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### EDITORIAL

- 6348** Biomarkers for gastrointestinal adverse events related to thiopurine therapy  
*Zudeh G, Franca R, Stocco G, Decorti G*

### REVIEW

- 6357** Fully covered metal biliary stents: A review of the literature  
*Lam R, Muniraj T*
- 6374** Intraoperative use of indocyanine green fluorescence imaging in rectal cancer surgery: The state of the art  
*Peltrini R, Podda M, Castiglioni S, Di Nuzzo MM, D'Ambra M, Lionetti R, Sodo M, Luglio G, Mucilli F, Di Saverio S, Bracale U, Corcione F*

### MINIREVIEWS

- 6387** Transcription factors specificity protein and nuclear receptor 4A1 in pancreatic cancer  
*Safe S, Shrestha R, Mohankumar K, Howard M, Hedrick E, Abdelrahim M*
- 6399** Artificial intelligence for the early detection of colorectal cancer: A comprehensive review of its advantages and misconceptions  
*Viscaino M, Torres Bustos J, Muñoz P, Auat Cheein C, Cheein FA*
- 6415** Faecal immunochemical test outside colorectal cancer screening?  
*Pin-Vieito N, Puga M, Fernández-de-Castro D, Cubiella J*

### ORIGINAL ARTICLE

#### Basic Study

- 6430** Fecal metabolomic profiles: A comparative study of patients with colorectal cancer vs adenomatous polyps  
*Nannini G, Meoni G, Tenori L, Ringressi MN, Taddei A, Niccolai E, Baldi S, Russo E, Luchinat C, Amedei A*

#### Retrospective Cohort Study

- 6442** High total Joule heat increases the risk of post-endoscopic submucosal dissection electrocoagulation syndrome after colorectal endoscopic submucosal dissection  
*Ochi M, Kawagoe R, Kamoshida T, Hamano Y, Ohkawara H, Ohkawara A, Kakinoki N, Yamaguchi Y, Hirai S, Yanaka A, Tsuchiya K*

#### Retrospective Study

- 6453** Effects of acute kidney injury on acute pancreatitis patients' survival rate in intensive care unit: A retrospective study  
*Shi N, Sun GD, Ji YY, Wang Y, Zhu YC, Xie WQ, Li NN, Han QY, Qi ZD, Huang R, Li M, Yang ZY, Zheng JB, Zhang X, Dai QQ, Hou GY, Liu YS, Wang HL, Gao Y*

- 6465** Magnetic resonance imaging-radiomics evaluation of response to chemotherapy for synchronous liver metastasis of colorectal cancer

Ma YQ, Wen Y, Liang H, Zhong JG, Pang PP

#### Observational Study

- 6476** Deep learning *vs* conventional learning algorithms for clinical prediction in Crohn's disease: A proof-of-concept study

Con D, van Langenberg DR, Vasudevan A

- 6489** Serum soluble suppression of tumorigenicity 2 as a novel inflammatory marker predicts the severity of acute pancreatitis

Zhang Y, Cheng B, Wu ZW, Cui ZC, Song YD, Chen SY, Liu YN, Zhu CJ

#### CASE REPORT

- 6501** Monomorphic epitheliotropic intestinal T-cell lymphoma presenting as melena with long-term survival: A case report and review of literature

Ozaka S, Inoue K, Okajima T, Tasaki T, Ariki S, Ono H, Ando T, Daa T, Murakami K

#### CORRECTION

- 6511** Correction to "Effect of probiotic *Lactobacillus plantarum* Dad-13 powder consumption on the gut microbiota and intestinal health of overweight adults". *World J Gastroenterol* 2021; 27(1): 107-128 [PMID: 33505154 DOI: 10.3748/wjg.v27.i1.107]

Rahayu ES

#### LETTER TO THE EDITOR

- 6513** Preservation of the superior rectal artery in laparoscopic colectomy for slow transit constipation: Is it really associated with better outcomes?

Parra RS, Feres O, Rocha JJR

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## Transcription factors specificity protein and nuclear receptor 4A1 in pancreatic cancer

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### Abstract

Specificity protein (Sp) transcription factors (TFs) Sp1, Sp3 and Sp4, and the orphan nuclear receptor 4A1 (NR4A1) are highly expressed in pancreatic tumors and Sp1 is a negative prognostic factor for pancreatic cancer patient survival. Results of knockdown and overexpression of Sp1, Sp3 and Sp4 in pancreatic and other cancer lines show that these TFs are individually pro-oncogenic factors and loss of one Sp TF is not compensated by other members. NR4A1 is also a pro-oncogenic factor and both NR4A1 and Sp TFs exhibit similar functions in pancreatic cancer cells and regulate cell growth, survival, migration and invasion. There is also evidence that Sp TFs and NR4A1 regulate some of the same genes including survivin, epidermal growth factor receptor, PAX3-FOXO1,  $\alpha$ 5- and  $\alpha$ 6-integrins,  $\beta$ 1-,  $\beta$ 3- and  $\beta$ 4-integrins; this is due to NR4A1 acting as a cofactor and mediating NR4A1/Sp1/4-regulated gene expression through GC-rich gene promoter sites. Several studies show that drugs targeting Sp downregulation or NR4A1 antagonists are highly effective inhibitors of Sp/NR4A1-regulated pathways and genes in pancreatic and other cancer cells, and the triterpenoid celastrol is a novel dual-acting agent that targets both Sp TFs and NR4A1.

