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A positive feedback regulation of ISL-1 in DLBCL but not in pancreatic β-cells

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ABSTRACT

Insulin enhancer binding protein-1 (ISL-1), a LIM-homeodomain transcription factor, has been reported to play essential roles in promoting adult pancreatic β-cells proliferation. Recent studies indicate that ISL-1 may also involve in the occurrence of a variety of tumors. However, whether ISL-1 has any functional effect on tumorigenesis, and what are the differences on ISL-1 function in distinct conditions, are completely unknown. In this study, we found that ISL-1 was highly expressed in human pancreatic β-cells, as well as in diffuse large B cell lymphoma (DLBCL), but to a much less extent in other normal tissues or tumor specimens. Further study revealed that ISL-1 promoted the proliferation of pancreatic β-cells and DLBCL cells, and also accelerated the tumorigenesis of DLBCL *in vivo*. We also found that ISL-1 could activate c-Myc transcription not only in pancreatic β-cells but also in DLBCL cells. However, a cell-specific feedback regulation was detectable only in DLBCL cells. This auto-regulatory loop was established by the interaction of ISL-1 and c-Myc to form an ISL-1/c-Myc transcriptional complex, and synergistically to promote ISL-1 transcription through binding on the ISL-1 promoter. Taken together, our results demonstrate a positive feedback regulation of ISL-1 in DLBCL but not in pancreatic β-cells, which might result in the functional diversities of ISL-1 in different physiological and pathological processes.

1. Introduction

Insulin enhancer binding protein-1 (ISL-1), also known as islet 1, is a LIM-homeodomain family transcription factor, with 100% conservation of amino acid sequences from rat, hamster and human species [1]. It has been previously described to play crucial roles in heart, motor neuron and pancreas development [2–4]. In addition to the traditional view that ISL-1 functions as an activator of insulin gene [5], our recent study reveals that ISL-1 can increase adult pancreatic β-cells proliferation and attenuate cell apoptosis against oxidative stress, which demonstrate that ISL-1 is an homeostasis regulator rather than an insulin gene activator [4]. Intriguingly, all of these studies have shown that ISL-1 is a strict tissue-specific gene: during embryo development, it localizes in second heart field (SHF), motor neuron and pancreas; whereas in adult, it is mainly expressed in pancreatic islet endocrine cells (α, β, γ, ε) as well in the central nervous system [2,4,6].

Recently, accumulated evidences reveal that the aberrant expression of ISL-1 has been detected in a variety of malignant tumors and may be closely related to the tumorigenesis [7–9]. In our pilot study, different human tumor specimens from breast, colon, liver, lung, esophagus, etc. were analyzed by immunohistochemical staining. A markedly higher level expression of ISL-1 was observed in human diffuse large B cell lymphoma (DLBCL) compared with that in the tumors from other organs as well reactive lymph nodes (unpublished data). However, whether ISL-1 is directly and specifically involved in human DLBCL and what its function and regulatory mechanism are, to date, entirely unknown.

It is reasonable to suspect that the abnormal expression of ISL-1 in different tissues or organs may profoundly relate to their dysfunctions. Hence, inevitable issues were raised: whether and why the high level expression of ISL-1 in normal pancreatic β-cells and tumor cells leads to distinct outcomes? We suspect that the function of ISL-1 on pancreatic β-cell homeostasis must be different from that involved in human cancers, and the role of ISL-1 in cancer cells may depend on the factors it interacts with. In this

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study, chosen pancreatic β-cell and DLBCL as experimental models, we aim to elucidate the different function and cell-specific regulatory mechanism of ISL-1 in different physiological and pathological processes.

