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Effect of *NR5A2* inhibition on pancreatic cancer stem cell (CSC) properties and epithelial-mesenchymal transition (EMT) markers

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Abstract

NR5A2 (aka *LRH-1*) has been identified as a pancreatic cancer susceptibility gene with missing biological link. This study aims to demonstrate expression and potential role of *NR5A2* in pancreatic cancer. *NR5A2* expression was quantified in resected pancreatic ductal adenocarcinomas and the normal adjacent tissues of 134 patients by immunohistochemistry. The intensity and extent of *NR5A2* staining was quantified and analyzed in association with overall survival (OS). The impact of *NR5A2* knockdown on pancreatic cancer stem cell (CSC) properties and epithelial-mesenchymal transition (EMT) markers was examined in cancer cells using RT-PCR and Western Blot. *NR5A2* was overexpressed in pancreatic tumors, the IHC-staining H score (mean±SE) was 96.4±8.3 in normal versus 137.9±8.2 in tumor tissues ($P<0.0001$). Patients with a higher *NR5A2* expression had a median survival time 18.4 months compared to 23.7 months for those with low IHC H scores ($P=0.019$). The hazard ratio of death (95% confidence interval) was 1.60 (1.07- 2.41) after adjusting for disease stage and tumor grade ($P=0.023$). *NR5A2* was highly expressed in pancreatic cancer sphere forming cells. *NR5A2*-inhibition by siRNA was associated with reduced sphere formation and decreased levels of CSCs markers *NANOG*, *OCT4*, *LIN28B* and *NOTCH1*. *NR5A2* knockdown also resulted in reduced expression of *FGB*, *MMP2*, *MMP3*, *MMMP9*, *SNAIL*, and *TWIST*, increased expression of epithelial markers E-cadherin and β -catenin, and a lower expression of mesenchymal marker Vimentin. Taken together, our findings suggest that *NR5A2* could play a role in CSC stemness and EMT in pancreatic cancer, which may contribute to the worse clinical outcome.

Keywords

Expression; Survival; Stem cell-like cancer cell; EMT

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Introduction

Recent genome-wide association studies have identified *NR5A2* (*nuclear receptor subfamily 5 group A member 2*, aka *Liver receptor homologue-1*) as a pancreatic cancer susceptibility gene [1]. *NR5A2* belongs to the orphan nuclear receptor superfamily, and plays vital roles in early development, cholesterol homeostasis, steroidogenesis and certain diseases, including cancer [2]. *NR5A2* has been linked with colon and gastric cancer through a synergistic action with β -catenin in regulating cell cycle and cell proliferation [3-5]. *NR5A2* may also play a role in breast cancer through regulation of in situ steroidogenesis [6]. Over-expression of *NR5A2* at both mRNA and protein levels have been reported in human pancreatic ductal adenocarcinoma (PDAC) [7]. Some studies in cell lines and in animal models have shown that over-expression of *NR5A2* promotes cell proliferation and tumor metastasis in pancreatic cancer [7,8]. However, another study reported that *NR5A2* was actually required to restore pancreatic homeostasis upon damage and to suppress *KRAS*-driven intraepithelial neoplasia progression as a tumor suppressor [9]. In the current study we examined the expression of *NR5A2* in tissues samples from PDAC patients in association with tumor characteristics and overall survival. We further investigated the possible molecular mechanisms that linking *NR5A2* with PDAC.

Emerging evidence suggests that most solid tumors may arise from cancer stem cells (CSCs) and CSCs have been identified in pancreatic cancer [10]. CSCs have the property of self-renewal, the ability to produce differentiated progeny, and increased expression of the developmental signaling molecules. *NR5A2* is expressed in embryonic stem (ES) cells, and maintains undifferentiated ES cells by controlling expression of two transcription factors, *OCT4* and *NANOG*. Somatic cells can be reprogrammed to induced pluripotent stem cells (iPSCs) with the introduction of *OCT4* (*organic cation/carnitine transporter4*), *SOX2* (*sex determining region Y-box 2*), *KLF4* (*Kruppel like factor 4*), and *c-MYC* (*v-myc avian myelocytomatosis viral oncogene homolog*) [11]. Several studies have demonstrated that *NR5A2* can substitute for *OCT4* in derivation of iPSCs and maintaining pluripotency [12,13]. These observations suggest that *NR5A2* may contribute to cancer development through a potential role in regulating CSCs. Yet, no study has addressed the effect of *NR5A2* on pancreatic cancer cells from this perspective.