**Key Words:** Specificity protein; Nuclear receptor 4A1; Pancreatic cancer; Transcription factors; Ligand inhibitors; Nuclear receptor 4A antagonists

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**Core Tip:** Specificity protein (Sp), transcription factors (TFs), Sp1, Sp3 and Sp4, and nuclear receptor 4A1 (NR4A1, Nur77) are highly expressed in pancreatic cancer cells and tumors. Results of gene silencing studies show that Sp TFs and NR4A1 are pro-oncogenic and regulate pathways/genes associated with cell proliferation, survival and migration/invasion. Bis-indole derived ligands (CDIMs) that bind NR4A1 act as NR4A1 antagonists and we discuss an important mechanism of gene regulation by NR4A1/Sp complexes which can be inhibited by NR4A1 antagonists.

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## INTRODUCTION

Pancreatic cancer is a devastating disease and it is estimated that in 2020, the number of diagnosed cases in the United States will be 57600 and 47050 will die because of this disease[1]. Despite advances in treating pancreatic cancer with surgical intervention and various therapeutic regimens, the five-year survival rate from pancreatic cancer is < 10%[1] and this is due, in part to the late detection of the tumors due to lack of symptoms and lack of early biomarkers of this cancer. There are many risk factors for pancreatic cancer and this includes pancreatitis, obesity and metabolic syndrome; due to the obesity crisis in many countries, it is estimated that by 2030, pancreatic cancer will be the second leading cause of cancer deaths[2]. Development of pancreatic cancer is associated with the temporal increases in expression of various oncogenes and inactivation of tumor suppressor genes resulting in enhanced cancer cell proliferation, survival, migration, invasion and metastasis[3-6]. Gemcitabine has largely replaced 5-fluorouracil as the major cytotoxic drug for treatment of pancreatic cancer and many other mechanism-based drugs targeting critical genes associated with various pro-oncogenic pathways in pancreatic cancer are being developed or are in clinical trials[7, 8]. The success of these agents in combination therapies targeting one or more pro-oncogenic pathways has been limited but promising. For example, one study using the vascular endothelial growth factor inhibitor bevacizumab in combination with leucovorin, nab-paclitaxel, oxiplatin and 5-fluorouracil and this resulted in a one-year survival of 82% for the treated patients[9]. In this review, we will focus on three transcription factors (TFs), namely specificity proteins (Sps) Sp1, Sp3 and Sp4, and nuclear receptor 4A1 (NR4A1, TR3, Nur77) and their overlapping pro-oncogenic roles in pancreatic cancers. We will also highlight an interesting convergence of these TFs and outline approaches for simultaneous targeting NR4A1 and Sp TFs by mechanism-based agents resulting in inhibition of cancer cell growth, survival, migration and invasion.

### Sp and NR4A1: Background

Sp TFs are members of the Sp-Kruppel-like factor family (Sp/KLF) of TFs which includes 9 Sp TFs and 17 KLF proteins[10-14]. Sp1-Sp4 genes have a similar domain structure, however, it is evident that among other Sp/KLF family of TFs, their domain structures are highly diverse with the most common features being the zinc finger DNA binding domain and its subsequent interactions with GC/GT-rich gene promoter sequences[10-14]. Sp1 was the first TF identified and characterized[15] and Sp1 knockout mouse embryos exhibited retarded development and other abnormalities, and embryo lethality is observed (day 11)[11]. Comparable studies on other Sp family members indicate that these TFs play important roles in early development and differentiation by their regulation of key genes involved in these functions. A detailed analysis of the age-dependent expression of individual Sp TFs is not available, however, there is evidence that for Sp1, there is an age-dependent decrease in expression in both rodent and human tissues[16-19]. Presumably the functions of Sp1 in aging animals are replaced by other TFs. The concern and subsequent focus on Sp1 and other Sp TFs (Sp3 and Sp4) in cancer emerged from studies showing that Sp1, Sp3 and Sp4 were overexpressed in cancer cells and tumors

