

## Original Article

# A novel NR4A2-HuR axis promotes pancreatic cancer growth and tumorigenesis that is inhibited by NR4A2 antagonists

Sneha Johnson<sup>1\*</sup>, Zuhua Yu<sup>1,2\*</sup>, Xi Li<sup>1</sup>, Mehrdad Zarei<sup>3,4</sup>, Ali Vaziri-Gohar<sup>3,4</sup>, Miok Lee<sup>1,5</sup>, Srijana Upadhyay<sup>1</sup>, Heng Du<sup>6</sup>, Mahsa Zarei<sup>1,6</sup>, Stephen Safe<sup>1</sup>

<sup>1</sup>Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, TX, USA; <sup>2</sup>Henan University of Science and Technology, Luoyang, Henan, P. R. China; <sup>3</sup>Department of Surgery, University Hospitals, Cleveland, OH, USA; <sup>4</sup>School of Medicine, Case Western University, Cleveland, OH, USA; <sup>5</sup>Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, USA; <sup>6</sup>Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, USA. \*Equal contributors.

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**Abstract:** Pancreatic ductal adenocarcinoma (PDAC) patients' express higher levels of the orphan Nuclear Receptor 4A2 (NR4A2, NURR1) compared to normal pancreas and NR4A2 is a prognostic factor for patient survival. Knockdown of NR4A2 by RNA interference (RNAi) inhibited cell proliferation, invasion, and migration. RNA sequencing performed in NR4A2<sup>(+/+)</sup> and NR4A2<sup>(-/-)</sup> MiaPaCa2 cells demonstrated that NR4A2 played a significant role in cellular metabolism. Human antigen R (HuR) and isocitrate dehydrogenase 1 (IDH1) were identified as NR4A2 target genes. HuR is a pro-oncogenic RNA binding protein and silencing of HuR by RNAi significantly downregulated expression of NR4A2. Expression of HuR and IDH1 were significantly downregulated after treatment with NR4A2 inverse agonist, 1,1-bis(3'-indolyl)-1-(p-chlorophenyl)methane resulting in significant inhibition of tumor growth in an athymic nude mouse xenograft model. This study demonstrates that NR4A2 and HuR regulate genes and signaling pathways that enhance tumorigenesis and targeting NR4A2 and HuR expression with an NR4A2 inverse agonist represents a novel regimen for treating PDAC.

**Keywords:** Pancreatic cancer, NR4A2, HuR, IDH1, NR4A2 antagonist treatment

## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the leading causes of cancer deaths, with five-year and one-year survival rates of 5-7 and 20% respectively [1]. PDAC is lethal due to poor clinical prognosis, late-stage diagnosis with surgical abscission being the only curative therapy (for <20% patients) [2, 3]. The common symptoms of PDAC including loss of weight, abdominal pain, nausea, and vomiting resemble symptoms of other diseases [4] and this often results in late-stage diagnosis. Out of the >350,000 people that are diagnosed annually with pancreatic cancer worldwide, more than 340,000 die from the disease [2]. Treatments of various solid cancers including pancreatic cancer have improved [1] however, this has not significantly impacted the high mortality

rates observed for PDAC patients. Despite the increasing data on genetic alterations and impairment of signaling pathways that lead to pancreatic tumorigenesis, this has not resulted in development of more effective therapies [5].

The orphan nuclear receptor 4A (NR4A) sub-family members of NR4A1 (Nur77), NR4A2 (Nurr1) and NR4A3 (Nor1) are stress/inflammation-induced immediate-early genes that play key functional roles in maintaining cellular homeostasis and in pathophysiology [6]. It has previously been reported that offspring from the crossing of NR4A1<sup>-/-</sup> and NR4A3<sup>-/-</sup> rapidly develop symptoms of acute myeloid leukemia and both receptors exhibit tumor suppressor-like activities in blood-derived cancers [7]. In contrast there is extensive data in solid tumors that both NR4A1 and NR4A2 are pro-oncogenic

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and exhibit tumor promoter-like activity [8]; NR4A3 has not been extensively studied in solid tumors but there is evidence for tumor suppressor activity or lack of any effects [9, 10]. Research in our laboratory has focused on the pro-oncogenic role of NR4A1 and NR4A2 in solid tumors and development of ligands for these receptors that act as inverse agonists that inhibit these receptors. Functional assays based on receptor knockdown studies show that both NR4A1 and NR4A2 play a role in solid tumor-derived cancer cell proliferation, survival, migration and invasion and regulation of genes and gene products that mediate these responses [6]. For example, NR4A1 regulates genes that maintain low cellular redox levels and knockdown of NR4A1 or treatment with bis-indole derived (C-DIM) NR4A1 inverse agonists increased ROS and ROS-dependent stress and inhibited mTOR signaling [11-15]. In pancreatic cancer cells, this response was due, in part, to downregulation of pro-reductant genes thioredoxin domain containing 5 (TXNDC<sub>5</sub>) and isocitrate dehydrogenase-1 (IDH1) [15]. NR4A2 also regulates glioblastoma and pancreatic cancer cell growth, survival, migration and invasion, and ongoing studies in these cell lines show that NR4A1 and NR4A2 regulate comparable pro-oncogenic pathways and some of the same genes [10, 15-17]. Our recent study also showed that NR4A2 was over-expressed in PDAC patients compared to non-tumor tissue and was also a negative prognostic factor for PDAC patient survival and regulated chemoresistance and autophagy in pancreatic cancer cell lines [17].

The RNA binding protein Hu-antigen R (HuR) exhibits many of the same pro-oncogenic functions reported for NR4A2, and also regulate some of the same genes including survivin,  $\beta$ -catenin, Slug, Zeb1 and IDH1 [15, 16, 18-25]. Since the functional activities of NR4A2 and HuR are comparable, the aim of this study was to investigate possible NR4A2-HuR interactions. Our results show that HuR and NR4A2 interact in pancreatic cancer cells and that NR4A2 regulates HuR expression and vice versa. Moreover, the NR4A2 ligand, 1,1-bis(3'-indolyl)-1-(4-chlorophenyl)methane (C-DIM12) downregulates HuR and thus targeting NR4A2 represents a novel chemotherapeutic approach for targeting HuR in cancer cells.

## Materials and methods

### *Cell lines and transfection with small-interference RNA and plasmids*

MiaPaCa2, HPNE and Panc1 pancreatic cancer cell lines were obtained from ATCC (American Type Culture Collection) and were cultured in in DMEM supplemented with 10% FBS (Gibco/Invitrogen), 1% L-glutamine (Gibco/Invitrogen), and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA) at 37°C in 5% humidified CO<sub>2</sub> incubators. CRISPR/Cas9-mediated knockout of NR4A2 in MiaPaCa2 cells was accomplished using guide RNAs targeting NR4A2, fused with CRISPR/Cas9 and GFP protein as previously described [17]. CRISPR Universal Negative Control plasmid (CRISPR06-1EA) were purchased from Sigma-Aldrich (St. Louis, MO). Cells were harvested after 48 hours of transfection and GFP positive cells were single sorted by using a FACS Calibur flow cytometer and cells which did not express NR4A2 were isolated as described [17]. The guide RNA sequences used were: NR4A2-1 (gatccccgggtcgtccacat), NR4A2-2 (gggcttgtagtaaacgacc). For all cell cultures experiments the mycoplasma detection kit MycoAlert (Lonza) was performed after each thawing and at least monthly to ensure mycoplasma free cells were used.

Cells were plated at 60% confluency in 6-well plates, and transient transfections were performed using Lipofectamine 3000 (Invitrogen), and Opti-MEM (Invitrogen) according to the manufacturer's protocol; 48 hours after transfection, cells were treated or analyzed, as described. Small interfering RNA (siRNA) oligos were purchased from Invitrogen and are summarized in [Supplementary Table 1](#).

### *Cell growth and Annexin V staining assays*

Cells were plated in 96-well plates at  $1 \times 10^3$  cells per well. After 5 days of incubation, cell growth was measured using Quant-iT™ PicoGreen™ dsDNA assay kit (Invitrogen). To estimate cell death, cells were trypsinized and counted after Trypan blue staining (Invitrogen) with a Hausser bright-line hemocytometer (Fisher Scientific). Annexin V/PI staining was performed using the Dead Cell Apoptosis Kit (ThermoFisher Scientific #V13245, Waltham, MA), according to the manufacturer's instructions. Staining was measured with an Accuri C6

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flow cytometer and analyzed with FlowJo Version 10.2 software.

### *Immunoblot analysis*

Cells were lysed using 1% Triton in TBS containing protease and phosphatase inhibitors. Tumors were lysed using 1X RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of total protein were separated by electrophoresis on a 4-12% Bis-Tris gel and transferred to a PVDF membrane. Blots were blocked in 5% BSA, and then probed with antibodies against anti-NR4A2 (sc-376984, SCB), anti-IDH1 (ab184615, Abcam), anti-HuR (sc-5261 clone 3A2, SCB), anti-Sp1 (ab124804, Abcam), Sp3 (sc-644, SCB), Sp4 (sc-645, SCB) and anti- $\alpha$ -tubulin (21445, Cell Signaling Technology). Chemiluminescent (32106, Thermo Fisher Scientific) signals were captured using a Syngene G-BOX iChemi XT imager.

