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Extra-nuclear activity of INSM1 transcription factor enhances insulin receptor signaling pathway and *Nkx6.1* expression through RACK1 interaction

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Abstract

INSM1 is an islet transcription factor essential for pancreas development. INSM1 functions as a transcriptional repressor of *NeuroD/β2* and *insulin* gene in pancreas. INSM1 also possesses extra-nuclear activities through binding to multiple cellular regulators such as cyclin D1 and RACK1. In this study, we report that the interaction of INSM1 and RACK1 is essential to enhance the insulin receptor (InR)-mediated signaling pathway. A proline-rich region in the N-terminus of INSM1 is required for RACK1 binding, which interrupts RACK1-InR interaction and enhances InR signal activation. Binding of INSM1 to RACK1 increases AKT phosphorylation. The INSM1-enhanced AKT phosphorylation can be inhibited by the PI3K inhibitor, LY294002. When INSM1 induces AR42J cell trans-differentiation, the *Nkx6.1* gene is activated through the InR-mediated signaling pathway and an elevation of the acetyl-H4 modification on the *Nkx6.1* gene promoter/enhancer is observed. The PI3K inhibitor interrupts *Nkx6.1* and *insulin* gene expression. Therefore, we conclude that the extra-nuclear activity of INSM1 by enhancing PI3K/AKT signaling pathway is important for pancreatic cell differentiation.

Keywords

INSM1; InR signaling; RACK1; PI3K/AKT; *Nkx6.1*

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Author contributions

T.Z. and M.S.L. conceived and designed experiments. T.Z., C.C., N.A.S., K.S. and M.S.L. performed experiments. T.Z., M.B.B. and M.S.L. analyzed the data. T.Z., M.B.B. and M.S.L. wrote and reviewed the manuscript.

1. Introduction

INSM1¹ is a zinc-finger transcription factor originally isolated from a human insulinoma subtraction library [8]. INSM1 function is critical for pancreatic endocrine cell differentiation, which sustains proper islet hormone secretion [14]. Our data support that in the pancreatic endocrine transcriptional network, *INSM1* is situated downstream of neurogenin3 (*Ngn3*) and upstream of *NeuroD/β2* in the islet transcription factor (ITF) cascade [2;3]. In trans-differentiation studies using pancreatic duct and acinar cells conversion into insulin-positive cells, we showed that INSM1 can not only promote endocrine differentiation, but also activates other downstream ITFs such as Pax6 and Nkx6.1 [28;29]. *Insm1* gene ablation studies demonstrated that INSM1 is essential for pancreatic endocrine cell development [7;14]. Since INSM1 is a transcriptional repressor [4], it is intriguing to investigate how the transient expression of INSM1 during pancreas development exerts its functional role in endocrine differentiation. The molecular mechanism underlying the functional effects of INSM1 in pancreas development is still largely unclear. In our previous trans-differentiation study, we discovered that INSM1 does not merely function as a transcription factor. It possesses additional extra-nuclear activities, which can couple to various signaling pathways. We have shown that INSM1 possesses extra-nuclear activity through binding to cyclin D1 to induce cell cycle arrest [27]. Additionally, we found that INSM1 displays an extra-nuclear function through its involvement in the insulin receptor (InR)-mediated signal transduction pathway. InR-mediated signaling is important for pancreatic endocrine cells [31]. This study is particularly meaningful since a growing body of evidence suggests that insulin and InR signaling play a key role in fueling tumors, thus unraveling the obesity-diabetes-cancer connection [23]. Multiple studies have shown that an insulin-lowering drug known as metformin was associated with the modulation of InR signaling and showed a significant decrease in cancer incidence [16]. Since INSM1 is closely associated with neuroendocrine differentiation and tumors, we believe the connection of INSM1 to the InR signaling is important. The current study reveals that INSM1 physically interacts with an adaptor protein RACK1 (Receptor of Activated C Kinase 1). RACK1 was originally identified as a 36-kDa intracellular receptor for protein kinase C (PKC). It contains seven WD40 repeats, and is a β subunit of G-protein homologue [6].

In the present study, we use an AR42J trans-differentiation model to study the molecular mechanisms of INSM1 promoting cell signaling and gene activation. We identified multiple fragments of the RACK1 sequence that interacts with the INSM1 bait-vector isolated from a yeast two hybrid library screen [13]. At least two WD domains of the RACK1 protein and the proline-rich sequence at the N-terminus of INSM1 are required for the INSM1-RACK1 interaction. The INSM1-RACK1 binding can interrupt the RACK1-InR binding and relieve the RACK1 interference of the InR signaling. Thus, INSM1 can physically pull RACK1 away and enhance InR-mediated signal transduction by promoting AKT phosphorylation. We also examine an ITF, *Nkx6.1* during AR42J cell trans-differentiation. An INSM1-MutN failed to bind to RACK1 and failed to enhance InR signaling and induce *Nkx6.1* gene activation. Overall, the present study demonstrates that INSM1 possesses an extra-nuclear function by enhancing InR-mediated signaling, which facilitates endocrine cell differentiation and *Nkx6.1* gene activation.