CSCs have also been linked to epithelial-to-mesenchymal transitions (EMT) in various solid tumors including PDAC [14,15]. Cancer cells that undergo EMT and acquire stem cell-like features are believed to prelude metastasis [16,17]. Indeed, circulating pancreatic cancer cells underwent EMT prior to dissemination in a genetically engineered mouse model [18]. Interestingly, *NR5A2* has been linked to EMT in breast cancer and over-expression of *NR5A2* promotes breast cancer motility and invasion by regulation of E-cadherin and MMP9 (matrix metalloproteinase 9) [19]. Therefore, *NR5A2* could be a key protein mediating properties shared by CSCs and EMT. The current study explored the role of *NR5A2* in regulating CSC properties and EMT markers of pancreatic carcinoma cells.

Materials and Methods

Cell lines and Human Tissues

Human pancreatic adenocarcinoma cell lines AsPC-1, Panc-1, MiaPaCa-2, Hs766T, and BxPC-3 cells were purchased from the American Type Culture Collection and cultured as described in their product information sheets. Colo357 and its fast growing (FG) subline, as well as the immortalized normal human pancreatic ductal epithelial (HPDE) cell line were gifts from Dr. Craig D. Logsdon (MD Anderson Cancer Center, Houston, TX) [20,21]. All cell lines have been authenticated by testing 14 polymorphic markers. Cancer cells were cultured in RPMI 1640 medium or Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. HPDE cell was maintained in keratinocyte serum-free medium supplemented with epidermal growth factor and bovine pituitary extract.

A tissue array of 134 pairs of surgically resected PDAC tumor tissues and their adjacent non-tumor tissues were obtained from MD Anderson Tissue Bank for immunohistochemistry. All tissue samples used in this study were residual surgical samples from patients undergoing tumor resection without pre-operative treatment at MD Anderson Cancer Center. A written informed consent was signed by each patient and the study was approved by the Institutional Review Board of MD Anderson.

Immunohistochemistry (IHC)

After deparaffinization, tissue sections were subjected to antigen retrieval and endogenous peroxidase activity blocking. The primary antibody used was rabbit NR5A2 antibody from Sigma Life Science (St. Louis, MO) at a dilution of 1:400. After treatment with the biotinylated secondary antibody, the antibody complex was detected using an avidin-biotin-peroxidase complex solution and visualized using 3,3'-diaminobenzidine (Zymed Laboratories, Inc., San Francisco, CA). A negative control was run in each experiment using a rabbit IgG Isotype (Santa Cruz). Images were evaluated by an experienced pathologist for staining patterns in different types of pancreatic cells, different cellular components, as well as in tumor and normal tissues. The staining intensity was scored as 0 for negative, 1 for weak, 2 for intermediate, and 3 for strong staining. The percentage of cells with positive staining were scored as 0 for none, 1 for 1-25%, 2 for 25-50%, and 3 for 50-100%. The final staining H score was the product of the intensity score and the percentage score. The difference in staining scores of the non-tumor and tumorous tissues was compared by paired *t* test.

Sphere Formation Assay

Sphere formation assay was conducted to assess the self-renewal capacity of CSC. Single cell suspensions of AsPC-1 and Panc-1 cells transfected with *NR5A2* or control siRNA were plated on ultra-low adherent wells of 6-well plate (Corning Life Sciences, Tewksbury, MA) at a density of 10^4 cells/ml in DMEM/F12 (Invitrogen, Carlsbad, CA) supplemented with B27 and N2 (Life Technology, Grand Island, NY). Single cell status was confirmed under microscope. Fresh medium was added every 2–3 days. After 6–7 days, the spheres termed as “pancreatospheres” were collected by centrifugation (300 ×g, 5 minutes). The sphere formation assay of secondary pancreatospheres was conducted by using primary

pancreatospheres. Briefly, primary pancreatospheres were harvested and incubated with Accutase (Sigma Life Science, St. Louis, MO) at 37°C for 5 to 10 minutes. Single-cell suspensions of pancreato-spheres were plated at 2000 cells per well in the sphere formation medium. Following 1-week incubation secondary pancreatospheres were collected by centrifugation as described above.