### *Chromatin immunoprecipitation assay*

Cells were cross-linked for 10 minutes at room temperature by addition of a solution containing 11% formaldehyde in 50 mM HEPES pH 7.4, 100 mM NaCl, 1 mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, followed by 5 minutes quenching with a solution of 2.5 M glycine. Cells were then washed with PBS (2X), the supernatant was aspirated, and the cell pellet was flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used. Antibody precipitation, reversing cross-links, sonication and DNA purification were carried out as described [10, 17]. The primers are summarized in [Supplementary Table 1](#).

### *Boyden chamber invasion assay and migration assay*

Cell suspension ( $50 \times 10^3$  cells in 500  $\mu\text{l}$  2.5% FBS DMEM) was added on the Corning™ Boyden Invasion chamber with Corning™ Matrigel Matrix (Life Sciences; 354480) disc and 500  $\mu\text{l}$  10% FBS DMEM was added to the well. Cells were incubated at  $37^{\circ}\text{C}/5\% \text{CO}_2$  and allowed to invade through the porous membrane. After 24 hours, the discs were taken out of the medium and placed in 500  $\mu\text{l}$  0.5% crystal violet for 10-15 minutes at room temperature. The discs were washed and dried for 24 hours. For migration assays, cells were seeded at 60-70% confluency in a 6-well plate. After 24 hours, cells were transfected with siN-

R4A2 or NR4A2 overexpression plasmid using Lipofectamine 2000 (Invitrogen; 11668-027) and Opti-MEM (Gibco; 31985-062). After 24 hours of seeding, the cell monolayers were scratched using micropipette tips and the photographs of the scratch on each plate were taken at 0 and after 48 hours by using EVOS FL microscope and the ratio of migrated cells was analyzed by using the ImageJ software (National Institutes of Health, Bethesda, MD).

### *Co-immunoprecipitation assay*

Co-immunoprecipitation assay was carried using Pierce Co-Immunoprecipitation kit (Pierce; 26149). The AminoLink Plus Coupling Resin provided in the kit was used for coupling the anti-NR4A2 and anti-Sp4 antibodies following the manufacturer's protocol. Protein lysate was extracted from MiaPaCa2 cells using the provided IP Lysis/Wash Buffer and all Co-IP steps were performed according to the manufacturer's protocol. The total cell lysate and Co-IP samples were then used to detect NR4A2 and Sp4 on SDS gel for western blot analysis using the anti-NR4A2 or anti-Sp4 antibodies.

### *Clonogenic assay*

Cells (1000-2000/well) were plated in a 6-well plate. The media was not changed during experiments unless indicated. Upon completion of the experiments, colonies were fixed in reagent containing 80% methanol and stained with 0.5% crystal violet. To determine relative growth, dye was extracted from stained colonies with 10% acetic acid and the associated absorbance measured at 600 nm using a Microplate Reader/Synergy HT BioTek plate reader.

### *Xenograft tumor growth studies*

Athymic nude mice (Female, Nude Foxn-1 nu, 6-8 weeks old) were ordered from EN-VIGO and housed in a pathogen-free environment with vented cages. The mice were housed in Texas A&M University's animal facility with the standard of AAALAC. The protocol of the study was approved by IACUC (#2020-0138), Texas A&M University. Mice were allowed to acclimatize for 1-2 weeks before the experiment. The animals (10 per treatment group) were fed with standard chow diet and water during the experiment. MiaPaCa2 cells were cultured at a den-

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sity ( $4 \times 10^6$ ) in 10% FBS DMEM. The cells were harvested and resuspended in 100  $\mu$ l of PBS with Matrigel. Mice (10 per treatment group) were injected with MiaPaCa2 cells ( $4 \times 10^6$ ) subcutaneously. In two weeks when tumors grew to a size of 100 mm<sup>3</sup>, mice were administered C-DIM12 (50 mg/kg) or vehicle control by intraperitoneal injections three times a week for a period of 6 weeks. Some of the mice were observed for their survival and tumor growth after the final injection.

Mice were also injected with wild-type and CRISPR/Cas9-derived NR4A2-knockout MiaPaCa2 cells and tumor growth was evaluated for 4 weeks. Some mice were observed for tumor-free survival after the end of the experiment. Tumor volumes and body weights were determined periodically; a Vernier caliper was used to measure the size of the tumor, and tumor volume was determined by the formula (Volume = Length  $\times$  Width<sup>2</sup>/2). At the end of the experiment, the mice were euthanized under deep isoflurane anesthesia. The tumors were resected and stored in 10% neutral buffer formalin for immunohistochemistry or stored at -80°C until processed for protein and RNA analysis.

### *siRNA and cDNA transfection*

MiaPaCa2 cell were seeded on a 6-well plate. After 24 hours the transfection mixture containing siNR4A2 (1  $\mu$ mol/L)/ siHuR (1  $\mu$ mol/L) or NR4A2 overexpression plasmid (1  $\mu$ g) along with Lipofectamine 2000 (Invitrogen; 11668-027) and Opti-MEM (Gibco; 31985-062) was added. After 48 hours, cells were harvested for protein and RNA analysis. Overexpression (NOE) and empty vector were used as previously described [17].

### *RNP-IP and RT-qPCR*

Cells were plated at 50% confluency in 100 mm dishes. The following day, immunoprecipitation was performed using either anti-HuR or IgG control antibodies as previously described [23]. For RT-qPCR, the RNA lysate was extracted from the cultured cells using RNeasy Mini Kit (Qiagen; 74104 and 74106) and cells were harvested according to the manufacturer's protocol. The cDNA was extracted by using High-Capacity cDNA Reverse Transcription Kits as per manufacturer's protocol (Applied Bio-

systems; 4368813, 4368814, 4374966, and 4374967). The RT-qPCR was performed by using Taqman™ Universal Master Mix II (Thermo Fisher Scientific; 4440038) with probe (NR4A2 probe- Thermo Fisher Scientific; 4331182, ELAVL1 probe- Life Technologies; 4453320, IDH1 probe- Thermo Fisher Scientific; 4351372) and cDNA and was analyzed by using the Bio-Rad CFX Maestro software. Oligonucleotides are summarized in [Supplementary Table 1](#).

### *RNA sequencing*

RNA quality was assessed via the Agilent 2100 Bioanalyzer (Agilent Technologies). Strand-specific RNA-seq library was prepared using NEBNext Ultra II Directional RNA Library Prep Kit (NEB, Ipswich, MA) according to the manufacturer's protocols. RNA-sequencing was performed using 150-bp paired-end format on a NovaSeq 6000 (Illumina) sequencer. RNA-seq quality was checked by running FastQC, and TrimGalore was used for adapter and quality trimming. Sequence reads were aligned to the hg19 human genome build using the STAR aligning program [26]. Quantification of all genes and their isoforms was performed using FPKM normalized values using Cufflinks v2.2.1, DESeq2 analysis with an adjusted *P*-value <0.05 was used to get a list of differentially expressed genes [27].

### *Immunohistochemistry*

IHC staining was performed on paraffin embedded tissue sections. Sections were incubated with HistoClear (National Diagnostics; HS-200) for 5 minutes (3X), 100% ethanol (2X) and in 95% ethanol (2X) for 10 minutes each, then with purified water (2X) for 5 minutes. Antigen retrieval was performed by boiling for 10 minutes in 10 mM sodium citrate pH 6.0, followed by washing in water, then 3% hydrogen peroxide for 10 minutes, then water. 1X TBST + 2.5% normal goat serum (Vector; MP-7451) was used as a blocking solution and the following primary antibodies were diluted in Signal Stain Antibody Diluent (Cell Signaling technology; 8112) and incubated overnight at 4°C: Ki67 (Abcam; ab16667), c-Casp3 (Cell Signaling Technology; 9664). Slides were then washed for 5 minutes in 1X TBST (3X) and incubated in Signal Stain Boost IHC Detection Reagent (Cell Signaling Technology; 8114) for 30 minutes at room temperature, followed by washes in 1X



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TBST (3X) for 5 minutes. The signal was detected using the AEC Peroxidase (HRP) Substrate Kit (Vector; SK-4200) according to manufacturer's instructions. Slides were counterstained with hematoxylin (Vector; H3401). Coverslips were mounted with Permount Mounting solution (Thermo Fisher Scientific; 00-4958-02). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed following the In-Situ Apoptosis Detection Kit (Trevigen; 4810-30-K) with 40-fold magnification as described [17].

### Statistical analysis

Data were expressed as mean  $\pm$  standard error of the mean (SEM) of at least three independent experiments. An unpaired, two-tailed student t test was used to determine the differences between groups (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ). ANOVA test was used for the analysis of tumor measurements among treated groups.