¹The abbreviations used are: INSM1, insulinoma-associated antigen-1; Ngn3, neurogenin 3; Pdx-1, pancreatic duodenal homeobox-1; MafA, musculoaponeurotic fibrosarcoma oncogene homolog A; ITF, islet transcription factor; NeuroD/β2, neurogenic differentiation factor D/beta 2; Pax6, pair-box 6; Nkx6.1, homeodomain transcription factor 6.1; InR, insulin receptor; IGF-R, insulin-like growth factor 1-receptor; DAPI, 4',6-diamidino-2-phenylindole; InR, insulin receptor; RACK1, receptor of activated C kinase 1; AR42J, a rat pancreatic acinar adenocarcinoma cell line; MOI, multiplicity of infection; GST, glutathione S-transferase; PBS, phosphate-buffered saline; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2. Material and methods

2.1 Cell lines and chemicals

A rat pancreatic acinar adenocarcinoma (AR42J), a mouse insulinoma cell line (MIN6), and an African green monkey kidney cell (Cos-7) were obtained from ATCC and maintained according to the manufacturer's recommendation. Rabbit anti-Flag and anti- β -actin antibodies were purchased from Sigma (St. Louis, MO). Pho-AKT signaling pathway kit and anti-insulin receptor (InR) antibodies were purchased from Cell Signaling Technology (Cambridge, MA). Mouse anti-HA (clone 16B12) and anti-Flag (M2) antibodies were purchased from Covance (Berkeley, CA) and Sigma. Mouse SP-1, and PDI antibodies were obtained from Santa Cruz Biotech. (Dallas, Texas). Nkx6.1 mouse antibody (F55A12) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Anti-RACK1 antibody was purchased from BD Bioscience (San Jose, CA). Rabbit anti-acetyl-H3/H4 antibodies were purchased from Upstate Biotech. (Lake Placid, NY). HRP conjugated secondary antibodies were purchased from Pierce (Rockford, IL). Fluorescence conjugated secondary antibodies were purchased from Southern Biotech (Birmingham, AL). Recombinant insulin was purchased from Sigma (St. Louis, MO).

2.2 Adenovirus infection and insulin induction

Recombinant adenovirus expressing Flag-tagged human INSM1 was generated using the AdEasy XL adenoviral vector system (Stratagene, La Jolla, CA). AR42J cells were infected with adenovirus expressing Ad-INSM1, Ad-INSM1-MutN, or Ad-LacZ (MOI 20) for the indicated time at 37 °C. After 24 hr, AR42J cells were synchronized in serum-free medium overnight. Then, 50 nM of insulin (final concentration) was added into the medium to stimulate InR signaling. AR42J cells were harvested at certain time points for further Western blot analyses or specific antibody staining.

2.3 RT-PCR and real-time PCR

Total RNA was isolated from the adenovirus infected cells using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA was treated with DNase I (Promega, Madison, WI) and precipitated using iso-propanol. A total of 2 μ g of the treated RNA was processed for reverse transcription using a cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. PCR conditions were 95 °C for 5 min, followed by 35–40 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C, and extension at 72 °C for 30 s. The PCR products were separated on 2% agarose gel. For real-time PCR, one microliter of the reverse-transcribed products obtained as described above was used in a 25 μ l reaction mixture including SYBR Green PCR Master Mix (BioRad, Hercules, CA) and 1 μ l 0.25 μ M primers for the rat *Nkx6.1* and *INSM1* gene. GAPDH was used as an internal control. The cycling conditions were hold at 95 °C for 5 min, followed by 45 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The fluorescent signals were analyzed using an ICycler IQ multicolor real-time detection system (BioRad, Hercules, CA). The PCR primer pairs and the product sizes are listed as follow, GAPDH (229 bp): 5'-GGTGAAGGTCGGTGTGAACG-3', 5'-CTCGCTCCTGGAAGATGGTG-3'; INSM1 (270 bp): 5'-AACTGTCCTTCGCTTGGA-3', 5'-ACGAGACAAACGCGTACAGCT-3'; Nkx6.1 (207 bp): 5'-TACCCCCCATCAAGGATCCATT-3', 5'-AGCCGCGTGCTTCTTCCTCCA-3'; Nkx6.1 (185 bp) for ChIP PCR, 5'-TGGAAGATGGGCGAGGAT-3', 5'-TGCGGAAAGGGAGCTAATG-3'.

2.4 Yeast transformation and selection

Bait-INSM1 (pGal4-BD-INSM1) and a series of vectors with various RACK1 fragments (pGal4-AD-RACK1) were isolated from a previous yeast two-hybrid screen [13] and co-

transformed into competent AH109 yeast cells according to the manufacturer's (Clontech) protocol. The positive clones were selected for their ability to grow in quadruple selection plate (Ade⁻/His⁻/Trp⁻/Leu⁻) with 5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside (X- α -Gal).