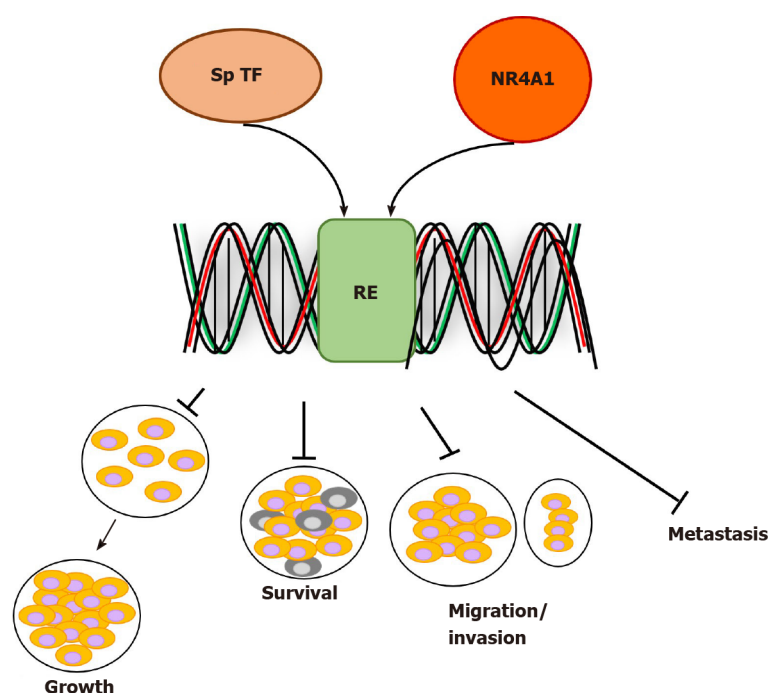
[20]. These TFs were also identified as negative prognostic factors for cancer patient survival from several cancers and functional studies demonstrated the pro-oncogenic activity for Sp TFs[20]. The overexpression and prognostic value of Sp TFs have primarily focused on Sp1 and one of the first studies showed that Sp1 was overexpressed in patients with pancreatic tumors and was a negative prognostic factor for patient survival[21]. Most studies on cancer patients have reported that Sp1 or Sp3 is overexpressed and/or is a negative prognostic factor for glioma, astrocytoma, colon, gastric, liver, prostate and head and neck cancers; in lung and breast cancers; in addition, there are some conflicting results[22-40]. An important linkage between Sp1 and cancer was observed in studies showing that carcinogen- or oncogene- induced transformation of human fibroblasts into fibrosarcomas was accompanied by an 8-18-fold increase in expression of Sp1. Moreover, the ability of fibrosarcoma cells to form tumors in athymic nude mice was abrogated after Sp1 knockdown[41]. Subsequent studies show that Sp TFs are pro-oncogenic and regulate pathways and genes associated with cell growth, survival, migration and invasion (Figure 1).

NR4A1 and two related receptors NR4A2 (Nurr1) and NR4A3 (Nor1) are orphan nuclear receptors with structures and endogenous functions that differ significantly from Sp TFs[42,43]. NR4A1 and NR4A3 knockout mice are viable whereas NR4A2<sup>-/-</sup> mice exhibit early mortality due to dopaminergic and other neuronal deficits[44-48]. Endogenous ligands for NR4A have not been identified, however, NR4A bind structurally diverse synthetic compounds and these receptors interact with nerve growth factor  $\beta$  response elements (NBREs: AAAGGTCA) and Nur response elements [NuRE: AAAT(GA)C/T/CA] as monomers and dimers respectively[49-51]. In addition, NR4A1 and NR4A2 form a heterodimer with retinoid X receptor and bind a DR5 motif[52,53]. NR4As are immediate early genes induced by diverse stressors and they play important roles in maintaining cellular homeostasis and in pathophysiology [42,43,54]. NR4A1 and Sp TFs are remarkably distinct in their structures and functions in maintaining cellular homeostasis, however, in pancreatic cancer and other tumor types, their functions are similar. Like Sp1, NR4A1 is overexpressed in solid tumors from patients with pancreatic, breast, liver, glioblastoma, ovarian, colon, melanoma, endometrial, cervical, rhabdomyosarcoma and gastric cancer[55-64]. Moreover, high expression of NR4A1 in tumors is a negative prognostic factor for lung, breast, colon and ovarian cancer patients[58-60,63]. The negative prognostic significance of NR4A1 overexpression is paralleled by studies showing that like Sp TFs, NR4A1 is pro-oncogenic and regulates pathways comparable to that described for Sp TFs (Figure 1). Thus, there is an interesting parallel in the expression, functions and prognostic values of Sp TFs (primarily Sp1) and NR4A1 in multiple tumor types. This review will focus on the individual roles and interactions of Sp TFs and NR4A1 in pancreatic cancer and this will include genes commonly regulated by NR4A1/Sp complex in which NR4A1/Sp1 and NR4A1/Sp4 interact at GC-rich gene promoter sites that bind Sp.

### Sp TFs and pancreatic cancer

Shi *et al*[65] first reported the overexpression of Sp1 in pancreatic cancer cell lines and in pancreatic tumors compared to non-tumor tissue and subsequent studies showed that Sp1, Sp3 and Sp4 were co-expressed in most pancreatic cancer cells[66]. The three structurally related TFs target similar GC-rich sequences in gene promoters and there is evidence for some genes, that Sp3 acts as a transcriptional repressor. A systematic study of the functions of Sp1, Sp3 and Sp4 was investigated in Panc1, MiaPaCa2 and L3.6pL pancreatic cancer cells and also in cell lines derived from lung, colon, kidney and breast tumors[66]. Figure 1 illustrates the functional effects of Sp1 knockdown which resulted in decreased pancreatic cancer cell growth, induced Annexin V staining (apoptosis) and decreased migration and this was accompanied by PARP cleavage, another marker of apoptosis. These results were somewhat surprising since it might be expected that the loss of Sp1 would be compensated for by Sp3 and Sp4. The functional properties of Sp3 and Sp4 were also determined in Panc1, L3.6pL and MiaPaca2 cells by knockdown and the results showed that like Sp1, knockdown of Sp3 or Sp4 decreased cell proliferation, induced apoptosis (increased Annexin V staining and cleaved PARP) and decreased cell invasion and similar results were observed in lung, breast, kidney and colon cancer cell lines[66]. These results indicated the importance of all three Sp TFs as independent pro-oncogenic factors that regulate pancreatic cell growth, survival and invasion, and related genes and it was suggested that Sp TFs were non-oncogene addiction genes[66].