### Results

#### *NR4A2 expression plays a key role in proliferation, invasion, and migration of pancreatic cancer cells*

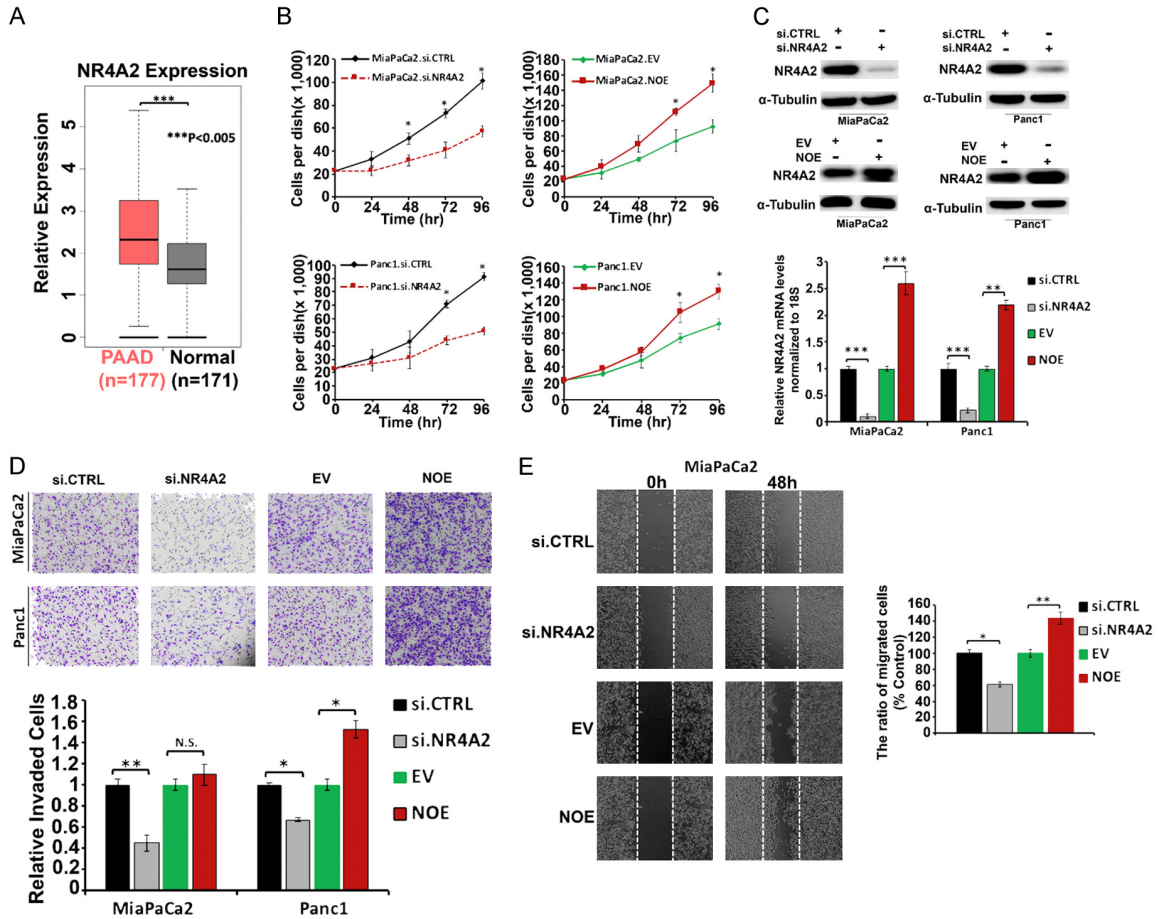
Analysis of the TCGA database showed that NR4A2 was highly expressed in tissues from PDAC cancer patients compared to normal donors, and the high expression of NR4A2 (**Figure 1A**) in tumors corresponded to poor patient survival ( $P < 0.05$ ) [17]. The functional effects of NR4A2 were determined by gene silencing or overexpression (OE). **Figure 1B** shows that silencing of NR4A2 by RNA interference resulted a significant decrease in proliferation of MiaPaCa2 and Panc1 pancreatic cancer cells after treatment for 72-96 hours whereas transfection of these cells with an NR4A2 overexpression plasmid significantly increased proliferation of both cell lines after 72 and 96 hours (**Figure 1B**). **Figure 1C** illustrates the effects of knockdown/overexpression on NR4A2 levels in MiaPaCa2 and Panc1 cells. Results obtained in Boyden chamber assay showed that NR4A2 silencing (**Figure 1D**) reduced invasion by 55% in MiaPaCa2 and by 40% in Panc1 cells (**Figure 1D**). Overexpression of NR4A2 increased the invasion in Panc1 cells however the effects on invasion of MiaPaCa2 cells was not significant (**Figure 1D**). In the

wound healing assay, NR4A2 silencing by RNA interference resulted in a significant decrease in migration whereas NR4A2 overexpression resulted in an increase in migration in Panc1 cells (**Figure 1E**). These data demonstrate that NR4A2 plays a significant pro-oncogenic role in pancreatic cancer cell proliferation, invasion and migration and these results are comparable to those previously observed after NR4A2 knockdown in pancreatic cancer and glioblastoma cells [10, 17].

#### *NR4A2 regulates HuR and IDH1 expression in pancreatic cancer cells*

To identify genes regulated by NR4A2 we performed RNA sequencing (RNA-Seq) in MiaPaCa2 cells. NR4A2 expression was deleted by gene editing using CRISPR/Cas9 system as previously described [14]. **Figure 2A** illustrates a volcano plot of the results and subsequent data analysis showed that loss of NR4A2 was associated with downregulation of IDH1 and HuR. According to Metabolite Set Enrichment Analysis pathways and gene associated with the tricarboxylic acid cycle and NADPH synthesis pathways were also affected by NR4A2 knockdown (**Supplementary Figure 1A and 1B**) and these are currently being investigated. Based on results of previous studies showing that NR4A1 and HuR regulated IDH1 expression [17, 20], we focused mechanistic analysis of both the HuR and IDH1 genes which were significantly downregulated in MiaPaCa2 cells after knockdown of NR4A2 by RNA interference. HuR encodes an RNA-binding protein that maintains mRNA stability and IDH1 encodes an enzyme that catalyzes the reversible conversion of isocitrate and  $\alpha$ -ketoglutarate, with the production of NADPH [18-25, 28]. To validate our RNA sequencing data, we showed that NR4A2, HuR and IDH1 levels were decreased in MiaPaCa2 cells after NR4A2 knockdown by RNA interference (**Figure 2B**). The western blot data was consistent with the RT-qPCR results and showed that NR4A2 knockdown decreased expression of HuR and IDH1 proteins in MiaPaca2 and Panc1 cells (**Figure 2B**). The western blot and RT-qPCR analysis of wild-type (NR4A2<sup>+/+</sup>) and 2 different NR4A2 knockdown (NR4A2<sup>-/-</sup>) (by RNA interference) cell lines also showed a significant downregulation of HuR and IDH1 mRNA and protein expression in the knockout cell lines (**Figure 2C**). Interactions

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**Figure 1.** NR4A2 plays an important role in pancreatic cancer cell survival and migration. A. Expression of NR4A2 in PDAC patient's tissues as compared to that in normal pancreas tissues, \*P<0.05 (data extracted from TCGA database). B. MiaPaCa2 and Panc1 cell proliferation after RNA silencing of NR4A2 (siNR4A2) and overexpression of NR4A2 (NOE) compared with controls (siCTRL and EV) was determined over time. C. Western blot and RT-qPCR analysis for NR4A2 expression in MiaPaCa2 and Panc1 cells after NR4A2 overexpression or knockdown. D. MiaPaCa2 and Panc1 cell images (4× magnification) and quantification of invaded cell numbers in Boyden chamber assay. E. MiaPaCa2 cell migration Images (4× magnification) and quantification of migrated cells in the scratch assay after NR4A2 knockdown or overexpression (NOE) in MiaPaCa2 cells. Quantitated results are means ± SE for at least 3 replicate determinations; significance, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