2.5 Nuclear and cytoplasmic fractionation

Adenovirus infected and insulin stimulated AR42J cells were subjected to nuclear and cytoplasmic fractionation using the NE-PER extraction kit (Thermo Scientific). Briefly, the cell pellet was suspended in CERI buffer vigorously for 15 sec. and the tube incubated on ice for 10 min. The ice-cold CERI was added to the tube, vortexed for 5 sec., and placed on ice for 1 min. The tube was vortexed 5 sec. and centrifuged for 5 min. at 14,000 $\times$ g. The cytoplasmic extract was immediately transferred to a clean tube for storage. The pellet was further extracted with ice-cold NER buffer with vortex every 15 sec. over a 10 min. interval for a total of 40 min. The nuclear extract was collected by centrifugation at 14,000 $\times$ g for 10 min.

2.6 Protein expression and purification in E. coli

cDNA constructs in pET41a(+) expression vector were transformed into BL21(DE3) competent cells (Novagen). Proteins were induced by isopropyl β -D-1-thiogalactopyranoside and purified using His-binding resin (Invitrogen) following the manufacturer's instructions. GST and recombinant GST-RACK1 protein expression were induced by IPTG at room temperature for 3 h.

2.7 GST pull-down assay

Recombinant GST-tagged RACK1 was incubated with cell lysate from Ad-LacZ, Ad-INSM1 wild type or Ad-INSM1-Mut N infected cells in 500 μ l of binding buffer (PBS, 1% bovine serum albumin, 0.4% Triton X-100, and 2 mM dithiothreitol) overnight at 4 $^{\circ}$ C. Then, glutathione-beads (Sigma) were added and incubated for 1 h at 4 $^{\circ}$ C. The beads were washed four times with 1 ml of binding buffer, separated by SDS-PAGE, and analyzed via Western blot. GST was used as negative control for non-specific binding.

2.8 Co-immunoprecipitation (Co-IP) and Western blot analysis

For Co-IP, AR42J cells were seeded in a 100 mm-diameter culture dish. Cells were infected with Ad-LacZ or Ad-INSM1 (MOI = 20) for 48 h. The infected cells were harvested at 48 h post-infection for Co-IP and Western blot analysis. Cell lysates were prepared using protein binding buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 0.1% (v/v), Triton-X 100 and 1 mM phenylmethylsulfonyl fluoride). For Co-IP, 300 μ g of the cell lysate was pre-cleared with recombinant Protein G-agarose and incubated overnight at 4 $^{\circ}$ C with an anti-RACK1 antibody, followed by incubation overnight at 4 $^{\circ}$ C with recombinant Protein G-agarose beads. The beads were washed five times with binding buffer, separated by SDS/PAGE, and transferred onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA, U.S.A.). RACK1 complexes with INSM1 or InR were detected by Western blot (1:1000) with anti-INSM1 (Flag-tag or HA-tag) or anti-InR antibody, respectively. The horseradish peroxidase-conjugated anti-mouse secondary antibody (1:4000) and chemiluminescent substrate (Pierce) were used for detection of INSM1 or InR. For Western blot of phosphorylated proteins, cells were lysed with 0.5 ml of ice-cold RIPA buffer containing 10mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 4 mg/ml each of leupeptin, pepstatin, and aprotinin, 5 mM NaF, and 2 mM sodium orthovanadate. Cell lysates (25 μ g/lane) were electrophoresed on 10% SDS-PAGE gel, transferred to nitrocellulose membrane, probed with specific antibody, and detected with the enhanced chemiluminescence system (Pierce).

2.9 Luciferase reporter assay

The MIN6 cell line was grown in low glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Luciferase assays were performed in a 24-well plate format using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Luciferase activities were normalized with a co-transfected TK-Renilla plasmid (Promega). *Nkx6.1* promoter (FLO1580⁺) linked reporter vector (pFOXLuc1) was obtained from Dr. M.S. German (UCSF, San Francisco, CA). Luciferase activities were measured using a Dual-Glo Luciferase Assay kit (Promega). Activities were averaged from three experiments.