Genomic analysis of altered gene expression after individual knockdown of Sp1, Sp3 and Sp4 in Panc1 pancreatic cancer cells further demonstrated their pro-oncogenic functions and unique effects of Sp1, Sp3 and Sp4 in this cell line. Figure 2A illustrates the total number of genes that are changed after individual knockdown of Sp1, Sp3



**Figure 1 Common functional properties of specificity protein transcription factors and nuclear receptor 4A1 in pancreatic and other cancer cells.** Specificity protein (Sp) transcription factors (TFs) (Sp1, Sp3 and Sp4) bind GC-rich gene promoter sequences to activate gene expression associated with enhanced cell proliferation, survival, migration, invasion and metastasis. Nuclear receptor 4A1 (NR4A1) binds as monomer or homodimers to NBRE and NuRE promoter elements and NR4A1 also acts as a cofactor for Sp1 and Sp4-regulated genes and NR4A1/Sp binds GC-rich promoter sequences. Sp: Specificity protein; TFs: Transcription factors; NR4A1: Nuclear receptor 4A1.

and Sp4, the number of uniquely modified genes and the number of genes commonly repressed or induced by Sp1-Sp3, Sp1-Sp4 and Sp3-Sp4. Knockdown of Sp3 and Sp4 resulted in the highest number (and percentage) of commonly induced or repressed genes. Causal Ingenuity Pathway Analysis (IPA) of the genes affected by knockdown were sorted into functional groups and **Figure 2B-D** illustrate the overlap in affected genes in the Sp1-Sp3, Sp1-Sp4 and Sp3-Sp4 groups associated cell growth, survival and cellular movement (migration and invasion). The overall number and percentage of commonly induced or repressed genes was highest for Sp3 and Sp4 in each functional category and this was similar to that observed for the total number of commonly induced or repressed genes (**Figure 2A**). Quantitative results from the causal IPA confirmed that the genomic changes observed in the array data were consistent with the functional changes observed after individual knockdown of Sp1, Sp3 and Sp4. This study also confirmed that knockdown of Sp1 alone or Sp1/3/4 (combination) in L3.6pL pancreatic cancer cells also decreased the growth of tumors in athymic nude mice bearing L3.6pL cells as xenografts[66].

In addition to the array studies noted above, there are a number of studies on Sp knockdown or overexpression in pancreatic cancer cells that have identified other Sp-regulated genes that contribute to the pro-oncogenic functions of Sp1, Sp3 and Sp4, and this includes regulation of pro-oncogenic long non-coding RNAs (MALAT1) and microRNAs (**Table 1**)[20,66-69]. These data were obtained only in pancreatic cancer cells; however, similar results for many of these genes have also been observed in other cancer cell lines[20]. Results summarized in **Table 1** were observed in multiple pancreatic cancer cell lines and for some genes, their regulation by Sp TFs is cell context-dependent and there are also differences in the roles of Sp1, Sp3 and Sp4. Some studies used a combination of oligonucleotides targeting the 3 TFs (siSp1/3/4) and the combined knockdown of Sp1, Sp3 and Sp4 by RNA interference in Panc28 and L3.6pL pancreatic cancer cells decreased expression of NFκB-p65 and NFκB-p50 proteins[70]. In the former cell line, individual knockdown of Sp1, Sp3 or Sp4 partially decreased expression of both NFκB subunits. In L3.6pL cells, Sp1 and to a lesser extent Sp3, but not Sp4 knockdown decreased NFκB-p65 whereas Sp4 knockdown was primarily responsible for decreased expression of NFκB-p50 protein.