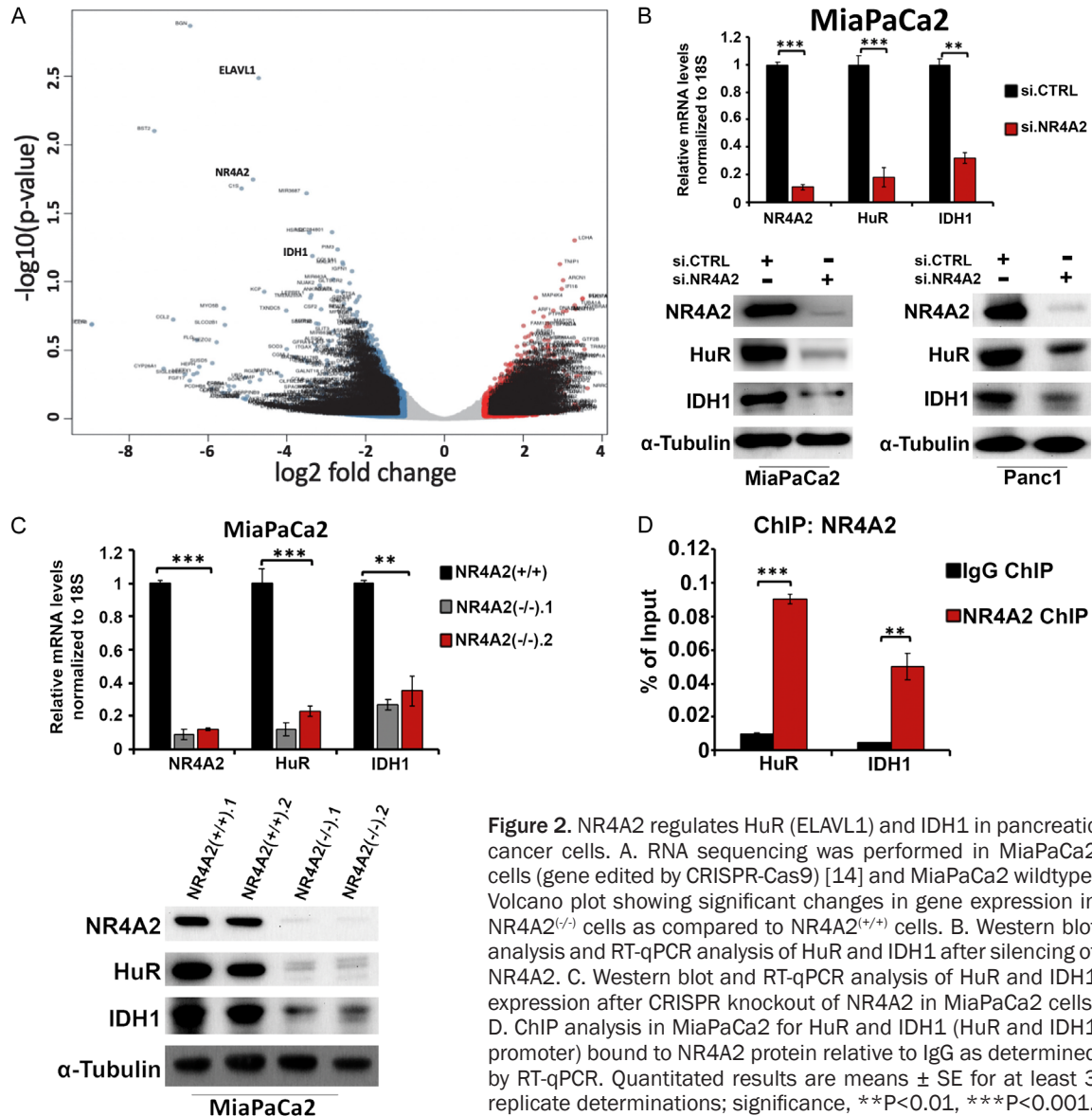
of NR4A2 with the HuR and IDH1 gene promoters was carried out by ChIP analysis in MiaPaCa2 cells. Immunoprecipitation with antibodies showed enrichment of NR4A2 on the HuR and IDH1 promoters (**Figure 2D**) and these results demonstrate that NR4A2 regulates expression of HuR and IDH1 in MiaPaCa2 cells.

## NR4A2 regulation of HuR and IDH1 through specificity protein (Sp) transcription factors in pancreatic cancer cells

There is evidence that NR4A2 and NR4A1 regulate similar functions and genes in pancreatic and other cancer cell lines and previous stud-

ies show that NR4A1 acts as a ligand-dependent co-activator of several Sp regulated genes including survivin, PAX3-FOXO1, β1- and β3-integrins and α5- and α6-integrins [16, 29-32]. Mechanism studies showed that these genes were regulated by NR4A1/Sp1 or NR4A1/Sp4 binding to GC-rich gene promoters. Both the HuR and IDH1 gene promoters contain GC-rich Sp binding sites and in the absence of cognate NR4A2 binding sequences we hypothesize that HuR and IDH1 may also be NR4A2/Sp1- or NR4A2/Sp4-regulated genes (**Figure 3A**). Knockdown of Sp1 in MiaPaCa2 cells was minimal (**Figure 3B**); similar results were observed with another oligonucleotide

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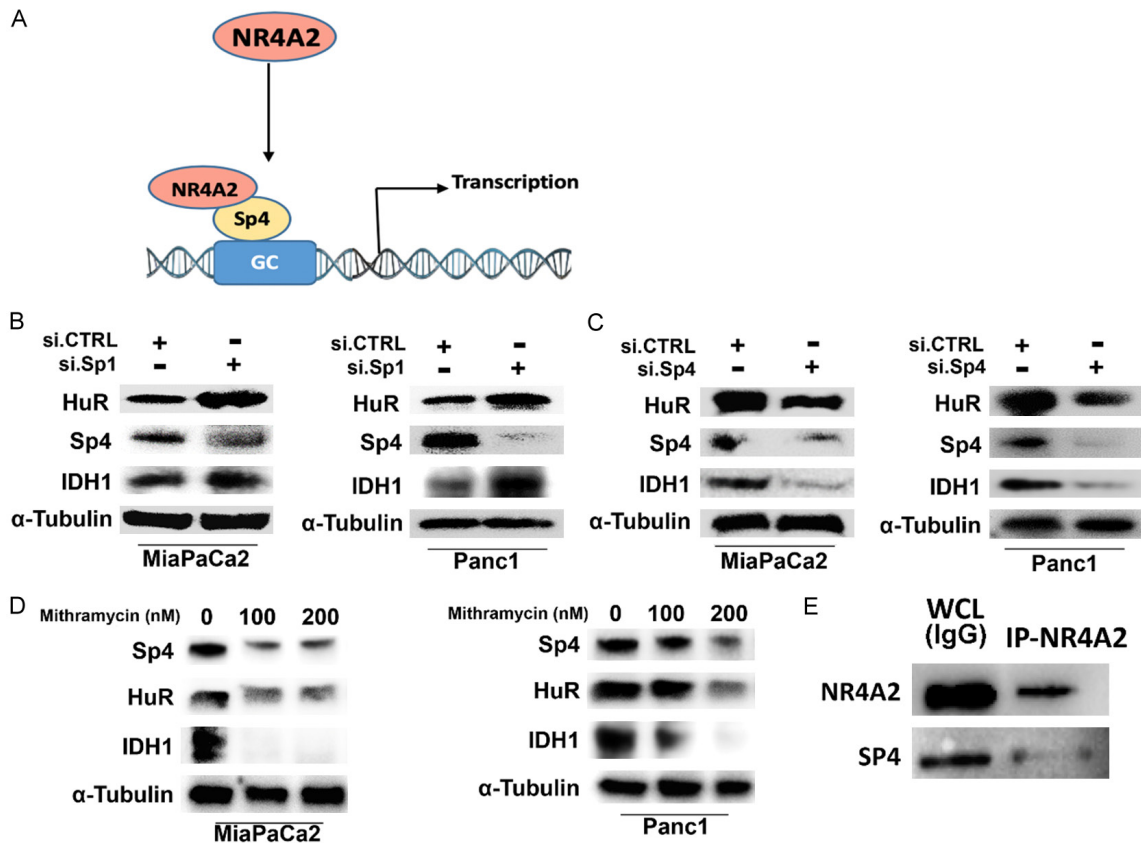


(data not shown) and levels of HuR and IDH1 were not decreased. Surprisingly, in Panc1 cells Sp1 silencing decreased Sp1 but increased levels of IDH1 and HuR. The reason for increased expression of IDH1 and HuR is not known however the results indicate that both genes are not regulated by NR4A2/Sp1. In contrast, knockdown of Sp4 in MiaPaCa2 and Panc1 cells decreased levels of Sp1, IDH1 and HuR gene products (**Figure 3C**) suggesting that NR4A2/Sp4 plays a role in regulation of IDH1 and HuR in MiaPaCa2 and Panc1 cells. This mechanism is further supported by the observation that mithramycin, which blocks binding of Sp1 and Sp4 to GC-rich sites [33] also decreased levels of Sp4, HuR and IDH1 in

MiaPaCa2 and Panc1 cells (**Figure 3D**). These results are consistent with previous studies on NR4A1/Sp4 regulation of other genes [12, 29-32]. We also observed in co-immunoprecipitation studies that NR4A2 antibodies co-immunoprecipitated both NR4A2 and Sp4 (**Figure 3E**); similar results were observed for Sp4 antibodies, however the NR4A2 band was very weak (data not shown).