2.10 Chromatin immunoprecipitation assay (ChIP Assay)

ChIP assays were performed using a ChIP kit (Upstate). The immunoprecipitated DNA templates were prepared according to the manufacturer's instructions. Briefly, cross-linking was performed by adding formaldehyde directly to the tissue culture media to a final concentration of 1 %. Fixation proceeded at 37 °C for 10 min. The fixed cells were rinsed twice with ice-cold PBS. The pooled cells were collected by centrifugation and re-suspended in SDS lysis buffer supplemented with protease inhibitor cocktail (Roche). After incubation on ice for 10 min, lysates were sonicated on ice to an average length of 200–1000 bp and centrifuged at 15,000 rpm at 4 °C for 12 min. The chromatin solution was pre-cleared with protein-A beads for 1 h at 4 °C. The pre-cleared chromatin was incubated with an anti-acetyl histone H3, H4 (Upstate), or normal control IgG (Southern Biotech Inc.). Input chromatin solution was used as a positive control. Following an overnight incubation with each of the corresponding antibodies, the antibody/chromatin mixtures were precipitated with protein-A beads and the beads were sequentially washed with ChIP wash buffer and TE buffer. The complexes were eluted twice with 250 µl of elution buffer (1% SDS and 100 mM NaHCO₃) for each ChIP reaction. Cross-links were reversed by adding 5M NaCl and incubated at 65 °C for 4 h. The DNA was de-proteinized by adding proteinase K at 45 °C for 2 h. The immunoprecipitated DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The PCR condition was initial hold at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72°C for 30 seconds. The fluorescent signals were analyzed using an ICycler IQ multicolor real-time detection system (BioRad, Hercules, CA).

2.11 Immunohistochemistry

After Ad-LacZ or Ad-INSM1 infection, AR42J cells were washed and fixed onto glass slides with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Tween 20. The slides were blocked with 5% BSA and 1% goat antiserum in PBS. *Nkx6.1* and INSM1 staining was performed using primary antibody overnight at 4 °C and a secondary antibody for 30 min at room temperature. The fluorescent signals were detected by a fluorescence microscope and the nucleus was stained with 4', 6-diamidino-2-phenylindole (DAPI). The slides were read using a laser scanning confocal microscope (LSM 510, Carl Zeiss).

2.12 Statistical analysis

Values were corrected and expressed relative to an untreated control group. All experiments were repeated three times. Results are presented as mean $\pm$ SD. Statistical analysis was performed using either the Student's t-test when only two groups were in the experiment or by an one-way ANOVA comparison of multiple groups using the Tukey-Kramer test with differences at *p* value of less than 0.05 being considered significant.

3. Results

3.1 INSM1 and RACK1 Interaction

Screening of a yeast two-hybrid library derived from a 45-day-old human fetus brain (highly expressing INSM1 message, obtained from the Central Laboratory for Human Embryology, University of Washington, Seattle, WA, USA), we obtained multiple RACK1-positive clones interacting with INSM1-Bait (pGal4-BD-INSM1). Further, we mapped the INSM1-binding domain in the RACK1 protein using a yeast two-hybrid system. The INSM1-bait vector and five different fragments of RACK1 clones were co-transformed into AH109 competent yeast cells and selected on a quadruple selection plate (Ade⁻/His⁻/Leu⁻/Trp⁻) with X- α -Gal. As shown in Fig. 1A, clones A, B, and C interact strongly with the INSM1-bait, clone D interacts weakly, and clone E does not interact with INSM1-bait. Since the D fragment contains the last two WD domains at the C-terminus of RACK1, this suggests that at least the last two WD domains from the RACK1 protein are required for INSM1 binding and that WD5-WD7 are critical for strong binding. The full-length recombinant RACK1 was expressed and purified in a GST bacterial expression system (Fig. 1C, left panel). We cloned full-length INSM1, the N-terminal fragment (1–263 amino acid), and the C-terminal fragment (263–510 amino acid) containing a HA-tag in a mammalian expression vector (Fig. 1B). The over-expressed INSM1 protein fragments in Cos-7 cells were subjected to a GST recombinant RACK1 protein pull-down assay. The bottom of Fig. 1C shows that only the full-length and N-terminal portion of INSM1 is responsible for RACK1 binding. The C-terminal portion of INSM1, which contains mainly the five zinc-finger motifs do not bind to the RACK1 protein.

3.2 INSM1 Co-localization with RACK1

INSM1 is a tissue-restricted transcription factor normally expressed in the nucleus, whereas RACK1 is an intracellular receptor for protein kinase C. RACK1 is ubiquitously expressed and has been implicated in a variety of cellular processes [1;11;18]. While the yeast two-hybrid screen and the above pull-down study revealed strong binding between INSM1 and RACK1 proteins, we further examine whether INSM1 and RACK1 co-localize in the cytoplasm or nucleus. We infected Ad-INSM1 or Ad-LacZ into AR42J cells (INSM1 negative), starved, and stimulated with insulin to activate InR-mediated signaling. As shown in Fig. 2A, anti-RACK1 staining is concentrated at the membrane in contrast to INSM1, which is mostly located in the nucleus. However, INSM1 is also partially detected in the cytoplasm and co-localizes with RACK1 suggesting a physical interaction between INSM1 and RACK1. Furthermore, we fractionated cellular lysates into cytoplasmic and nuclear extracts. The co-localization of INSM1 and RACK1 was confirmed by using protein disulfide isomerase (PDI) and SP-1 as markers to identify the proper cellular compartments. A small amount of INSM1 can be detected in the cytoplasmic extract and be co-precipitated with a RACK1 antibody (Fig.2B). This result supported that INSM1 and RACK1 interact with each other in the cytoplasm.