SiRNA Blocking

Small interfering RNA (siRNA) transfections were performed using siPORT™ NeoFX™ Transfection Reagent according to the manufacturer's protocol (Life Technologies, Grand Island, NY). Briefly, in this reverse transfection, adherent cells of AsPC-1 and PANC-1 were trypsinized, suspended and diluted in DMEM/F12 growth medium supplemented with B27 and N2. Next, siPORT™ NeoFX™ Transfection Agent with a negative siRNA control (si control) or siRNA directed against *NR5A2* (siRNA1 or siRNA2) were complexed, and dispensed into ultra-low adherent six-well plate or flask. At last, cell suspensions were overlaid onto the transfection complexes and mixed by gently tilt the plate or flask. The optimal concentration of siRNA for transfection was 10 nM and the final density of suspension cells were 10^4 cells/ml. Two different siRNA duplexes targeting NR5A2 (NM_003822) were tested. The sequences of the siRNAs are as follows: 5'-GGAAGGAAUAAGUUUGGGCtt-3' (siRNA1), 5'-GGUUGAUGACCAAAUGAAGtt-3' (siRNA2). After the transfections, cells were incubated and the medium was replaced every two days by carefully aspirating the old medium and adding fresh medium. Pancreatic CSCs were prepared for analyses at 6-9 days after transfection.

RNA extraction, cDNA Synthesis, and Real time PCR

Total RNA was isolated from the cells using Trizol reagent according to manufacturer's protocol (Ambion, Austin, TX). Complementary DNA was synthesized from 1.0 µg of total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Quantitative RT-PCR (qRT-PCR) was performed in triplicate samples using predesigned primers and probe sets (Hs00167041-m1, Life Technologies, Grand Island, NY). The primer sequences are available in Supplementary Tables 1 and 2. The RT-PCR results were first normalized to the threshold cycle (Ct) of GAPDH, referred to as Ct. The fold change in expression of genes in the test group compared to that in the control group was expressed as $2^{-\Delta Ct}$, in which ΔCt equals the Ct of the test group minus the Ct of the control group, which was normalized to 1.

Western Blotting

Cells were lysed with cell lysis buffer (Cell Signaling Technologies, Danvers, MA). Cleared lysates were fractionated by polyacrylamide gel electrophoresis and transferred to a Trans-Blot® Turbo™ Mini PVDF membrane using a Trans-Blot Turbo transfer system (Bio-Rad Laboratories, Hercules, CA). The membrane was incubated overnight at 4°C with primary antibodies, which were used at the following working dilutions: NR5A2 (1:1000 dilution, Santa Cruz; 1:3000 dilution, LS-C139026, Abcam, Cambridge, MA), CD44 (CD44 molecule), EPCAM (epithelial cell adhesion molecule), NANOG (Nanog homeobox), OCT4, NOTCH1, EZH2 (enhancer of zeste 2 polycomb repressive complex 2 subunit) and β-Catenin (1:1000 dilution, Cell Signaling Technologies, Beverly, MA); E-Cadherin and

Vimentin (1:1000 dilution, BD, San Jose, CA). Beta-actin at 1:3000 dilutions was used as the loading control. After incubation with appropriate secondary antibodies conjugated to horseradish peroxidase, the membranes were exposed to ECL Western Blotting detection reagent. Membranes were stripped for 30 minutes at 55°C in a buffer containing 2% SDS, 62.5 mM Tris (pH6.7), and 100 mM 2-mercaptoethanol for staining of multiple proteins. The results were quantified by densitometric analysis using the Image-Quant software.

Flow Cytometry Analysis

The CSC markers were measured by flow cytometry using the following FITC antibodies: mouse anti-human CD24 Clone ML5 (cat# 560992) and mouse anti-human CD44 Clone G44-26 (cat# 555478) from eBioscience (San Diego, CA), anti-human c-MET (HGF receptor) (cat#11-8858-42) and anti-ALDH1A1 (aldehyde dehydrogenase 1 family member A1, cat# mAbcam61293) from Abcam (Cambridge, MA). Alexa Fluor® 488 Mouse IgG1 κ Isotype Control (cat# 557721, eBioscience) was used as control.

Cells were suspended and washed with Stain Buffer and then pelleted with centrifugation. The cell pellets were re-suspended with cold Stain Buffer to a final concentration of 2×10^7 cells/mL. Non-specific Fc-mediated interaction was blocked with Fc Block at a concentration of 2.5 μ g/ 10^6 cells. Incubation with antibodies was on ice and protected from light for 75 minutes. Cells were then washed, pelleted and re-suspended in 0.5 mL of Stain Buffer. Flow cytometry was performed in duplicates for each marker using BCI Gallios flow cytometry and FCS Express Software (Beckman Coulter Life Science, Indianapolis, IN). The average number ($\pm$ SD) of two runs was presented in the data analysis.