### *Bis-indole derived C-DIM12/NR4A2 inverse agonist inhibits pancreatic cancer cell proliferation and migration*

Previous studies showed that 1,1-bis(3'-indolyl)-1-(p-substituted phenyl) methane (C-DIM)

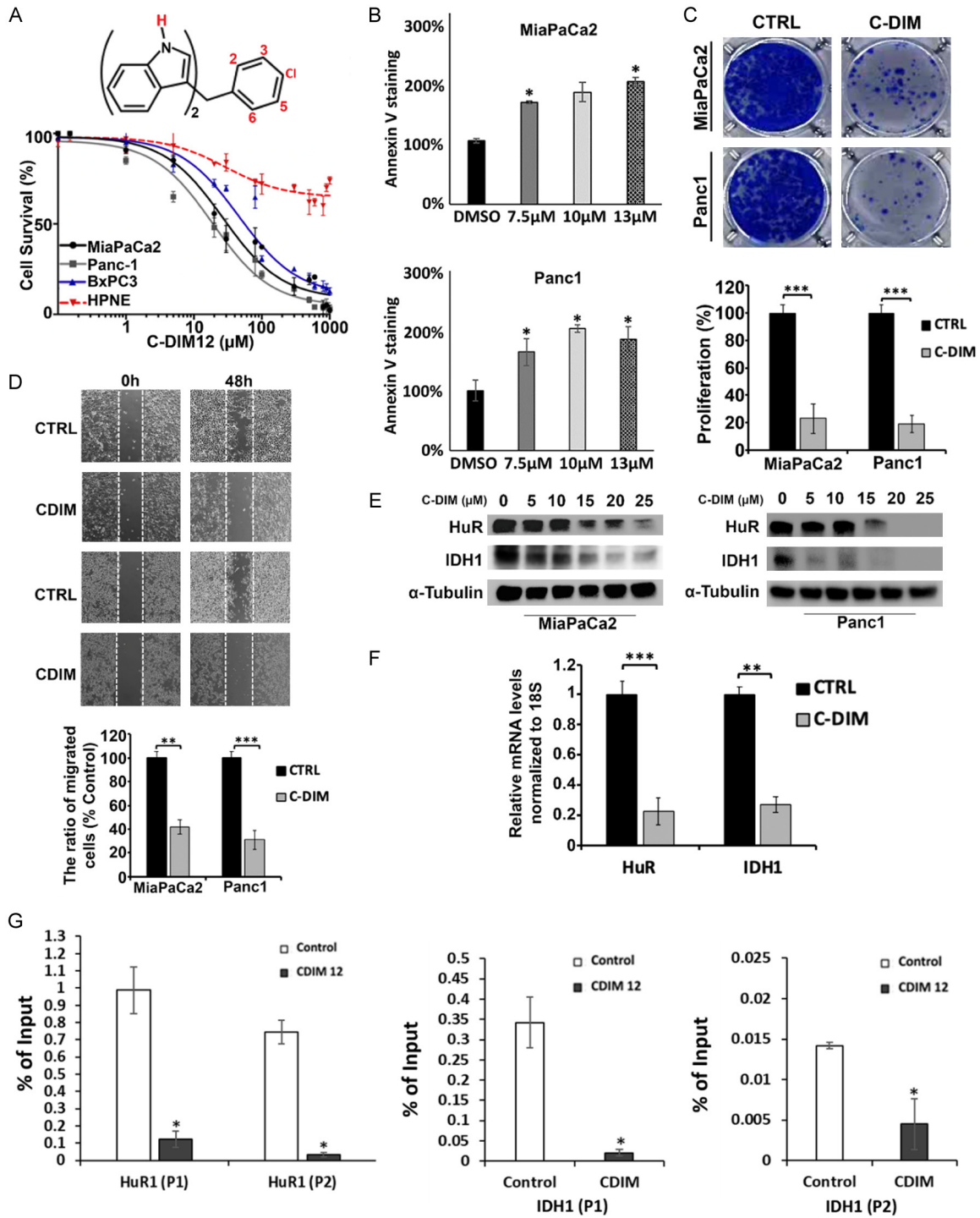


**Figure 3.** NR4A2 regulated expression of HuR and IDH1 involves interactions with Sp transcription factors. (A) Schematic representation of NR4A2 binding to Sp4 and regulating gene transcription. (B) Expression of gene products after RNA silencing of Sp1, (C) Sp4 and (D) treatment with mithramycin in pancreatic cancer cell lines (MiaPaCa2 and Panc1). (E) Co-immunoprecipitation of NR4A2 and Sp4 using NR4A2 antibodies and subsequent western blot analysis as outlined in the methods. Sp4 antibodies also co-immunoprecipitated Sp4 and NR4A2 however the NR4A2 band was weak (data not shown).

analogs inhibited growth and induce apoptosis in cancer cell lines by acting as NR4A1 or NR4A2 inverse agonists [7]. Specifically, 1,1-bis(3'-indolyl)-1-(*p*-chlorophenyl) methane (DIM-C-*p*PhCl or C-DIM12) acts as an NR4A2 inverse agonist in cancer cells [10, 17, 34] and treatment of MiaPaCa2, Panc1, BxPC3 pancreatic cancer cells and HPNE normal pancreatic ductal cells with C-DIM12 (1-1000  $\mu$ M) preferentially decreased % survival in the cancer cell lines in a dose-dependent manner whereas this decrease was attenuated in non-transformed HPNE cells (Figure 4A). We also observed that treatment with 15  $\mu$ M DIM12 induced MiaPaCa2 and Panc1 cell death as indicated by increased Annexin V staining (Figure 4B). A soft agar clonogenic assay in MiaPaCa2 and Panc1 cells showed that C-DIM12 (15  $\mu$ M) inhibited anchorage-indepen-

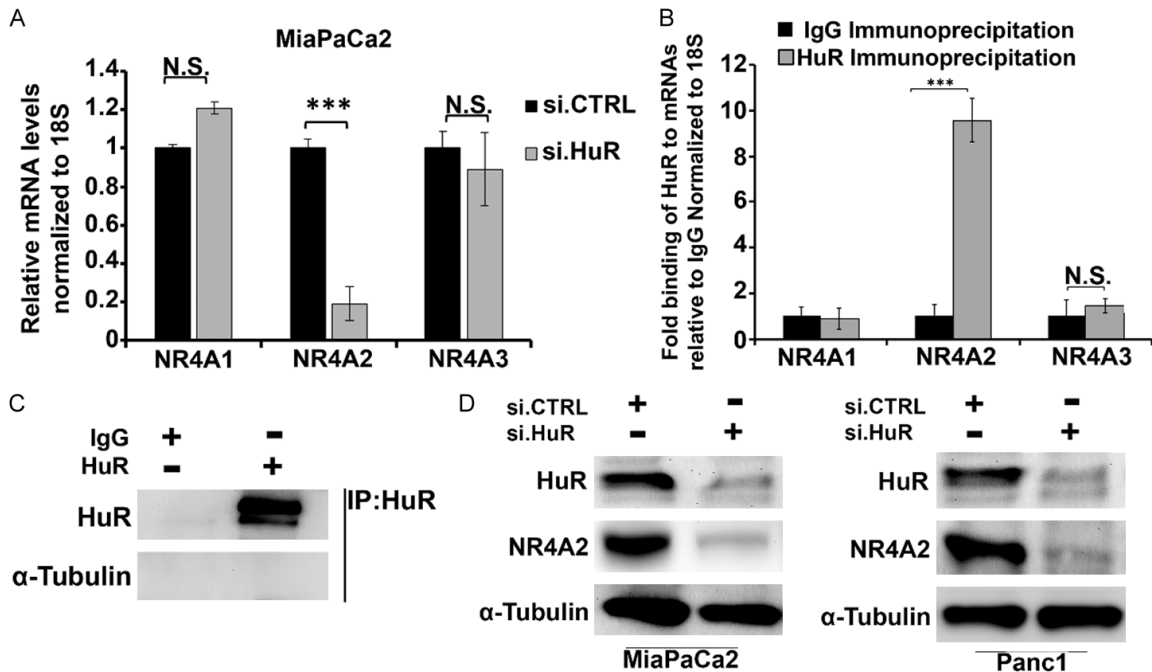
dent growth over a period of 4 weeks (Figure 4C) and in a scratch assay 15  $\mu$ M C-DIM12 decreased migration of MiaPaCa2 and Panc1 cells by 60-70% over a period of 48 hours (Figure 4D). Western blot analysis showed that C-DIM12 (5-25  $\mu$ M) downregulated expression of NR4A2 targets, HuR and IDH1 proteins in MiaPaCa2 and Panc1 cells (Figure 4E) and mRNA in Panc1 cells (Figure 4F). Moreover, we show in Figure 4G that treatment with C-DIM12 decreases NR4A2 interaction with the HuR and IDH1 gene promoter (2 primer sets for each gene) in a ChIP assay. These results demonstrate that the NR4A2 inverse agonist C-DIM12 inactivates NR4A2-dependent transcriptional activity and the effects of this compound in terms of function and gene expression (HuR and IDH1) overlaps those observed after NR4A2 knockdown (Figures 1-3).





**Figure 4.** C-DIM12 inhibits cell proliferation and migration and downregulates expression of HuR and IDH1. (A) The structure of DIM-pPhCl (C-DIM12) and percentage of cell survival after treatment with 0-1000 μM of C-DIM12 for 24 hours. (B) MiaPaCa2 and Panc1 cells were treated with 15 μM C-DIM12 for 24 hours and apoptosis was measured by Annexin V staining (images were taken at 4× magnification). (C) Soft agar colony formation assay was determined in pancreatic cancer cells treated with 15 μM C-DIM12 for 4 weeks. (D) Cell migration images and quantification of migrated cells in a scratch assay after treatment of pancreatic cancer cells with C-DIM12 for 0 or 48 hours. (E) Western blot and (F) RT-qPCR analysis of HuR and IDH1 levels in pancreatic cancer cells after treatment with C-DIM12 for 24 hours. (G) ChIP assay results showing that C-DIM12 decreases interaction of NR4A2 with the HuR and IDH1 gene promoter (2 primer sets used for each gene). Quantitated results are means ± SE for at least 3 replicate determinations; significance, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

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**Figure 5.** HuR regulates the expression of NR4A2. (A) Relative mRNA levels of NR4A1, NR4A2 and NR4A3 in MiaPaCa2 cells after transfection with siCTRL or siHuR. (B) Immunoprecipitation analysis in MiaPaCa2 for NR4As mRNAs bound to HuR protein relative to IgG and (C) preferential immunoprecipitation of HuR by HuR antibodies and not IgG. (D) Western blot analysis of HuR and NR4A2 expression in MiaPaCa2 and Panc1 cells transfected with siCTRL (control/non-specific) and siNR4A2. Quantitated results are means  $\pm$  SE for at least 3 replicate determinations; significance, \*\*\* $P < 0.001$ .