3.3 Interaction of INSM1 and RACK1 enhances the InR-mediated signaling pathway

RACK1 and INSM1 protein interaction is shown in Fig.1. It has been reported that RACK1 can bind to the InR and modulates its signaling pathway [12]. Using a Co-IP assay, we examined whether binding of INSM1 to RACK1 affects its InR binding. In Fig. 3A, expression of INSM1 in AR42J cells favors INSM1-RACK1 binding but greatly reduces RACK1's ability to pull-down InR. We further measured the phosphorylation state of InR-mediated downstream component, AKT. In the presence of INSM1, phosphorylated AKT was increased 20 min after insulin stimulation (Fig. 3B). This result suggests that the expression of INSM1 in AR42J cells binds favorably to RACK1, interrupts InR binding, and subsequently enhances AKT phosphorylation.

3.4 Removal of RACK1 is essential for InR-mediated signal activation

We identified that the N-terminal sequence (1–263 amino acid) of INSM1 is required for RACK1 binding (Fig. 1C). There are two proline-rich regions at the N-terminal sequence of INSM1. Proline-rich sequences usually contribute to protein-protein interactions. Thus, we constructed two mutant constructs (within the INSM1 full-length) and transfected them into Cos-7 cells separately as shown in Fig. 4A. As compared to the wild type, mutant N but not mutant C lost the ability to bind to RACK1 protein in a Co-IP assay suggesting the proline-rich sequence in mutant N is required for RACK1 binding. We transferred the mutant N into adenoviral vector in parallel with the wild type INSM1 and the control Ad-LacZ. Only the wild type INSM1 is capable of being pulled-down by RACK1 and competes with InR binding (Fig. 4B). Both Ad-LacZ and Ad-INSM1-MutN failed to compete with RACK1 for InR binding. Similarly, the phosphorylation state of AKT was not altered since mutant N is incapable of binding to RACK1. In order to examine whether the AKT phosphorylation is connected to InR through PI3K activation, the phosphorylation of AKT was further examined using a PI3K specific inhibitor, LY294002. Addition of the LY294002 inhibitor suppresses AKT phosphorylation in the presence of INSM1 suggesting that InR signaling is mediated through the PI3K/AKT pathway (Fig. 4C).

3.5 Extra-nuclear activity of INSM1 activates *Nkx6.1* gene through RACK1 binding and the InR signaling pathway

During treatment of AR42J cells with exogenous INSM1 to promote AR42J cell trans-differentiation [28], we noticed a consistent activation of *Nkx6.1* gene (Fig. 5 A and B). Since INSM1 is a transcriptional repressor, it is unlikely that INSM1 activates the *Nkx6.1* promoter directly. In an effort to rule out this possibility, we used the *Nkx6.1* promoter/enhancer (–1580/+1000 base pair) linked to the luciferase reporter gene [25] and failed to demonstrate any direct activation by INSM1 in MIN6 cells (Fig. 5B, right panel). In contrast, some inhibition of the *Nkx6.1* promoter activity was observed and further ruled out that INSM1 can directly activate the *Nkx6.1* promoter. Therefore, we examined whether the *Nkx6.1* gene expression is connected to the extra-nuclear activity of INSM1 and mediated through InR signaling using both the PI3K inhibitor and INSM1 mutant N construct (Fig. 5C). The result shows that the expression of *Nkx6.1* gene directly correlates with the RACK1 binding to INSM1 and InR signal transduction, since INSM1 mutant or the addition of LY inhibitor failed to activate *Nkx6.1* gene.

Gene activation is usually mediated through the modification of histone acetylation on the promoter/enhancer sequence. We performed a ChIP assay to further analyze a β -cell specific enhancer region of the *Nkx6.1* promoter (–895 to –710 base pair), which was known to regulate β -cell specific *Nkx6.1* gene expression using antibodies specific for acetyl-H3/H4 [25]. In Fig. 6A, acetyl-H4 is clearly increased with wild type INSM1 expression in contrast to the Ad-LacZ control, PI3K inhibitor, or Ad-INSM1-MutN infected AR42J cells. The result suggests that INSM1 binding to RACK1 activates the InR-mediated signaling pathway which triggers the *Nkx6.1* enhancer modification and gene activation. In order to further support that INSM1 not only activates *Nkx6.1* gene expression through the InR-mediated signaling pathway but also contributes to the trans-differentiation of AR42J cells into insulin positive cells, we treated AR42J cells with Ad-Pdx-1/INSM1/NeuroD1 in the presence or absence of the PI3K inhibitor (LY294002). This trans-differentiation model was previously studied and shown to be much more effective than using Ad-INSM1 alone [28]. Indeed, the LY294002 inhibitor interfered with both the *insulin* and *Nkx6.1* gene expression (Fig. 6B).