2. Materials and methods

2.1. Immunohistologic analysis

All human samples were obtained from the Department of Pathology, Peking University with the informed consent and with the approval of the Research Ethics Committee of Peking University (IRB 00001052-13014). All specimens were subject to immunohistochemistry (IHC) analysis using the Enovision Detection Kit/DAB (DAKO A/S, Denmark) according to the manufacturer's protocol with anti-ISL-1 (ab86472, Abcam, China). The slides were examined under microscopy by two researchers independently. Images were acquired with a Leica DM25000B microscope (Leica, Germany).

2.2. Cell culture and cell transfection

HIT-T15 (purchased from ATCC), SUDHL16 and Ly3 (gifts from Prof. Zhu J., Peking University Cancer Hospital), EC190, U937 and HL60 cells (preserved in our laboratory) were grown in RPMI 1640 containing 10% fetal bovine serum. MCF7 (gifts from Prof. Shang Y.F., Peking University School of Basic Medical Sciences), HT29, HepG2, A549, HeLa and 293T cells (preserved in our laboratory) were cultured in Dulbecco's modified eagle medium supplemented with 10% fetal calf serum.

The stably transfected HIT-T15 cells with ISL-1-overexpression or knockdown were previously constructed by our laboratory [4]. To establish stable overexpressing ISL-1 or c-Myc DLBCL cell lines, cells were transfected with 10 μg pcDNA3.1-ISL1 or pcDNA3.1-c-Myc plasmid, or a control pcDNA3.1 plasmid, at a density of 1×10^7/ml, in a 0.2 cm cuvette (BTX) using an BTX ECM803 Electroporator (Genetronics, USA) at 130 V, 20 ms. G418 selection (1000 μg/ml) was performed 48 h after transfection. The cells were cultured for about 21 days to obtain stable cell lines. To establish stable ISL-1-knockdown Ly3 cell line, Ly3 cells were electroporated with pLL3.7-ISL1-siRNA or pLL3.7-nonsilencer, respectively. 48 h after transfection, cells were cultured with a puromycin-containing medium (0.5 μg/ml) for about 21 days and then expanded for further experimentation. HIT-T15 and Ly3 cells were also transfected with siRNA duplex oligonucleotides targeting c-Myc (S#1: 5′-CAGAAAUG dTdT-3′, S#3: 5′-AAGGACUAUCCUGCUGCCAAG-3′), or negative control siRNA (NC) (Ribobio, Guangzhou, China) according to the manufacture's protocol.

2.3. Cell proliferation and cell cycle assays

The cell proliferation was detected using a CCK-8 cell proliferation kit (Dojindo Laboratories, Japan), according to the instruction from the supplier. Cell cycle assessment was carried out referring to our previous report [4].

2.4. Animal experiments

A xenograft mouse tumor model was established in 5-week-old female Beige mice. SUDHL16 cells stably transfected with pcDNA3.1-ISL1 (ISL-1) or pcDNA3.1 (Control) (1×10^7) were resuspended in 200 μl PBS and injected s.c. into the left (ISL-1 cells) or right (Control cells) dorsal flanks of 6 mice, respectively. Tumors were monitored 2 times per week with calipers, and tumor volumes were calculated as $1/2 \times \text{length} \times \text{width}^2$.

The expression prolife of ISL-1 was analyzed in different organs or tissues isolated from 8-week-old SD rats. The rats were anesthetized with 5 mg/100 g body weight of sodium pentobarbital and different organs or tissues were removed for mRNA preparation. All animals were purchase from the Department of Laboratory Animal Science of Peking University and all protocols were approved by the Animal Care and Use Committee of Peking University (LA 2010-066).

2.5. Luciferase assays

The plasmid transfection and luciferase activity detection were performed as described before [10]. *ISL-1-luc* W (wild type with the ISL-1 binding site) and *ISL-1-luc* D (the ISL-1 binding site was deleted) plasmids were constructed previously in our laboratory [10].

2.6. RT-PCR and real-time RT-PCR

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. RT-PCR and real-time RT-PCR amplifications were performed as descripted before [4,10].