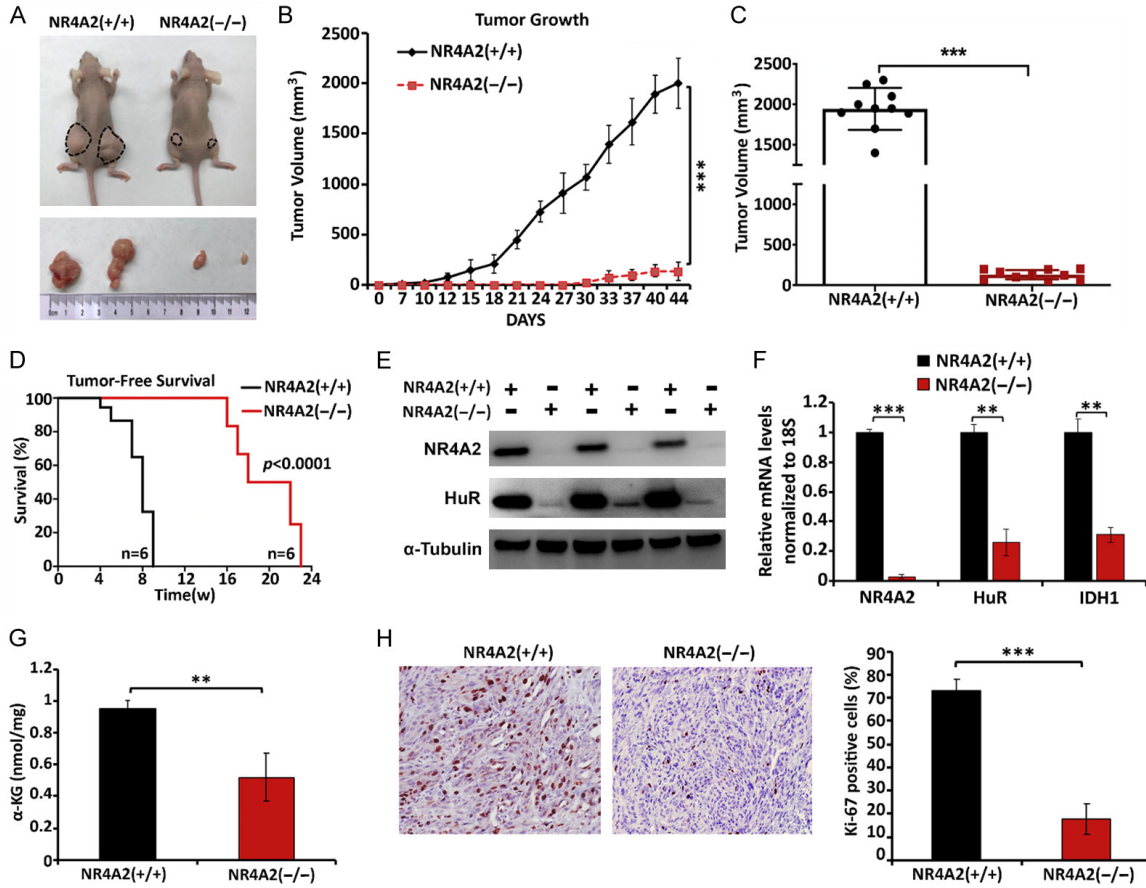
### HuR regulates NR4A2 expression

Since HuR also plays an important role in regulation of gene expression, we investigated the effects of HuR silencing on expression of NR4A family members NR4A1, NR4A2 or NR4A3 and observed that NR4A2 but not NR4A1 and NR4A3 expression were affected by HuR knockdown (**Figure 5A**). Immunoprecipitation of MiaPaCa2 cell lysate with HuR antibodies showed NR4A2 but not NR4A1 or NR4A3 mRNAs were enriched in the precipitate (**Figure 5B**) and the antibody specificity is indicated in **Figure 5C**. This confirms that among NR4A proteins NR4A2 preferentially is targeted by and interacts with HuR. Knockdown of HuR by RNAi also significantly decreased NR4A2 protein in MiaPaCa2 and Panc1 cells (**Figure 5D**). Bioinformatics analysis shows that there are 2-4 HuR binding sites on the 3'UTR of NR4A2 however studies in MiaPaCa2 cells with the 3'-UTR of NR4A2 did not reveal any functional interactions in HuR with the NR4A2-3'-UTR and the mechanism of NR4A2 regulation by HuR are currently being investigated.

### NR4A2 knockout suppresses tumor growth in vivo

To determine the effect of NR4A2 expression on pancreatic tumor growth in vivo, we used female athymic nude mice bearing wild-type and CRISPR/Cas9-derived NR4A2-knockout MiaPaCa2 cells as xenografts. Mice bearing NR4A2 knockout cells exhibited a 12.6-fold decrease in tumor volume ( $150 \text{ mm}^3$ ) compared to tumors derived from wild type cells ( $1200 \text{ mm}^3$ ) (**Figure 6A-C**). At the end of the experiment, a second group of mice were monitored until tumors reached a size of approximately  $2000 \text{ mm}^3$  and the results show that loss of NR4A2 significantly enhanced tumor free survival (**Figure 6D**). Immunoblot analysis of the tumor lysates validated NR4A2 knock-down in NR4A2<sup>(-/-)</sup> group vs NR4A2<sup>(+/+)</sup> group and this was accompanied by downregulation of HuR which is consistent with our in vitro data (**Figures 6E and 2D**). Results of NR4A2 knock-down also decreased expression of HuR and IDH1 mRNA in tumors (**Figure 6F**) and this was also accompanied by decreased expression of

# NR4A2-HuR axis is a novel target for treating pancreatic cancer



**Figure 6.** NR4A2 knockout inhibits tumor growth in vivo. (A) Mice were injected with wild-type and CRISPR/Cas9-derived NR4A2 knockout MiaPaCa2 cells and tumor growth was monitored for 6 weeks. Images represent the tumor size at the end of the experiment. (B) Tumor growth associated with wild-type and NR4A2 knockout MiaPaCa2 cells as xenografts and (C) tumor volumes determined 6 weeks after initial injection. (D) Mice with tumors were examined for tumor free survival until the tumors were palpable. (E) Western blot analysis of HuR expression in tumors derived from wild-type (NR4A2<sup>+/+</sup>) and knockout (NR4A2<sup>-/-</sup>) MiaPaCa2 cells as xenografts. (F) Relative mRNA levels of NR4A2, HuR and IDH1 normalized to mRNA levels of 18S and (G)  $\alpha$ -ketoglutarate levels in tumors derived from wild-type and NR4A2 knockout MiaPaCa2 cells. (H) Immunohistochemistry images and Percentage analysis of Ki-67 in 10% neutral buffer formalin fixed and paraffin embedded (FFPE) tissue sections of tumors derived from wild-type and NR4A2 knockout MiaPaCa2 cell xenografts. Quantitated results are means  $\pm$  SE for at least 3 replicate determinations; significance, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