### NR4A1 and pancreatic cancer

The pro-oncogenic role of NR4A1 has been investigated in multiple solid tumor



**Table 1 Specificity protein regulated genes in pancreatic cancer cells**

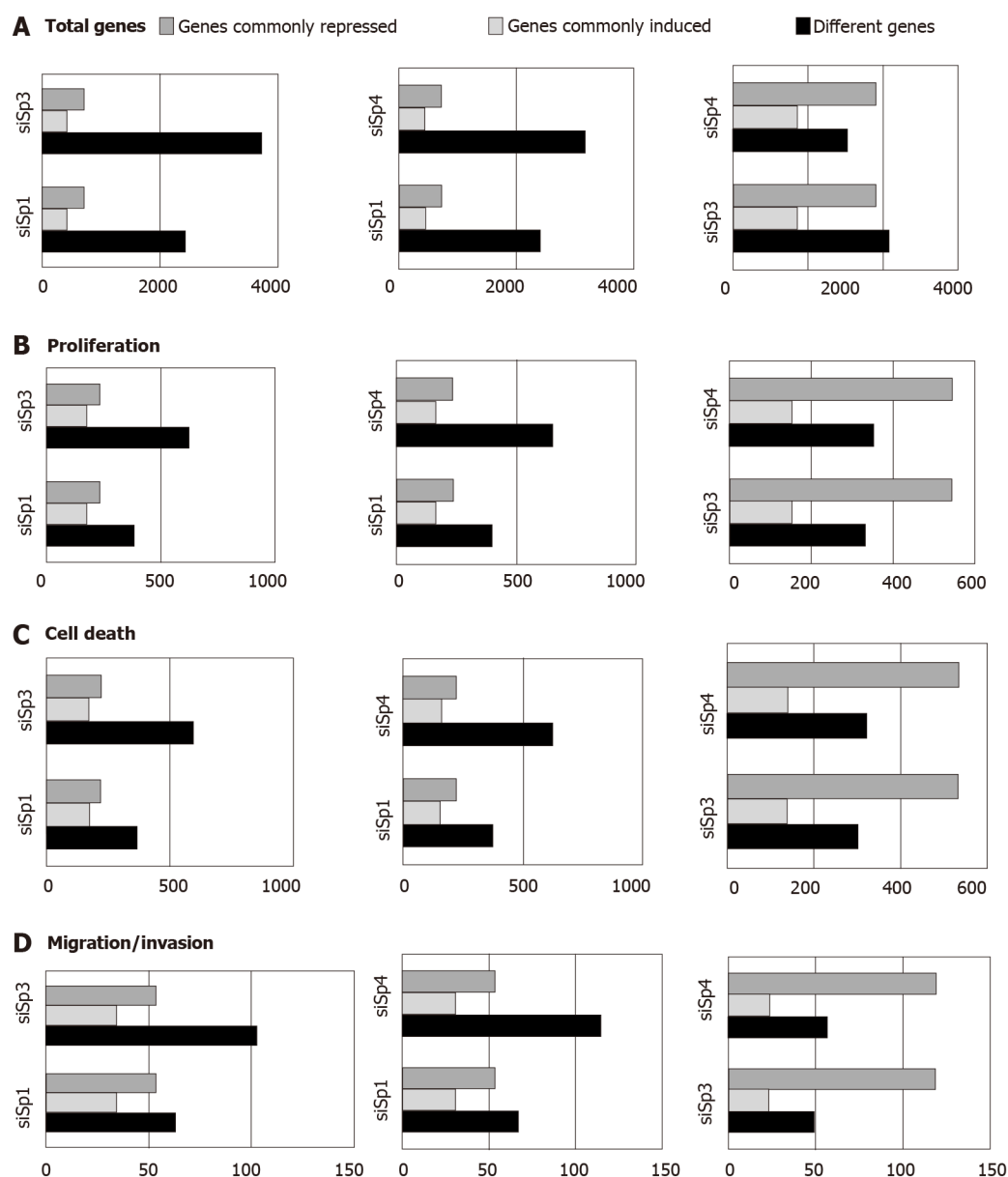
Functional	Sp-regulated gene
Cell proliferation and cell cycle progression	EGFR, IGFR, <i>cyclin D1</i> , PIK3R1, <i>pmTOR</i> , 4-EBP, <i>pS6RP</i> , STAT3, <i>pSTAT3</i> , NfκB- <i>p65/950</i>
Survival and apoptosis	<i>Survivin</i> , <i>bcl-2</i> , PARP cleavage, APAF1, ATM, TNFRSF25, COX2, NfκB- <i>p65/5</i>
Cell migration/movement EMT	<i>B1-integrin</i> , <i>α2-integrin</i> , MTSS1, <i>vimentin</i>
Angiogenesis	VEGF, VEGFR1, VEGFR2, COX2
Other pro-oncogenic factors	TNFα, BRCA1, ARHGEF2, <i>Ajuba</i> , MALAT-1, RAS-6TP, FAS

A detailed analysis of specificity protein (Sp)-regulated genes (Sp1, Sp3 and Sp4) in Panc1 cells based on RNAseq after Sp knockdown has been reported [66] and reviewed[20]. Sp: Specificity protein; EMT: Epithelial-mesenchymal transition.