2.7. Immunoprecipitation and Western blot analysis

Cell lysates preparation, immunoprecipitation and Western blot analysis were carried out as described before using the indicated antibodies [11]. Mouse monoclonal anti-ISL-1 (H00003670-M05, Abnova, China), rabbit monoclonal anti-ISL-1 (3727-1, Eptomics, China), rabbit monoclonal anti-c-Myc (#5605), and rabbit monoclonal anti-GAPDH (#2118) were all purchased from Cell Signaling Technology (Beverly, USA).

2.8. Chromatin immunoprecipitation (ChIP) and ChIP-re-IP assays

ChIP and ChIP-re-IP experiments were performed according to the method described by Zhang et al. [12] using following primers: P0 (covering −994 to −722 of ISL-1 promoter region): F: 5′-CCTTTCCTCCCACCAACGTTTTTA-3′, R: 5′-GCTT GGTTTGGTCCCCACG-3′; P1 (covering −613 to −373 of ISL-1 promoter region): F: 5′-AGGAGCACGCCACAGGAG-3′, R: 5′-ATTATCATATTTC AGCCTCGCCGC-3′.

2.9. Statistical analysis

Statistical analyses were carried out using the statistical software SPSS 17.0. The data are expressed as means ± standard deviation (S.D.). Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. ISL-1 is highly expressed in both pancreatic β-cells and DLBCL

In order to exam the functional diversities of ISL-1 in different physiological and pathological conditions, the expression profile of ISL-1 was firstly analyzed based on the public Genecards Database and RT-RCR assay. The results showed a high level of ISL-1 expression in postnatal pancreatic β-cells than that in other organs or tissues of both human (Fig. S1) and rat (Fig. 1A). In addition, a series of cancer cell lines, representing the cancers in human breast (MCF7), colon (HT29), esophagus (EC109), uterine cervix (HeLa), lung (A549) and liver (HepG2), as well leukemia (HL60 and U937) and lymphoma cell lines (SUDHL16 and Ly3) were applied