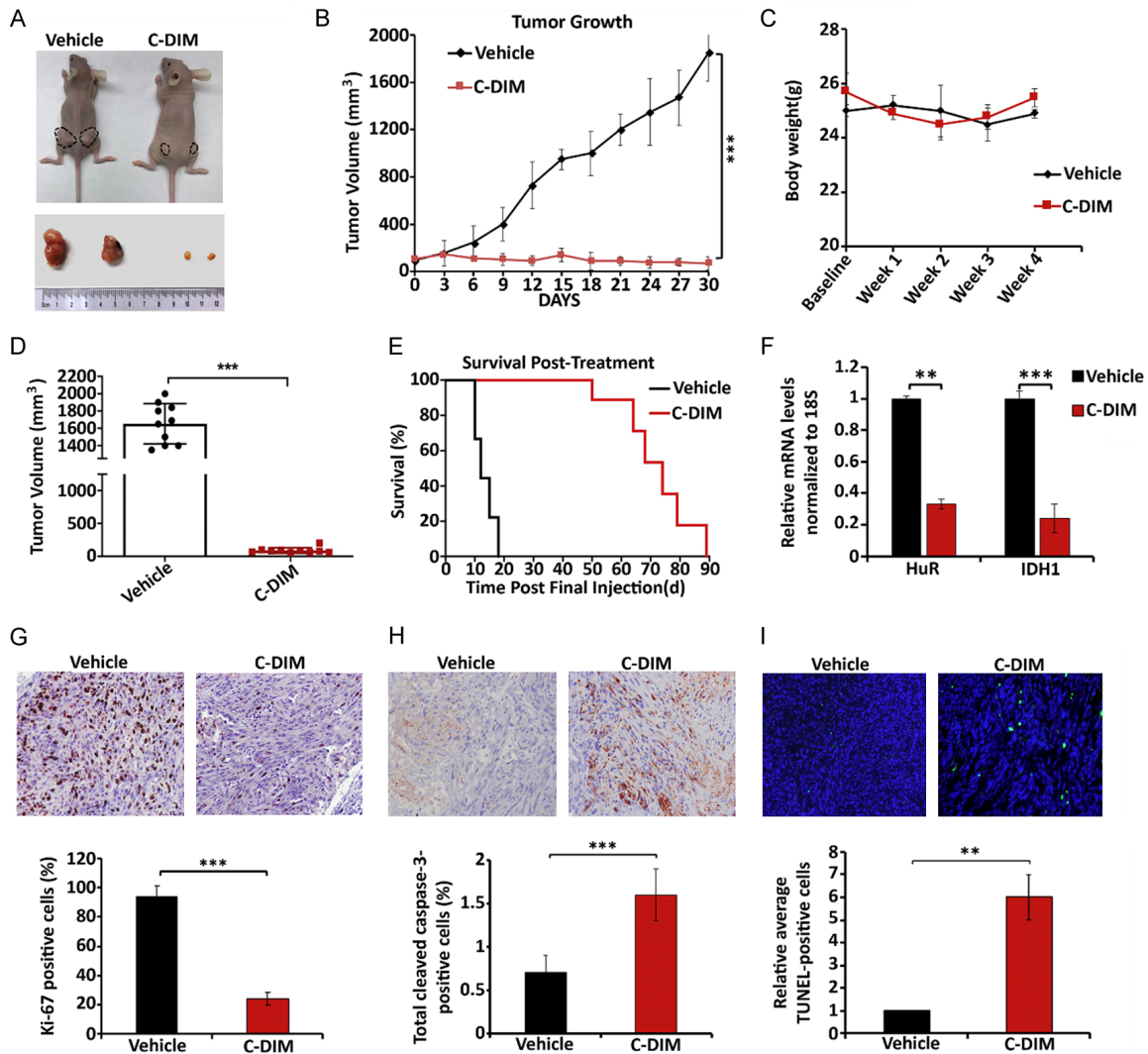
$\alpha$ -ketoglutarate ( $\alpha$ K6) (Figure 6G) which is dependent on IDH1-mediated conversion of isocitrate to  $\alpha$ -ketoglutarate. Immunohistochemical analysis of the Ki-67, marker for cell proliferation showed that knockdown of NR4A2 also decreased the percentage of Ki-67 positive cells (Figure 6H) indicating that NR4A2 plays a role in tumor growth.

*NR4A2 inverse agonist has a potent effect on pancreatic cancer cells, in-vivo*

We also investigated effects of the NR4A2 inverse agonist C-DIM12 on pancreatic tumors in an athymic nude mouse model. Mice bearing

MiaPaCa2 cells as xenografts were injected (ip) with C-DIM12 (50 mg/kg) and vehicle (corn oil) three times a week. Over the course of the experiment, C-DIM12 significantly decreased tumor growth (Figure 7A and 7B) but did not affect body weight (Figure 7C); organ weights were also unchanged and pathology was unchanged. C-DIM12 also decreased tumor volumes (Figure 7D) and in a longer-term survival experiment where the drug treatment was terminated after 30 days, mice in the treatment group exhibited longer survival than the control mice (Figure 7E). We did not observe any changes in organ weights or organ damage,

## NR4A2-HuR axis is a novel target for treating pancreatic cancer



**Figure 7.** C-DIM12 inhibits tumor growth in vivo. (A) Mice were injected with MiaPaCa2 cells and were treated with vehicle and C-DIM12 (50 mg/kg) when tumors reached the size of 100 mm<sup>3</sup>. Images represent the tumor size at the end of the experiment. (B) Tumor growth curve for MiaPaCa2 xenografts in nude mice, treated with 50 mg/kg C-DIM12 or vehicle control 3 times a week and (C) body weight changes in nude mice, treated with 50 mg/kg C-DIM or vehicle control 3 times a week over a period of 4 weeks and (D) scatter plots showing tumor volumes in treated and control group determined 6 weeks after initial injection. (E) Survival was evaluated after the last injection of C-DIM12. (F) Relative HuR and IDH1 mRNA levels in tumors derived from MiaPaCa2 cell xenografts (vehicle control and C-DIM12 treated). (G) Percentage of Ki-67 positive cells in tumors from vehicle control and C-DIM12 treated mice bearing MiaPaCa2 cells xenografts and Ki-67 positive cells in tumors. (H) Percentage of caspase-3 positive cells in vehicle control and C-DIM12 treated mice and (I) relative average TUNEL-positive cells in vehicle control and C-DIM treated mice. Quantitated results are means  $\pm$  SE for at least 3 replicate determinations; significance, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

and this is consistent with results of previous studies with this compound [10, 17, 34-36]. Treatment with C-DIM12 downregulated expression of HuR and IDH1 (Figure 7F) and immunohistochemical analysis showed that C-DIM12-dependent inactivation of NR4A2 decreased expression of Ki-67 (Figure 7G). Tumors from the C-DIM12 treated mice exhibited

an increased percentage of cleaved Caspase-3 positive cells (Figure 7H) and enhanced TUNEL staining (Figure 7I) indicating that the tumor growth inhibiting effects of C-DIM12 were also due to induced apoptosis. Results for the NR4A2 antagonist C-DIM12 paralleled those observed after NR4A2 knockdown in MiaPaCa2 cells (Figure 6).



### Discussion

PDAC is one of the deadliest tumors and is typically diagnosed in the later stages of tumor development and treatment options in terms of surgery and chemotherapy have limited effectiveness [1, 4]. The sequence of oncogene expression and mutations of tumor suppressor genes that lead to formation and metastasis of pancreatic tumors has been determined and some of these genetic alterations and their functions have been confirmed in mouse models [38-40]. However, clinical development of specific agents that target pro-oncogenic factors expressed in pancreatic cancer has not been robust and despite its limited effectiveness gemcitabine remains the first line treatment for pancreatic cancer. It has previously been demonstrated that the orphan nuclear receptor NR4A1 is highly expressed in multiple solid tumors including pancreatic cancer and NR4A1 knockdown results in decrease tumor growth, survival migration and invasion (rev. in 8). There is also evidence that NR4A2 exhibits comparable pro-oncogenic functions in solid tumor-derived cell lines [41-49] and a recent report shows that NR4A2 regulates growth, survival, migration, and invasion of patient derived glioblastoma cells [10].

Analysis of genomic data showed that NR4A2 is more highly expressed in PDAC vs normal pancreas and patients with high expression of NR4A2 exhibit decreased overall survival (Figure 1A). Knockdown and overexpression of NR4A2 in pancreatic cancer cells demonstrate that NR4A2 is pro-oncogenic as observed in glioblastoma and other cell lines [10, 41-49] and these results complement our recent study on the role of NR4A2 in pancreatic cancer [17]. NR4A1 and NR4A2 are primarily pro-oncogenic factors in solid tumors whereas results of breeding of NR4A1<sup>+/-</sup> with NR4A3<sup>-/-</sup> mice show that NR4A1 is a tumor suppressor in blood-derived tumors [7] and the reasons for differences in the roles of NR4A sub-family members in blood-derived and solid tumors is unknown. However, in pancreatic cancer cells both NR4A2 and NR4A1 exhibit pro-oncogenic activity and regulate cell growth survival and invasion/migration and ligands for both receptors inhibit these responses. Thus, both NR4A2 and NR4A1 ligands that act as receptor inhibitors or inverse agonist in pancreatic cancer

cells represent novel mechanism-based anti-cancer agents with potential for clinical applications for treating patients overexpressing the orphan receptors.