Results

Expression of *NR5A2* in pancreatic cancer cells and tumor tissues

Using quantitative RT-PCR analyses, we showed that *NR5A2* mRNA was overexpressed in several pancreatic cancer cell lines (Fig. 1A). The highest level of *NR5A2* expression was detected in AsPC-1, followed by Panc-1, Hs766T and MIAPaCa-2 cells. Western blot analysis showed consistency in the protein and mRNA expression levels, i.e. a higher expression of *NR5A2* protein in AsPC-1, PANC-1 and Hs766T and MIAPaCa-2 cells (Fig. 1B).

IHC analysis revealed that *NR5A2* was overexpressed in pancreatic tumors compared to normal adjacent tissues (Fig. 2, upper panels). Cytoplasmic and nuclear staining for *NR5A2* was observed in 70.9% and 14.9% of the normal adjacent tissues and 88% and 3% of the tumor tissues, respectively (Supplementary Table 3). The total IHC staining score for both cytoplasmic and nuclear staining (mean $\pm$ SE) was 96.4 $\pm$ 8.3 versus 137.9 $\pm$ 8.2 in normal versus tumor tissues ($P<0.0001$). Furthermore, patients with a higher *NR5A2* expression in the tumors had a significantly reduced OS. Using the median value of the total IHC score as the cutoff, the median survival time was 19.4 vs 23.7 months for those with a high or low IHC score, respectively ($P=0.021$, Figure 2, lower panel). The hazard ratio of death (95% confidence interval) was 1.61 (1.07- 2.42) after adjusting for disease stage and tumor grade

($P=0.023$). The IHC score was not significantly related to tumor grade, disease stage, or any other clinical characteristics of the study population (data not shown).

Characterization of CSCs in pancreatic cancer cell lines

To investigate the potential role of *NR5A2* in pancreatic cancer, we conducted sphere formation assay and examined the impact of *NR5A2* knock-down on CSC properties. We successfully obtained spheres in cell lines (AsPC-1 and PANC-1) that expressed higher levels of *NR5A2* (Fig. 3A). Importantly, spheres formed by AsPC-1 and PANC-1 could be serially passaged to form secondary (also referred as P2) and tertiary (P3) spheres (data not shown).

Using qRT-PCR method, we found a 1.30 to 12.83-fold higher mRNA levels of the transcription factors *OCT4*, *NANOG*, *LIN28B* (*lin-28 homolog B*), *EED* (*embryonic ectoderm development*), *EZH2* (*enhancer of zeste 2 polycomb repressive complex 2 subunit*), *HEY1* (*hes related family bHLH transcription factor with YRPW motif 1*), *SALL2* (*spalt like transcription factor 2*), *SOX17* (*SRY-box 17*) and *NR5A2* in sphere cells than in control adherent cells (Fig. 3B). Western blot analysis also demonstrated a significantly higher level of protein expressions of CD44, EPCAM, NANOG, OCT4 and *NR5A2* in spheres compared with their corresponding adherent cells (Fig. 3C and 3D).

Flow cytometry analysis showed a significantly increased expression of CSC markers in sphere-forming cells (Fig. 3E). CD24 was expressed in $51.0 \pm 0.03\%$ (mean $\pm$ SD) of adherent and $92.5 \pm 9.9\%$ sphere-forming AsPC-1 cells. Similarly, CD44 was expressed in $44.1 \pm 5.4\%$ vs $94.8 \pm 6.6\%$, cMET in $37.4 \pm 7.2\%$ vs $93.2 \pm 9.6\%$, and ALDH in $43.3 \pm 8.4\%$ vs $91.9 \pm 11.8\%$ of the adherent and sphere-forming cells, respectively.