4. Discussion

We found that INSM1 interacts with RACK1 protein and facilitates InR-mediated signaling. RACK1 is a cellular adaptor protein as the receptor for protein kinase C [6]. Both IGF-1R and InR were identified as RACK1-interacting proteins and the interaction modulates their signaling pathways. RACK1 inhibits the PI3K pathway by down-regulating AKT phosphorylation. However, the inhibition does not correlate with the IGF-1R and InR tyrosine kinase activity, but it requires serine 1248, which is also present in a conserved amino acid stretch in the InR (serine 1262) [12]. Other serine residues or domains in the receptor may also be involved in the RACK1 interaction in addition to the serine 1248, where mutation of this single amino acid is sufficient to disrupt the RACK1 association. Our study shows the proline-rich sequence at the N-terminus of INSM1 binds to RACK1 and pulls RACK1 away from the InR and relieves its inhibitory effect on the InR signaling.

Complex interactions among a number of ITFs play an important role in pancreas development. The combination of Pdx-1, Ngn3, and MafA can convert mouse acini into insulin-positive cells *in vivo* [30]. Pdx-1 is a critical factor when endodermal cells commit to the pancreatic lineage and it subsequently modulates *insulin* gene expression in mature islets, whereas Ngn3 controls endocrine lineage differentiation [9;20;21]. MafA functions as a transcription factor in mature β -cells and is critical for activation of the *insulin* gene [26]. A multitude of other ITFs also contribute greatly to the development of the pancreas as well as endocrine cells at different stages during development [15]. A zinc-finger transcription factor, INSM1 is essential for the development of the endocrine pancreas [7]. *INSM1* is transiently expressed downstream of *Ngn3* and upstream of *NeuroD* β 2 [2;3]. *INSM1* is activated by Ngn3/E47 through binding of the E-box in the promoter region and functions as a transcriptional repressor with the specific target sequence located in the promoter regions of *NeuroD* β 2, *insulin*, and *INSM1* itself (self-regulated) [2;4;24]. The components associated with INSM1 that act on pancreatic cells during pancreas development are not totally understood. Our previous studies showed that INSM1 possesses an extra-nuclear activity when bound to cyclin D1 to induce cell cycle arrest in non-neuroendocrine cells [27]. Additionally, in the present study we showed INSM1 binds to RACK1 and induces InR-mediated signaling to result in an indirect activation of the *Nkx6.1* gene expression during pancreatic acinar cell trans-differentiation [28]. Since RACK1 is capable of binding and regulating multiple growth factor receptors [5;10;12;17], we further examined whether the binding of INSM1 to the RACK1 molecule can modulate the InR-mediated signaling pathway. We used AR42J cells as a model since it was previously shown that INSM1 induced trans-differentiation of AR42J into endocrine cells with concomitant activation of *Nkx6.1* and *insulin* gene expression. Our data reveals that RACK1 co-precipitates the InR readily in AR42J cells. However in the presence of INSM1, the InR binding to the RACK1 decreases dramatically suggesting that when INSM1 binds to the RACK1, it could interrupt the RACK1-InR association. The interaction of INSM1-RACK1 actually pulls RACK1 away from the InR and facilitates the InR-mediated signaling upon insulin stimulation. Further analysis of the downstream InR-mediated signaling molecules revealed that INSM1 indeed promotes PI3K signaling and AKT phosphorylation. It has been demonstrated that activation of the class 2 PI3K pathway and AKT phosphorylation is a unique signal from the InR to regulate glucose metabolism [22]. These results help us pinpoint how the INSM1 transcription factor contributes to the InR signaling pathway and initiates cellular differentiation.

INSM1 plays an important role in modulating multiple ITFs either directly through the binding on the promoter region of the target gene or indirectly through growth factor stimulated signaling pathways [28;29]. Particularly, *Nkx6.1* is activated by INSM1 and could contribute greatly to β -cell differentiation [19]. We showed that INSM1 is a

transcriptional repressor and cannot activate the *Nkx6.1* gene promoter/enhancer directly. However, *Nkx6.1* gene activation by INSM1 could be attributed to the INSM1 promotion of pancreatic cell trans-differentiation. Therefore, we examined whether the involvement of INSM1 in the growth factor induced signaling pathway could actually contribute to the *Nkx6.1* gene expression. Since INSM1 functions as a neuroendocrine differentiation factor, it is believed that INSM1 inhibits cellular proliferation in a cell-type specific manner and is involved in switching between proliferation and differentiation pathways when it interacts with cyclin D1 and/or RACK1. In pancreatic cells, INSM1 induces duct cell differentiation into insulin-positive cells when the right conditions and stimulus were applied [29]. Whereas INSM1 in the wrong cell type will inversely push cells into cell cycle arrest and apoptosis. RACK1, the receptor for activated C kinase, binds and regulates InR signaling [12]. Thus, it is logical to ask whether the INSM1-RACK1 interaction modulates the InR signaling pathway that controls cell differentiation and gene activation. Specifically, we investigate whether the activation of the InR-mediated signaling pathway could contribute to the *Nkx6.1* gene activation. The homeobox gene *Nkx6.1* lies downstream of *Nkx2.2* in the major pathway of β -cell formation in the pancreas [19]. In this study, we have shown a direct connection between INSM1 and the InR through the physical interaction with the RACK1 molecule. Association of INSM1 and RACK1 modifies the acetyl-H4 level on the *Nkx6.1* enhancer/promoter region and induces gene expression. INSM1 imposes a direct interruption of RACK1 and InR binding and activates down-stream AKT phosphorylation. In contrast, a PI3K inhibitor or the INSM1-MutN blocks the InR-signaling and RACK1 binding and failed to activate the *Nkx6.1* gene. Thus, the functional effect of INSM1 binding to RACK1 affects the downstream components of InR-mediated signaling pathway such as PI3K, AKT and promotes cellular differentiation as well as *Nkx6.1* gene activation. We provide evidence that INSM1 can connect to signaling pathways specific for cellular events such as trans-differentiation and islet cell gene activation. The information obtained from this study will be valuable to expand the knowledge of how INSM1 exerts its functional effects in pancreas development. Therefore, we concluded that the transient expression of INSM1 during pancreas development is critical to facilitate pancreatic cell differentiation.