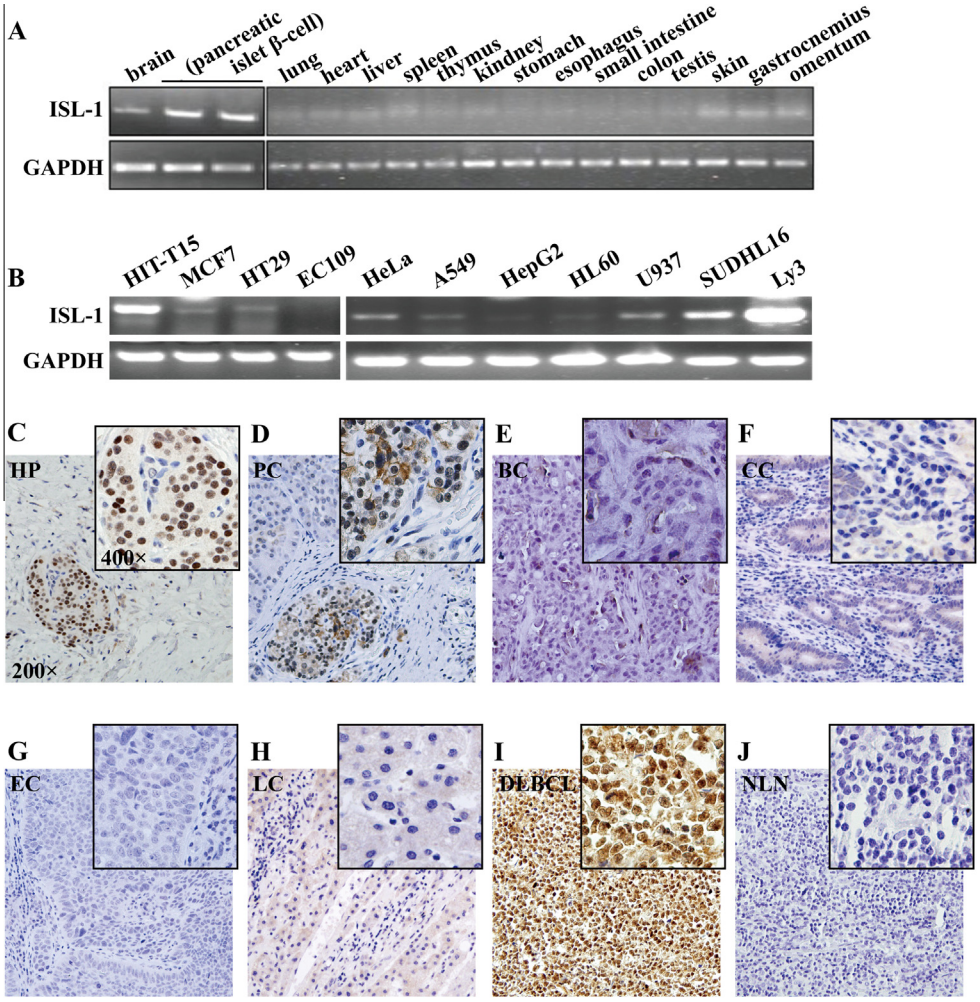

Fig. 1. ISL-1 is highly expressed in both pancreatic β-cells and DLBCL. (A) and (B) The expression profile of ISL-1 in different organs or tissues of rat (A) and human cancer cell lines (B) was analyzed by RT-PCR. (C)–(J) Representative immunohistochemical staining images of ISL-1 on human pancreas (C), a series of human cancers (D–I) and human normal lymph node (J) are shown. (HP: human pancreas; PC: pancreatic cancer; BC: breast cancer; CC: colon cancer; EC: esophagus cancer; LC: liver cancer; DLBCL: diffuse large B cell lymphoma; NLN: normal lymph node.)

for ISL-1 mRNA expression detection and a hamster pancreatic β-cell line (HIT-T15) was used as a positive control cell line. As shown in Fig. 1B, ISL-1 was predominantly detected in SUDHL16 and Ly3 cells. Although the expression level in SUDHL16 cells was lower than that in Ly3 cells, it was still comparable to the level in HIT-T15 cells. The expression of ISL-1 at the protein level in healthy human pancreas and different types of tumor samples (20 cases per tumor type) was further compared by immunostaining analysis. We found that ISL-1 was highly expressed in normal human pancreatic islets (Fig. 1C) and was also specifically found in DLBCL (Fig. 1I), but its expression was almost undetectable in normal lymph node (Fig. 1J) and other types of tumor specimens (Fig. 1D–H). Therefore, we choose human pancreatic β-cells and DLBCL cells, representing two different physiological and pathological conditions, for further analysis of the function and regulation of ISL-1.

3.2. ISL-1 promotes the proliferation of pancreatic β-cells and DLBCL cells, and also accelerates the tumorigenesis of DLBCL in vivo

Our previous research show that ISL-1 is required for maintaining pancreatic homeostasis and can promote adult pancreatic β-cells proliferation [4]. In order to determine the effects of ISL-1 in DLBCL cells, the cell growth rate was analyzed in HIT-T15 and DLBCL cells stably transfected with pcDNA3.1-ISL1 or pLL3.7-ISL1-siRNA. Western bolt analysis showed that ISL-1 expression level was effectively enhanced in HIT-T15 and SUDHL 16 cells transfected with pcDNA3.1-ISL1, while the expression level was significantly knocked-down by pLL3.7-ISL1-siRNA in HIT-T15 and Ly3 cells (Fig. S2). CCK-8 results showed that the overexpression of ISL-1 obviously promoted HIT-T15 and DLBCL cell growth, whereas ISL-1 knockdown significantly inhibited the cell growth (Fig. 2A and B).