Blocking *NR5A2* by specific siRNA inhibited sphere formation

Next we investigated the impact of *NR5A2* specific siRNA on sphere formation and gene expression at the transcript and protein levels in pancreatic CSCs. Two different siRNAs targeting *NR5A2* (siRNA1 and siRNA2) were transfected into AsPC-1 and PANC-1 cells and cells were harvested at 2, 3, 6, 7, 8 and 9 days after the transfection. The maximum inhibition of *NR5A2* expression was detected at 7 days at the mRNA level and at 9 days at the protein level (data not shown). *NR5A2* knockdown reduced the number of sphere formation (Fig. 4A). The *NR5A2* mRNA expression was inhibited by the two siRNAs by $76.07 \pm 5.22\%$ and $70.10 \pm 7.10\%$ in AsPC-1 CSCs, and $76.68 \pm 4.36\%$ and $66.80 \pm 3.82\%$ in PANC-1 CSCs (Fig. 4B). Consistently, Western blot analysis showed substantially reduced expression of *NR5A2* protein in CSCs treated with specific siRNAs compared to that in cells treated with negative control siRNA (Fig. 4C). Because siRNA1 showed a higher efficiency in inhibiting the *NR5A2* expression than siRNA2, we chose the former for the following experiments.

Knockdown of *NR5A2* reduced the expression of pancreatic CSCs markers

Given the essential role of *NR5A2* in sphere formation, we further tested the impact of *NR5A2* knockdown on pancreatic CSC markers. Using the qRT-PCR method, we found that *NR5A2* knockdown significantly down-regulated the expression of CSC markers, such as

transcription factors *Sox2*, *NANOG*, *OCT4* and *Lin28B* (Fig. 5A and 5B). Concomitantly, the expression of downstream targets of these transcription factors such as *ZIC2* (Zic family member 2), *ZIC3*, *SALL2* (*spalt like transcription factor 2*) and *SALL4* were also significantly down-regulated (Fig. 5A and 5B). To further confirm the results, we have conducted Western blot analysis of selected genes. Western blot analysis also demonstrated that the expressions of *NANOG* and *OCT4* proteins were significantly lower in *NR5A2*-inhibited pancreatic CSCs compared with the control cells (Fig. 5C and 5D). Polycomb group proteins are known to be involved in the regulation of gene repression through chromatin modifications, which is essential for the maintenance of the embryonic and adult stem cells [22].

Polycomb repressive complex 2 (PRC2) contains *EED*, *EZH2* and *SUZ12* (*SUZ12* polycomb repressive complex 2 subunit) subunits and functions in the embryonic and adult stem cells to repress developmental genes that are preferentially activated during differentiation [23]. In our experiments, we found that the expression levels of *EED*, *EZH2* and *SUZ12* mRNA were decreased in *NR5A2*-inhibited pancreatic CSCs compared with the control (Fig. 5A and 5B). Western blot also confirmed reduced expression of *EZH2* protein in these cells (Fig. 5C and 5D).

NOTCH signaling has been shown to play important roles in pluripotency and self-renewal capacity of both embryonic and adult stem cells. The qRT-PCR experiments also showed a significantly lower expression level of *NOTCH1*, *NPTCH2* as well as *NOTCH* downstream targets such as *hES* (*hes family bHLH transcription factor*) and *HEY* (*hairy ears, Y-linked*) mRNAs in *NR5A2*-inhibited pancreatic CSCs compared with the control (Fig. 5A and 5B). Likewise, Western blot analysis as shown in Fig. 5C and 5D confirmed the lower protein expression of *NOTCH1* in *NR5A2*-knockdown cells.

Knockdown of *NR5A2* regulated the expression of EMT markers in CSCs

Epithelial-mesenchymal transition (EMT) is defined by loss of epithelial cell polarity, disappearance of differentiated junctions, reorganization of the cytoskeleton and changes in migration abilities. During this process, epithelial vimentin, and fibronectin are over expressed. Several transcription factors such as *SNAIL*, *SLUG* and *TWIST* are usually up regulated, and proteins such as metalloproteinases (MMP) 2, 3 and 9, which are involved in basement membrane degradation, are also over expressed during EMT^{24, 25}.

To determine whether *NR5A2* could affect EMT in pancreatic cancer, we next examined the expression of epithelial and mesenchymal marker in *NR5A2*-inhibited pancreatic CSCs. We found that *NR5A2* knockdown suppressed mRNA expression of *FGB* (*fibrinogen β chain*), *FN1* (*fibronectin 1*), *MMP2*, *MMP3*, *MMMP9*, *TWIST* (*twist family bHLH transcription factor 1*), *SNAIL* (*snail family transcriptional repressor 1*), *ZEB1* (*zinc finger E-box binding homeobox 1*), but not *ZEB2* (Fig. 6A and 6B). At the protein level, *NR5A2* knockdown increased the expression of E-cadherin and total β -catenin but decreased expression of vimentin (Fig. 6C and 6D). Furthermore, the increased expression of β -catenin was confirmed by flow cytometry (Fig. 6E and 6F). These data suggested that silencing of the *NR5A2* gene may negatively modulates EMT process, and has the potential to reverse the EMT in pancreatic CSCs.