Conclusions

INSM1 islet transcription factor is essential for pancreatic endocrine cell differentiation and promotes acinar cell trans-differentiation. INSM1 modulates *Nkx6.1* gene expression via IR-mediated signaling pathway. INSM1 and RACK1 interaction promotes PI3K/AKT signaling and *Nkx6.1* gene activation. The extra-nuclear activity of INSM1 is unique and critical for pancreatic cell differentiation.

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Highlights

- INSM1 interacts with RACK1 to facilitate InR/PI3K/AKT signaling pathway.
- Binding of INSM1 to RACK1 increases AKT phosphorylation.
- InR/PI3K/AKT signaling pathway activates *Nkx6.1* promoter through H4-acetylation.
- The PI3K inhibitor interrupts *Nkx6.1* and *insulin* gene expression.
- The extra-nuclear activity of INSM1 is critical for pancreatic cell differentiation.

Figure 1. Interaction of INSM1 and RACK1

(A) Yeast two-hybrid binding. Schematic diagram at the right shows five different clones of the RACK1 protein. The RACK1 sequences in pGADT7 vector were co-transformed with pGBKT7-INSM1 bait-vector for the yeast two-hybrid screen. Fragment E failed to grow on the quadruple selection plate indicating that the last two WD domains at C-terminus are required for INSM1 binding. (B) INSM1 diagram shows full-length, N-terminal (1–263 amino acid), and C-terminal (263–510 amino acid) fragments. The line boxes represent the proline-rich region and the shaded boxes represent the five-zinc finger motifs. (C) Recombinant GST and GST-RACK1 (upper left panel) were purified by a glutathione affinity column. Cos-7 cells were transfected with HA-tagged full length, N-terminal, or C-terminal portions of the INSM1 cDNA, and the cellular extracts were subjected to Western blot analysis (upper right panel). Pull-down experiments were conducted using recombinant

GST-RACK1 or GST control mixed with INSM1 transfected extracts. The reaction mixtures were precipitated with glutathione beads and Western blotted with HA antibody (lower panel).

Figure 2. Co-localization of INSM1 and RACK1 in AR42J cells

(A) AR42J cells were infected with Ad-LacZ or Ad-INSM1 for 48 h. The infected AR42J cells were cultured in serum-free medium overnight then induced with 50 nM insulin on cover glasses. The AR42J cells were stained with anti-Flag (INSM1-red) and anti-RACK1 antibodies (green). Most of the INSM1 staining is located in the nucleus, whereas some of the INSM1 staining is localized in the cytoplasm and overlaid with RACK1 protein (yellow, arrows). The RACK1 proteins are concentrated at the vicinity of membrane. The nucleus was stained with DAPI (blue). (B) Detection of INSM1 in both cytoplasmic and nuclear extracts of AR42J cells. The cytoplasmic (Cyto-) and nuclear extracts (NE) from either Ad-

LacZ or Ad-INSM1 infected AR42J cells as described above were fractionated into Cyto- and NE fractions. Both fractions were subjected to direct Western blot or immuno-precipitation for INSM1, RACK1, protein disulfide isomerase (PDI), and SP-1 transcription factor. PDI serves as cytoplasmic marker, whereas SP-1 serves as nuclear marker. The translocation of INSM1 into the cytoplasmic fraction was confirmed by the co-precipitation by RACK1 antibody (left panel).

Figure 3. INSM1 interrupts RACK1 and InR binding and enhances AKT phosphorylation
(A) Co-IP of RACK1 with INSM1 or InR in AR42J cells. Ad-INSM1 or Ad-LacZ infected AR42J cell lysate was precipitated using an anti-RACK1 antibody. The immune-complex was Western blotted with either anti-INSM1 (anti-Flag) or anti-InR antibody. Co-precipitation data shows that INSM1 interrupts RACK1 and InR binding. **(B)** INSM1 modulates InR-mediated signaling pathway. AR42J cells were infected with Ad-LacZ or Ad-INSM1 for 24h and treated with serum-free starvation overnight. The starved cells were stimulated with insulin (50 nM) for 0, 5, 10, or 20 min. The cell lysates were subjected to Western blot analyses with specific antibodies. Anti-actin antibody was used as gel loading control.