To analyze how the growth of HIT-T15 and DLBCL cells was regulated by ISL-1, cell cycle dynamics were analyzed by flow cytometry. We found that in ISL-1-overexpressing HIT-T15 and SUDHL 16 cells, the cell population in the S and G_2/M phases was significantly increased compared with the control cells transfected with pcDNA3.1 plasmid, and the cell population in the G_1 phase was remarkably decreased. Consistently, the G_1 population was significantly increased in the ISL-1-knockdown cell lines with a corresponding decreased population in the S and G_2/M phases (Fig. 2C and D). The above results indicate that ISL-1 is a powerful cell proliferation regulator, even in tumor cell lines. Therefore, it was rational to suspect that ISL-1 may have potential oncogenic functions on the tumorigenesis of DLBCL.

To testify this hypothesis, a mouse xenograft model was used to study the impact of ISL-1 on DLBCL development. As shown in

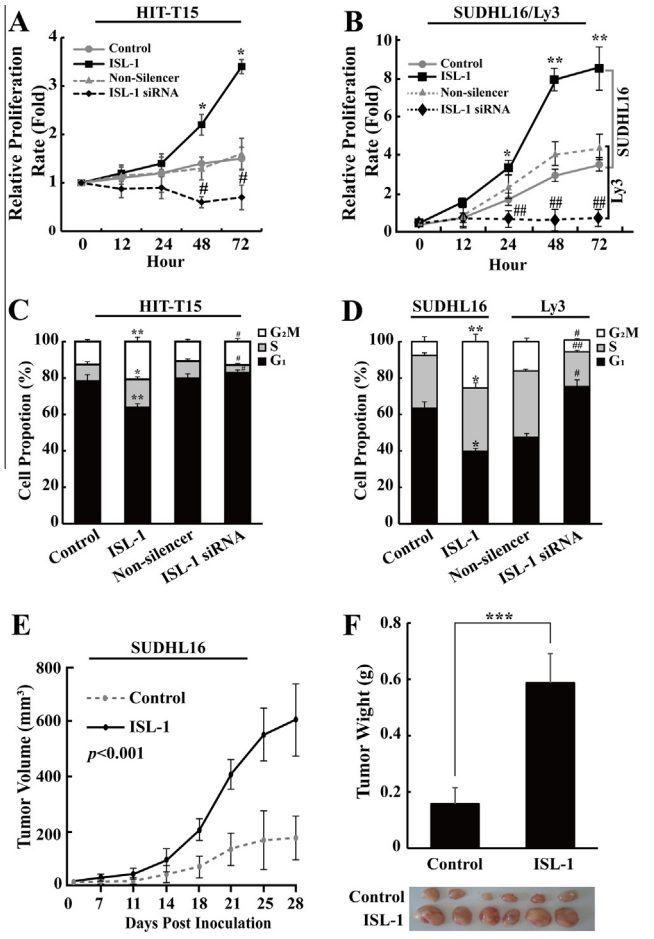

Fig. 2. ISL-1 promotes the proliferation of pancreatic β-cells and DLBCL cells, and also accelerates the tumorigenesis of DLBCL *in vivo*. (A) and (B) The relative proliferation rate of HIT-T15 cells (A) and DLBCL cells (B) was measured using CCK-8 analysis at indicated time post-seeding. (C) and (D) Cell cycle analysis of HIT-T15 cell (C) and DLBCL cells (D) was performed by flow cytometry assay. (E) Beige mice were injected s.c. with SUDHL16 cells stably transfected with pcDNA3.1 (Control), or pcDNA3.1-ISL-1 (ISL-1) construct. The tumor volume was monitored at indicated days post inoculation. (F) The isolated tumors are shown and the tumors weight was measured. Statistical analysis was carried out with Student's *t*-test (A–D) or 2-way ANOVA (E–F). ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs the control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs the non-silencer.)