Discussion

In this study we have shown that *NR5A2* gene is overexpressed in pancreatic cancer cell lines and in human PDAC tumor tissues compared to normal adjacent tissues. Higher levels of NR5A2 protein expression in the tumors were associated with significantly reduced overall survival and increased risk of death in patients with PDAC. We have further shown that *NR5A2* knockdown in pancreatic CSCs inhibited sphere formation, downregulated the expression of CSC markers, and regulated the expression of EMT markers. These findings suggest a potential role of *NR5A2* in maintaining stem cell properties and in the regulation of EMT in pancreatic CSCs, which are consistent with and adding new perspectives to the previously reported oncogenic activity of NR5A2 in pancreatic cancer [7,8].

NR5A2 has been identified as a susceptibility gene for pancreatic cancer [1]. However, the biological function of NR5A2 in pancreatic cancer is not clear. Studies in animal models reported controversial roles of *NR5A2* as an oncogene [24] or a tumor suppressor gene [9] in pancreatic cancer. *NR5A2* was overexpressed in human pancreatic cancer cell lines and in human pancreatic tumor tissues [7]. The current study has further demonstrated in a tissue array of 134 resected PDAC tumor samples that overexpression of *NR5A2* was associated with significantly reduced survival and increased risk of death in PDAC patients. This finding provides supporting evidence that *NR5A2* may play an oncogenic role in pancreatic cancer. Although no significant association between *NR5A2* expression and tumor characteristics was observed, tumor tissues showed a significantly lower proportion of nuclear staining than that of the normal adjacent tissues. The biological significance of the cellular localization of this protein needs further investigation.

The mechanisms of *NR5A2*-related pancreatic carcinogenesis may involve proliferation of dedifferentiated pancreatic cancer cells and the reprogramming events in the adult pancreas that lead to its transformation [7]. In vitro experiments have shown that knockdown of *NR5A2* by siRNA inhibited pancreatic cancer cell proliferation and the inhibition was tracked to the attenuation of the *NR5A2*'s transcriptional targets controlling cell growth, proliferation, and differentiation [7]. NR5A2 is also a known critical factor in the reprogramming of murine somatic cells to pluripotent cells [12,13], but little is known regarding the expression and function of *NR5A2* in CSCs. In this study, we performed the sphere formation assay and showed that spheres of pancreatic cancer AsPC-1 and PANC-1 cell lines expressed higher levels of *NR5A2* and CSCs marker genes, such as *CD24*, *CD44*, *c-MET*, *ALDH*, *EPCAM*, *EZH2*, *NANOG* and *OCT4* compared to their corresponding adherent cells (Figure 3). *NR5A2* knockdown by siRNA blocked the sphere formation of pancreatic CSCs (Figure 4) and decreased CSC marker expression (Figure 5). Furthermore, *NR5A2*-inhibited sphere cells showed decreased expression of *STAT3*, *SALL2*, *SALL4*, *ZIC2* and *ZIC3*, downstream targets of *NANOG*, *OCT4* and *SOX2* [25], polycomb repressor complex 2 (PRC2) consisting genes *EZH2*, *SUZ12* and *EED*, as well as NOTCH signaling pathway genes (Figure 5). *OCT4* functions as a master switch during differentiation by regulating the pluripotent potential in stem cells [26]. Previous studies have suggested that NR5A2 regulates *OCT4* expression by directly binding to the proximal enhancer and the proximal promoter regions of the *OCT4* gene [12]. In addition, a genome-wide binding analysis of NR5A2 revealed that it co-localizes with the OCT4–SOX2–NANOG cluster of

transcription factors [25], suggesting that NR5A2 may have a significant role in the maintenance of stem cells. Moreover, studies have shown that PRC2 target genes are co-occupied by stem cell regulators such as OCT4, Sox2 and NANOG. Likewise, NOTCH1, NOTCH2 as well as NOTCH downstream targets such as hES and HEY also play important roles in maintenance of pluripotent stem cells. Congruent with these data, we observed that the diminished levels of *NR5A2* and its transcriptional targets correlate with significant inhibition of the pancreatic CSC markers. These observation for the first time showed that *NR5A2* may play a crucial role in maintaining self-renewal and stem-like properties in pancreatic CSCs.

