \cdot 10^{-3} \]

Control
CC-cond. medium

P<0.05

Nodal ActivinA

p=0.11

C

\[ 2 \cdot 10^{-3} \]

Control
PSC-cond. medium

Nodal ActivinA
Figure 4 – Lonardo et al.

A

Number of spheres

\[ \begin{array}{cccc}
A6L & 185 & 354 & L3.6pl \\
\text{Control} & \text{PSC-cond. medium} & \text{PSC-cond. medium} & \text{PSC-cond. medium}
\end{array} \]

\* p<0.05 vs Control

B

% Invasion

\[ \begin{array}{cccc}
A6Ls & 185s & 354s & L3.6pl \\
\text{Control} & \text{PSC-cond. medium} & \text{PSC-cond. medium} & \text{PSC-cond. medium} + \text{SB431542}
\end{array} \]

\* p<0.05 vs Control

\** p<0.05 vs PSC-c.m.

C

24h

Migration in µm

\[ \begin{array}{cccc}
0h & \text{Control} & \text{PSC-cond. medium} & \text{Control} + \text{SB} & \text{PSC-c.m.} + \text{SB}
\end{array} \]

\* p<0.05 vs Control

\** p<0.05 vs PSC-c.m.
Figure 5 – Lonardo et al.

A

![Graph showing the number of cells over time with control and CUR199691 treatments. The graph indicates a significant difference (*p<0.005 vs Control) between the two treatments.]

B

![Bar graph showing fold change in expression for various factors with CUR199691 treatment. The graph indicates that CUR199691 has a significant effect on the expression of these factors.]

C

![Flow cytometry plots for DMSO and CUR199691 treatments at 48h and 72h, showing annexin V expression levels.]

D

![Immunohistochemistry images for GFAP and Hematoxylin staining in 185 Gem and 185 Gem+SB+CUR conditions.]
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