Figure 4. INSM1 and RACK1 binding is required to trigger InR signaling pathway

(A) We determined the proline-rich region in the INSM1 protein is essential for RACK1 binding. The top panel diagram shows two mutants (N- and C-) were constructed from INSM1 full-length by changing the proline residues into leucine residues. The HA-tagged wild type (full-length INSM1), mutant N, or mutant C construct was transfected into Cos-7 cells. Co-immunoprecipitation was performed using an anti-RACK1 antibody to precipitate the INSM1 full-length, N-terminal mutant, or C-terminal mutant. The presence of INSM1 and RACK1 in the cell lysates was confirmed by Western blot analysis (lower two blots). Co-IP assay using anti-HA antibody shows that wild type and Mut C, but not Mut N binds to

the RACK1 suggesting that the proline-rich region of the INSM1 mutant N is essential for RACK1 binding. **(B)** INSM1 mutant N is incapable of interrupting RACK1 binding with the InR. We constructed Ad-INSM1 mutant N in parallel with wild type Ad-INSM1 and tested its effects on AR42J cells, since AR42J cells are resistant to transfection when various transfection reagents were used including Lipofectamine 2000 and Fugene 6. The AR42J cells were readily infected with Ad-LacZ, Ad-INSM1, or Ad-INSM1-MutN for 48 hours. The cell lysates were starved and stimulated with insulin for 20 mins before collected for co-immunoprecipitation using an anti-RACK1 antibody to precipitate LacZ control, INSM1 full-length, and N-terminal mutant. The presence of INSM1 and RACK1 in the cell lysates was confirmed by Western blot analysis (lower two blots). Co-IP assay shows INSM1 wild type, but not Mut N binds to the RACK1. Additionally, INSM1 mutant N does not interrupt RACK1 and InR binding. INSM1-Mut N can no longer enhance AKT phosphorylation (lower panel). The ratios of pho-AKT/total AKT are shown underneath (**, $p<0.01$, *, $p<0.05$). **(C)** PI3K inhibitor LY294002 was used to block AKT phosphorylation enhanced by INSM1. The ratios of pho-AKT/total AKT are shown underneath (**, $p<0.01$, *, $p<0.05$).

Figure 5. INSM1 activates Nkx6.1 in AR42J cells

(A) Ad-INSM1 induces AR42J cells trans-differentiation into insulin-positive cells in 4 days [28]. The infected cells were harvested for immunostaining and RT-PCR. Infected cells were immunostained with anti-Flag (INSM1, red) or anti-Nkx6.1 antibody (green). DAPI is used for nuclear staining (blue). (B) *Nkx6.1* gene expression in AR42J cells was activated by Ad-INSM1 infection using RT-PCR assay. GAPDH was used as control (B left panel). INSM1 failed to activate the *Nkx6.1* promoter activity (right panel). The *Nkx6.1* promoter (−1580/+1000 bp)-linked reporter with or without INSM1 cDNA were co-transfected into MIN6 cells for 24 h. The relative luciferase activity was normalized using co-transfected

renilla activity. (C) Real-time quantitative comparative RT-PCR analysis of *Nkx6.1* gene expression in Ad-LacZ, Ad-INSM1, Ad-INSM1 plus LY294002, and Ad-INSM1-Mut-N infected AR42J cells. Relative *Nkx6.1* mRNA was compared against wild type INSM1 infected AR42J cells (***, $p<0.001$). Representative gel images are shown underneath.

Figure 6. ChIP assay for *Nkx6.1* promoter

(A) The adenoviral infected AR42J cells were fixed with 1% formaldehyde at 37 °C for 10 min. The harvested cells were subjected to ChIP assay for *Nkx6.1* promoter. Quantitative comparative analysis via real-time PCR for histone H3/H4 acetylation on the *Nkx6.1* promoter (–895 to –710 bp) in Ad-LacZ, Ad-INSM1, Ad-INSM1 plus LY294002, or Ad-INSM1-Mut N infected cells. Input (Inp) DNA as positive control and normal IgG as negative control. Acetyl-H4 ChIP assays were calculated against wild type INSM1 infected AR42J cells (***, $p < 0.001$). Representative gel images are shown underneath. (B) When AR42J cells were induced to differentiate into insulin-positive cells, we tested whether the

differentiation event is directly linked to the PI3K/AKT signaling pathway using LY294002 inhibitor. Three islet transcription factors (Pdx-1, INSM1, and NeuroD/β2) were added to the AR42J cells with or without LY294002. RT-PCR revealed that both insulin and Nkx6.1 expression levels are dramatically reduced. Actin serves as a control.