EMT is an important process contributing to the invasion, metastasis, chemo-resistance, [16,17,28] and the propagation of cancer stem cells [14,15] in solid tumors including pancreatic cancer [29-31]. The EMT process is associated with loss of epithelial dedifferentiation and switching toward mesenchymal phenotype, thus decreased expression of epithelial markers such as E-cadherin and increased expression of mesenchymal markers such vimentin. In the current study, we found that in pancreatic CSCs transfected with *NR5A2* siRNA, epithelial markers (E-Cadherin) was highly over expressed whereas mesenchymal markers vimentin was under expressed at both the RNA and protein levels (Figure 6). Furthermore, the increased expression of E-Cadherin coincided with the reduced expression of mesenchymal markers such as the members of the SAIL/SLUG family of zinc-finger transcription factors as well proteins that are involved in basement membrane degradation (Figure 6). Consistent with our findings, a previous study had shown that NR5A2 co-activates nuclear β -catenin/Tcf4 to promote intestinal cell proliferation by stimulating the expression of the G1 cyclins D1 and E1 [14]. In addition, NR5A2 promoted the motility and invasiveness of breast cancer, which was associated with decreased E-cadherin mRNA expression, the post-translation cleavage of mature 120 kDa E-Cadherin to its inactive 97 kDa form, as well as induced MMP9 mRNA expression[19]. All these experimental evidence support a role of NR5A2 in promoting EMT.

On the other hand, NR5A2 silencing has the potential to reverse the EMT in pancreatic cancer cells⁸. The current study has demonstrated that *NR5A2* knockdown in pancreatic CSCs increased the expression of E-Cadherin (as shown by Western blot) and β -catenin (as shown by both Western blot and flow cytometry). Although an inverse correlation between E-cadherin and Wnt/ β -catenin signaling has been reported in other studies [32,33], increased level of total β -catenin, which was attributable to an increase in the E-cadherin-associated membrane fraction of β -catenin has been associated with differentiation in colon cancer cells [34]. We used whole-cell lysate not nuclear extracts in the Western blot assay, which measured the total β -catenin. The flow cytometry analysis showed a high level of membrane-associated β -catenin. These data suggest that silencing of *NR5A2* may play a role in inducing cell differentiation, enhancing cell-cell adhesion and suppressing EMT [35].

In summary, we have shown that *NR5A2* is overexpressed in human pancreatic adenocarcinoma and the overexpression of *NR5A2* was associated with significantly reduced survival and increased risk of death. Using the *in vitro* sphere formation assay, we have shown an enrichment of *NR5A2* in pancreatic CSCs, and the expression levels of

NR5A2 and its transcriptional targets correlated with pancreatic CSCs' sphere formation. Considering that sphere-generating cells are highly aggressive *in vivo* when compared with adherent cells [36], our data suggest that *NR5A2* might be a functional pancreatic CSC marker. Findings from *NR5A2*-inhibition experiments suggest a possible role of *NR5A2* in maintaining CSC properties and in EMT. Further investigations are necessary to corroborate these preliminary but interesting findings and to better understand the role of *NR5A2* in pancreatic carcinogenesis.

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Abbreviations

CSC	cancer stem cell
ES	embryonic stem
EMT	epithelial-mesenchymal transition
OS	overall survival
PDAC	pancreatic ductal adenocarcinoma

Figure 1.

NR5A2 mRNA expression in human pancreatic ductal epithelial (HPDE) and carcinoma cell lines. Expression of mRNA was measured by RT-PCR (top panel) and protein by Western blot (lower panel). For Western blot the samples were treated with primary antibody (1:1000 dilution, Santa Cruz), imaged by enhanced chemiluminescence, and the results showed normalized expression of NR5A2 protein in the corresponding cells. *GAPDH* and β -actin was used as internal control for RT-PCR and Western blot, respectively. The mRNA and protein expression levels were consistent and the highest level was seen in AsPC-1 and PANC-1 cells.

Figure 2.

Representative micrographs of NR5A2 in pancreatic adenocarcinoma cancerous tissue or its surrounding non-tumor tissue by immunohistochemistry. Images (A and B) show light cytoplasmic and nuclear expression of NR5A2 in normal pancreatic tissues. Images (C and D) show high level of cytoplasmic expression of NR5A2 in pancreatic ductal adenocarcinoma tumor tissues. Magnifications: 40× for A and C; 200× for B and D. (E) Patients with a higher NR5A2 expression in the tumors (n=68) had a significantly reduced OS compared to those with a lower level of expression (n=66). Using the median value of the total IHC score as the cutoff the median survival time was 19.4 vs 23.7 months for those with a high (solid line) or low (dashed line) IHC score, respectively ($P=0.021$).