derived cancer cell lines (rev. in 70), however, the number of publications on the functions of NR4A1 in pancreatic cancer is limited[55,71,72]. Knockdown of NR4A1 in pancreatic cancer cells inhibits cancer cell and tumor growth in athymic nude mouse xenograft studies, induces apoptosis and inhibits migration/invasion. Thus, the pro-oncogenic functions of NR4A1 and Sp TFs in pancreatic cancer cell lines are similar (Figure 1) and comparable results were obtained after their knockdown. For example, knockdown of NR4A1 by RNA interference in pancreatic cancer cells decreased expression of *bcl-2* and *survivin* and this was accompanied by markers of apoptosis including increased TUNEL staining, caspase-3 and PARP cleavage[55,71]. Proteome analysis confirmed that loss of NR4A1 enhanced cell death and decreased gene products associated with cell proliferation and this was accompanied by increased reactive oxygen species (ROS) and ROS-induced endoplasmic reticulum (ER) stress genes including CHOP, Grp78, ATF4 and XBP-1s[71]. In addition, genes such as thioredoxin domain containing 5 (TXNDC5) and isocitrate dehydrogenase 1 (IDH1) that maintain high reductant levels are also NR4A1 regulated genes and their loss after receptor knockdown triggers the ROS-ER stress response. Knockdown of NR4A1 also decreased cell migration and expression of β1- and α5-integrins which play a role in NR4A1-regulated pancreatic cancer cell migration[72]. Thus, there is overlap between the function of Sp TFs and NR4A1 in pancreatic cancer cell lines and studies in other cancer cell lines show that many of the Sp-regulated genes summarized in Table 1 are also regulated by NR4A1[70]. Regulation of the same genes by Sp TFs and NR4A1 was recently reported in breast cancer cells where knockdown of Sp4 or NR4A1 decreased expression of α6- and β1-integrins in MDA-MB-231 and SKBR3 breast cancer cells. The effects of Sp3 and Sp1 knockdown on expression of these integrins was gene- and cell context-specific.

### **Mechanistic convergence of NR4A1 and Sp TFs**

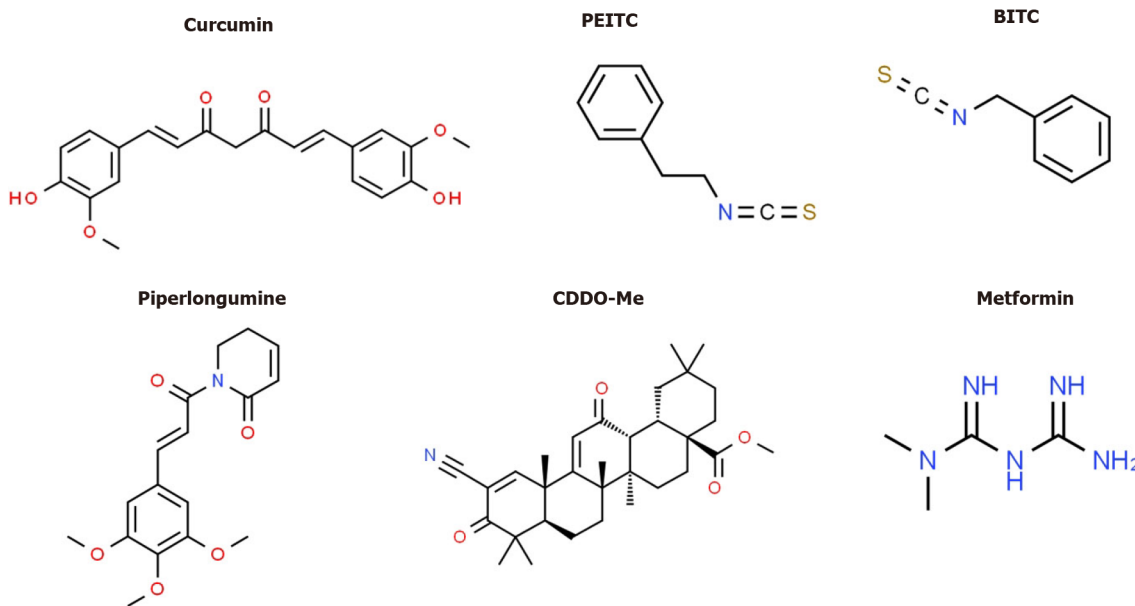
Both NR4A1 and Sp TFs are nuclear proteins that interact with defined cis-elements in their target gene promoters. Mechanistic studies showing cooperative activation of common genes by NR4A1 and Sp1 was first observed in pancreatic cancer cells and subsequently investigated and confirmed in other cancer cell lines[55,72]. The *survivin* gene has a GC-rich promoter that binds Sp TFs and has been extensively characterized as an Sp-regulated gene[73,74]. Knockdown of NR4A1 or treatment with NR4A1 antagonists decreased expression of *survivin* protein and mRNA and also decreased luciferase activity in pancreatic cancer cells transfected with GC-rich *survivin* promoter-luciferase constructs[55]. Further analysis of *survivin* regulation in pancreatic cancer cells showed that *survivin* is regulated by a p300/NR4A1/Sp1 complex where Sp1 binds the GC-rich *survivin* promoter, NR4A1 acts as a cofactor/coactivator and p300 is also part of the complex. The identification of this functional NR4A1/Sp complex is consistent with previous reports showing many other nuclear receptors also activate gene expression through interactions with Sp1. Studies in other cancer cell lines have identified other NR4A1-regulated genes that are regulated by NR4A1/Sp1, NR4A1/Sp4 or NR4A1/Sp1/4 and these include β1-, β3- and β4-integrins, α5- and α6-integrins, TXNDC5 and the PAX3-FOXO1 fusion oncogene and the checkpoint gene PD-L1 (rev. in 70). Thus, like *Ajuba*[75], NR4A1 acts as a cofactor for Sp TFs in pancreatic and other cancer cell lines and they coregulate some of the same genes (EGFR, *survivin* and IGF1R). The possible interactions of NR4A1 and *Ajuba*, a coregulator of Sp-regulated genes needs to be further investigated.