NR4A2 regulated genes that dictate the pro-oncogenic functions of this receptor include genes associated with autophagy [17] and changes in gene expression after knockdown of NR4A2 were further analyzed in MiaPaCa2 cells. Metabolite Set Enrichment Analysis indicated that NR4A2 regulated genes are involved in multiple pathways including the tricarboxylic acid cycle. Previous studies reported that both HuR and IDH1 were highly expressed in pancreatic tumors and regulation of IDH1 by HuR plays a role in pancreatic cancer cell survival [5, 18, 20, 49, 50]. Results of RNAseq show that both IDH1 and HuR are decreased after NR4A2 knockdown, and we further investigated the mechanisms of NR4A2 regulation of HuR/IDH1 and the potential for targeting both pro-oncogenic factors with the NR4A2 inverse agonist C-DIM12. The results of RNA interference studies show that NR4A2 directly regulates HuR and IDH1 gene expression through interactions of NR4A2 with their respective promoters (Figure 2). Moreover, treatment of pancreatic cancer cells with C-DIM12 mimicked the effect of NR4A2 knockdown and decreased interaction of NR4A1 with the HuR and IDH1 promoters (Figure 4) demonstrating that the NR4A2 ligands can be used to simultaneously target both HuR and IDH1 in pancreatic cancers. These results complement previous studies in pancreatic cancer cells showing the NR4A1 also regulated IDH1 expression and an NR4A1 inverse agonist decreased expression of this gene [16]. Mechanistic studies show that NR4A1 regulation of several genes in pancreatic and other cancer cell lines involves NR4A1/Sp interactions at GC-rich promoter sites where NR4A1 acts as a nuclear cofactor of Sp1 or Sp4 [12, 16, 29-32]. This type of gene regulation has been observed for many other nuclear receptors which enhance Sp (Sp1 or Sp4) regulated gene expression by targeting cis-acting GC-rich elements in target gene promoters [51]. Results of Sp knockdown studies and treatments with mithramycin that blocks Sp-DNA interactions confirm that NR4A2/Sp4 regulates expression of HuR and IDH1 by direct interactions with GC-rich sequences in the HuR and IDH1 promoters (Figure 3). Thus, IDH1 expression is inhibited directly by the NR4A2

inverse agonist and indirectly by parallel down-regulation of HuR. Kang and coworkers previously showed the NF- $\kappa$ B regulated expression of HuR in gastric cancer cells [52] whereas C-DIM12/NR4A1 inhibited NF- $\kappa$ B in neuronal cell lines [53]. In this study we did not investigate the role of NF- $\kappa$ B in the NR4A2-dependent inhibition of HuR expression in pancreatic cancer cells. Differences in the effects of NF- $\kappa$ B on regulation of HuR could be due to several possibilities including cell context dependent variability of nuclear factors and chromatin structure and these will be investigated in future studies.

Previous studies have characterized the important functions of HuR in pancreatic cancer which include a role in enhancing cell growth, migration, and invasion [5, 18, 20, 52]. Since these functions overlap with those observed for NR4A2 in this study we hypothesized that the functions of HuR may be due, in part, to regulation of NR4A2 expression. This hypothesis was confirmed by studies showing that HuR specifically regulates and interacts with NR4A2 but not NR4A1 or NR4A3 expression in pancreatic cancer cells (**Figure 5**) however the mechanisms of HuR regulation of NR4A2 were not determined and are currently being investigated. In this study we also demonstrate the crosstalk between NR4A2 and HuR and that drugs targeting either gene represent a unique approach for treating pancreatic cancer. These results complement our recent study which showed that NR4A2 regulated autophagy through the autophagic genes ATG7 and ATG12, and enhanced gemcitabine resistance and these could be reversed by knockdown of NR4A2 or treatment with C-DIM12 [17]. Thus, we have further demonstrated the efficacy of targeting NR4A2 with the NR4A2 inverse agonist C-DIM12 which is a potent inhibitor of pancreatic tumor growth (**Figure 7**). Our results in this study and our previous report showing that C-DIM12 decreased gemcitabine resistance in pancreatic cancer cells [17] suggest that the bis-indole derived NR4A2 ligands in combination with gemcitabine may be a highly effective combination therapy for treating pancreatic cancer patients.

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### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Stephen Safe, Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, USA. Tel: 979-845-5988; E-mail: ssafe@cvm.tamu.edu

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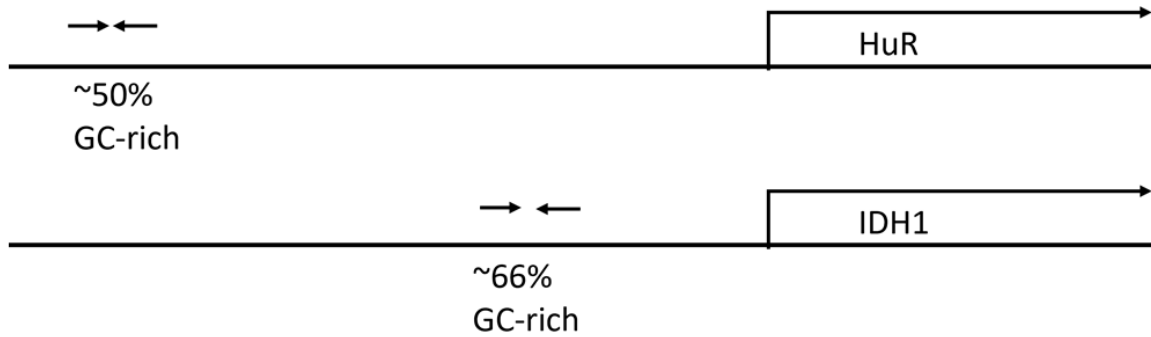


# NR4A2-HuR axis is a novel target for treating pancreatic cancer

**Supplementary Table 1.** Sources of oligonucleotides

A. Sources of siRNAs (life technologies)	
siRNA oligos	Catalog
siNR4A2	s9787
siELAVL1/HuR	s4609
siSp1	s13319
siSp3	s13325
siSp4	s13327
siCTRL	AM4635

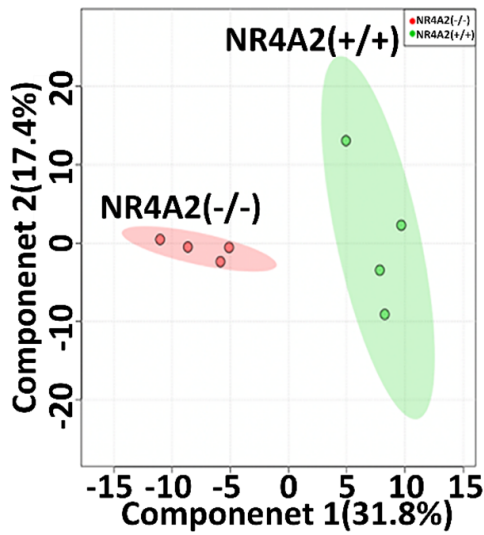
B. ChIP primers	
IDH1	HuR
ChIP F1: GAGCAAGGCCCTGTCTTAAA	ChIP F1: GCAAGACCTCATCGCTACAA
ChIP R1: GTTACCTGCCGGGATGATATG	ChIP R1: CTCCCAAAGAGCTGGGATTAC
ChIP F2: TGGAAAATCGAGGGGTGGAA	ChIP F2: CATCCAGGTGAGGCGGTG
ChIP R2: TATCACCCGTCCTATGGCC	ChIP R2: GTAGCTGGCCCCACTCTC
ChIP Primer 1:	ChIP Primer1:
Forward: GAGCAAGGCCCTGTCTTAAA	Forward: GCAAGACCTCATCGCTACAA
Reverse: GTTACCTGCCGGGATGATATG	Reverse: CTCCCAAAGAGCTGGGATTAC



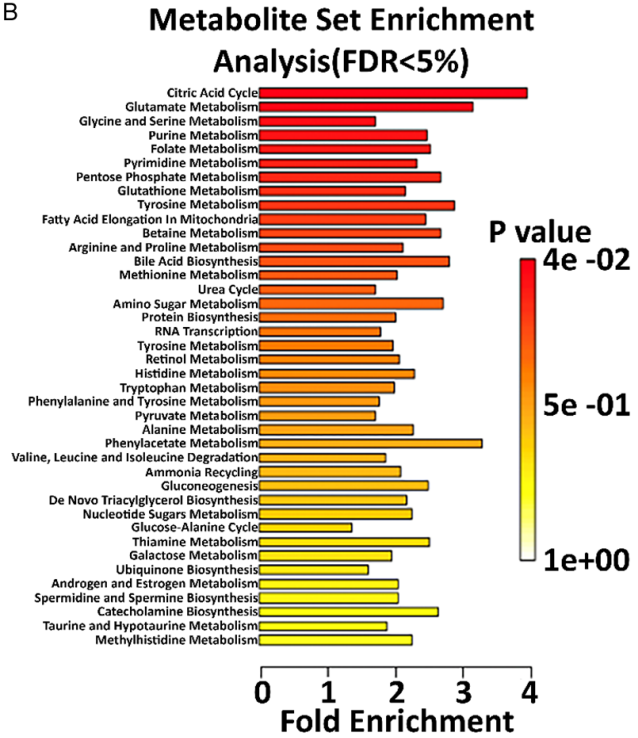
C. RT-PCR primers (life sciences)	
NR4A2	Forward: CGACATTCTGCCTTCTCC Reverse: GGTAAGTGTCCAGGAAAAG
IDH1	Forward: CTATGATGGTGACGTGCAGTG Reverse: CCTCTGCTTCTACTGTCTTGCC
HuR	Forward: AACGCCTCCTCCGGCTGGTG Reverse: GCG GTA GCC GTTCAGGCTGG

NR4A2-HuR axis is a novel target for treating pancreatic cancer

A



B



**Supplementary Figure 1.** NR4A2 knockout induces alterations in multiple genes and pathways in MiaPaCa2 cells. A. Partial least squares discriminant analysis (PLS-DA) plot analyzed by LC-MS/MS performed on NR4A2<sup>(+/+)</sup> and NR4A2<sup>(-/-)</sup> cells. B. Metabolite set enrichment analysis in NR4A2 knockout MiaPaCa2 cells.