Figure 3.

Characterization of CSCs in pancreatic cancer cell lines. (A) Microscopy images of adherent cells and corresponding spheres in PANC-1 and AsPC-1 cells, Magnifications: 100× (B) Fold difference of mRNA expression levels of stem cell markers in sphere as compared to that of adherent PANC-1 and AsPC-1 cells. (C) Western blots of adherent cells and corresponding spheres in PANC-1 and AsPC-1 cells. (D) Densitometric analysis showed increased expression of CD44, EPCAM, OCT4 and NANOG proteins in PANC-1 and AsPC-1 spheres as compared to that in the corresponding adherent cells. The expression levels of proteins are normalized to those of β -actin. (E) Flow cytometry analysis for CD24, CD44, c-MET and ALDH expression in AsPC-1 adherent (upper panels) and sphere-forming cells (lower panels). (F) CD24 was expressed in $51.0 \pm 0.03\%$ (mean $\pm$ SD) of adherent and $92.5 \pm 9.9\%$ sphere-forming AsPC-1 cells. Similarly, CD44 was expressed in $44.1 \pm 5.4\%$ vs $94.8 \pm 6.6\%$, c-MET in $37.4 \pm 7.2\%$ vs $93.2 \pm 9.6\%$, and ALDH in $43.3 \pm 8.4\%$ vs $91.9 \pm 11.8\%$ of the adherent and sphere-forming cells, respectively.

Figure 4.

Blocking NR5A2 by specific siRNA in pancreatic CSCs. Adherent cells of AsPC-1 and PANC-1 were trypsinized and suspended in DMEM/F12 growth medium supplemented with B27 and N2. siPORT™ NeoFX™ Transfection Agent with 10 nM negative siRNA control (si control) or 10 nM anti-NR5A2 siRNAs (siRNA1 or siRNA2) were complexed and dispensed into ultra-low adherent six-well plate, then cell suspensions overlaid onto the transfection complexes and gently tilt the plate to mix. The final density of suspension cells were 10^4 cells/mL. (A) Microscopy images showing spheres 7 days after NR5A2 knockdown in AsPC-1 and PANC-1 CSCs. Magnifications: 40×. (B) Quantitative RT-PCR showing NR5A2 mRNA expression in spheres transfected with negative control siRNA (si control) and anti-NR5A2 siRNAs (siRNA1 and siRNA2) at 6 days following the transfections. (C) Western blots showing NR5A2 protein expression in spheres transfected with control and anti- NR5A2 siRNAs. Data are shown at 9 days after transfection. Expression of β-actin was used as an internal control.

Figure 5.

Gene expression profiling of stem cell markers in pancreatic CSCs. Panels A and B: reduced mRNA expression of stem cell marker genes in *NR5A2*-inhibited PANC-1 and AsPC-1 CSCs as determined by RT-PCR. Relative mRNA levels were normalized to GAPDH. Panels C and D: significantly decreased expressions of OCT4, NOTCH, NANOG and EZH2 proteins in *NR5A2*-inhibited PANC-1 and AsPC-1 CSCs. Expression of β -actin was used as an internal control.

Figure 6.

NR5A2 regulates EMT-related marker genes in pancreatic CSCs. Panel A and B showed decreased mRNA expression of EMT-related transcription factor such as *FGB*, *FN1*, *MMP2*, *MMP3*, *MMP9*, *TWIST*, *SNAIL*, and *ZEB1* and the mesenchymal marker *Vimentin*, and increased expression of the epithelial marker *E-cadherin* in NR5A2-inhibited PANC-1 and AsPC-1 CSCs compared with controls. Relative mRNA levels were normalized to GAPDH. Panel C and D showed reduced protein expression of the mesenchymal marker Vimentin and increased expression of the epithelial marker E-cadherin and β-catenin in NR5A2-inhibited PANC-1 and AsPC-1 CSCs compared with controls. Expression of β-actin was used as an internal control. Panel E showed the increased expression of membrane β-catenin in NR5A2-inhibited AsPC-1 sphere-forming cells compared to that in control siRNA-transfected cells by flow cytometry.