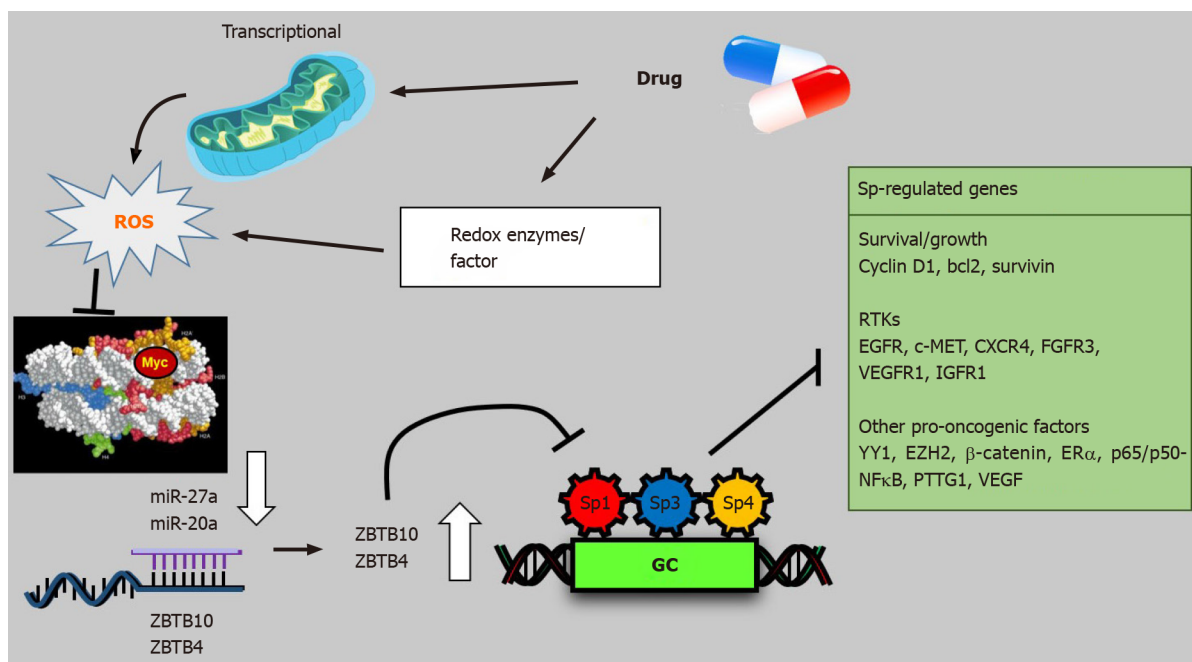
**Figure 2 Genomic analysis of Sp1, Sp3 and Sp4 regulated genes in pancreatic cancer cells.** A: The number of total genes regulated by specificity protein (Sp) transcription factors (TFs) in Panc1 cell was determined by individual Sp knockdown (siSp1, siSp3 and siSp4) and the number of unique (Different genes) and commonly repressed (Genes commonly repressed) and induced (Genes commonly induced) genes are indicated; B-D: Ingenuity Pathway Analysis analysis of genes that regulate cell proliferation (B), cell death (C) and migration/invasion (D) compares the number of unique and commonly repressed/induced genes by Sp1 and Sp3, Sp1 and Sp4 and Sp3 and Sp4 as described[66].

## DRUGS THAT TARGET SP TFS AND NR4A1

Several drugs that target Sp TFs in pancreatic cancer cells have been identified and these include tolferamic acid and structurally related compounds, COX2 inhibitors curcumin, metformin, piperlongumine, methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate (CDDO-Me), benzylisothiocyanate (BITC) and phenethylisothiocyanate (PEITC) (Figure 3)[20]. All of these compounds downregulate Sp1, Sp3 and Sp4 through different pathways. For example, tolferamic acid and COX2 inhibitors induce proteasome-dependent degradation of Sp1, Sp3 and Sp4 whereas metformin induces the dual specificity phosphatases MPK-1 and MPK-5 which are important for Sp downregulation. The phosphatases downregulate miR-27a which results in upregulation of ZBTB10, a Sp repressor which competitively displaces Sp TFs from GC-rich sites resulting in gene inactivation since ZBTB10 Lacks a transactivation domain. ROS-inducers including PEITC, BITC, piperlongumine, CDDO-Me and curcumin also decrease Sp-regulated gene expression through an ROS-mediated pathway which also involves induction of Sp repressors namely ZBTB4, ZBTB10 and ZBTB34[20] (Figure 4). This pathway involves ROS-dependent remodeling of repressor complexes



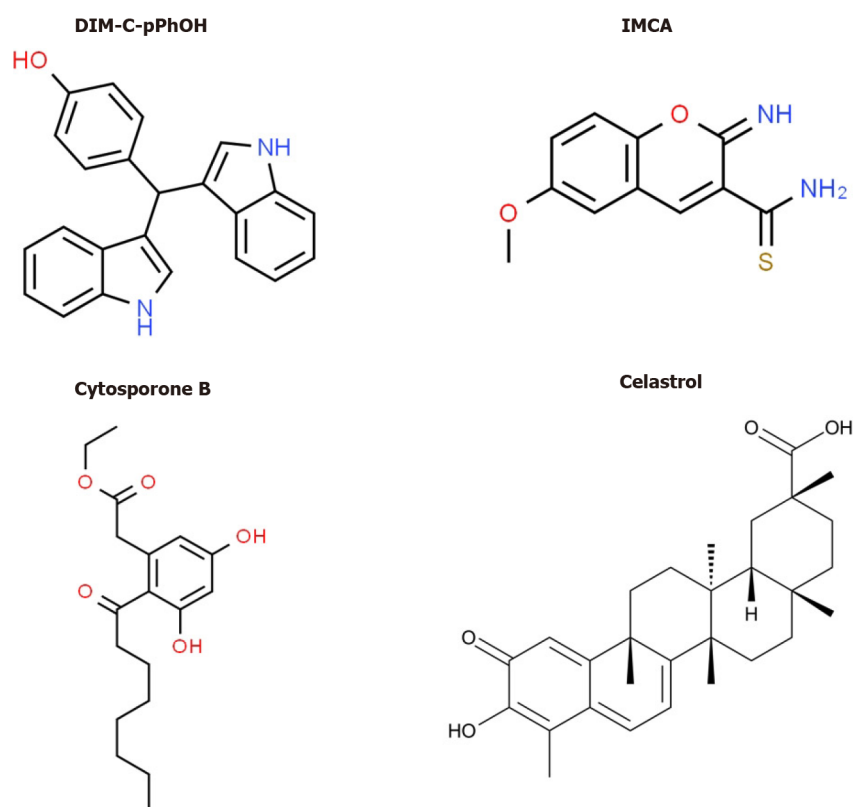
**Figure 3** Structures of agents that target downregulation of Sp1, Sp3 and Sp4 in pancreatic and other cancer cell lines[68]. The structurally diverse compounds act through multiple pathways in different cancer cell lines.



**Figure 4** Mechanism of reactive oxygen species-dependent downregulation of specificity protein transcription factors. Induction of reactive oxygen species results in epigenetic suppression of Myc, downregulation of microRNAs, induction of ZBTB suppressors and decreased expression of specificity protein (Sp) transcription factors and Sp-regulated genes[20]. ROS: Reactive oxygen species; Sp: Specificity protein.

which results in downregulation of cMyc and cMyc-dependent microRNAs (miR-27a, miR-17-92) and induction of ZBTBs. These compounds (Figure 4) and others induce similar patterns and mechanisms of Sp downregulation in other cancer cell lines[20], however, there are compound-specific differences in mechanisms that are cell context-dependent.

Bis (3'-indolyl)-1-(p-hydroxyphenyl) methane (DIM-C-pPhOH) (Figure 5) was identified as an NR4A1 Ligand that acts as a receptor antagonist and mimics the effects of NR4A1 knockdown in pancreatic and other cancer cell lines[70]. DIM-C-pPhOH and several 3,5-disubstituted phenyl analogs are inhibitors of cell proliferation, survival and migration/invasion and they inhibit expression of NR4A1/Sp- and other NR4A1-regulated genes. Moreover, many of the 3,5-disubstituted analogs of



**Figure 5 Nuclear receptor 4A1 ligands.** Structures of compounds that directly bind nuclear receptor 4A1 include DIM-C-pPhOH, IMCA, cytosporone B and celastrol[76-80].

DIM-C-pPhOH inhibit tumor growth in mouse xenograft models at doses < 5 mg/kg per day and ongoing studies have identified ligands that inhibit tumor growth at doses < 1mg/kg per day. Interestingly, several other classes of compounds bind and modulate NR4A1-dependent pathways/genes and these include cytosporone B and structurally related compounds, IMCA (2-imino-6-methoxy-2H-chromene-3-carbothiomide) and the triterpenoid celastrol and analogs[76-80] (Figure 5). Fascinatingly, celastrol also activates ROS-dependent downregulation of Sp1, Sp3 and Sp4 in bladder cancer cells[80] (*e.g.*, Figure 4) and uniquely acts as a bifunctional agent targeting both Sp TFs and NR4A1. Ongoing studies in this laboratory have identified other dual targeting agents with potential for clinical applications for pancreatic cancer therapy.

## CONCLUSION

In summary, it is evident that both Sp TFs and NR4A1 are pro-oncogenic factors that regulate pancreatic cancer cell and tumor growth and interactions of NR4A1 and Sp TFs are also important for regulating many critical genes (Figure 1) involved in pro-oncogenic functions. Although the functional importance of NR4A1/Sp regulated genes in cancer cell growth, survival, migration and invasion has been established in pancreatic and other cancers, clinical applications of drugs targeting Sp and NR4A1 are lacking. Several compounds have been identified that induce Sp downregulation or inhibit/antagonize NR4A1. Current studies in our laboratories are focused on identifying agents like celastrol that act simultaneously as NR4A1 antagonists and Sp downregulators as a new class of drugs that will enhance the effectiveness of current chemotherapies used for clinical treatment of pancreatic and other cancers.

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