Fig. 2E, the growth of tumors initiated by ISL-1-overexpressing cells was significantly faster than that initiated by the control cells. At 4 weeks post inoculation, the tumors were isolated and weighed. We found that ISL-1-overexpressing cells produced significantly larger tumors than the control cells (Fig. 2F). Whereas, to our knowledge, ISL-1 does not have oncogenic effect in normal adult pancreatic β-cells although its expression level is extremely high.

Collectively, these results show that ISL-1 overexpression promotes the proliferation of pancreatic β-cells and DLBCL cells, and also accelerates the tumorigenesis of DLBCL *in vivo*, which demonstrate that the role of ISL-1 in DLBCL is similar to, but actually to a larger extent distinct from that in pancreatic β-cells. However, the regulatory mechanisms are not yet clarified.

3.3. ISL-1 positively influences c-Myc expression in pancreatic β-cells and DLBCL while c-Myc only has positive effect on ISL-1 in Ly3 cells but not in pancreatic β-cells

Our previous reports show that ISL-1 can promote adult pancreatic β-cells proliferation through activating c-Myc transcription [4]. c-Myc overexpression has also been recognized to contribute to the development of DLBCL, cell cycle regulation, and could also be linked to adverse prognosis [13–15]. In order to explore the mechanism of ISL-1-stimulated DLBCL cell proliferation and identify the different roles of ISL-1 in pancreatic β-cells and DLBCL, whether the expression level of ISL-1 and c-Myc could influence each other was first analyzed. The results showed that ISL-1 positively influenced c-Myc expression, i.e. the level of c-Myc was up-regulated with ISL-1 overexpression or down-regulated with ISL-1 knockdown in both HIT-T15 cells (Fig. 3A and B) and Ly3 cells (Fig. 3C and D).

However, our further study showed that c-Myc overexpression or knockdown did not affect the expression level of ISL-1 in HIT-T15 cells (Fig. 3E and F). Whereas, in Ly3 cells, up- or down-regulated c-Myc could significantly increase or reduce the expression level of ISL-1 at both mRNA and protein levels (Fig. 3G and H). Moreover, the effects of siRNA were more significant than overexpression of c-Myc on ISL-1 regulation, probably because c-Myc was already expressed at a relative high level in DLBCL and knockdown would therefore produce more obvious effects. These results indicate that the functional diversity of ISL-1 in pancreatic β-cell and DLBCL may depend on the different regulation or interaction between ISL-1 and c-Myc.

3.4. ISL-1 establishes a positive feedback regulatory loop through interacting with c-Myc in DLBCL rather than in pancreatic β-cells

In order to explore the difference and exact mechanism underlying c-Myc-mediated ISL-1 transcription in both pancreatic β-cell and DLBCL, MatInspector bioinformatic analysis was used to identify potential c-Myc binding sites on the transcriptional regulatory region of ISL-1. However, the results did not show any specific c-Myc binding site on the core transcriptional regulatory region (CTRR) of ISL-1. Interestingly, we found that there was a conserved ISL-1 binding sequences (Y**TAAT**GR) [4] (Fig. 4A top panel) located between −442 and −428 of ISL-1 CTRR [10]. Therefore, we suspect that ISL-1 may be a transcriptional regulator of itself, and form a positive feedback loop to contribute to its own up-regulation.

To testify these speculations, luciferase assay was performed in 293T cells. As shown in Fig. 4B, ISL-1 transfection could significantly stimulate the luciferase activity of *ISL-1-luc* W (with the ISL-1 binding site) but not with *ISL-1-luc* D (the ISL-1 binding site was deleted). To determine if ISL-1 could occupy its own transcriptional region *in vivo*, ChIP assays were performed with a specific primer containing the ISL-1 binding site (P1) and a control primer covering the region outside of the ISL-1 binding site (P0) (Fig. 4A bottom panel). Not beyond our expectation, the results showed that there was no specific ISL-1 or c-Myc recruitment at the ISL-1 binding region in HIT-T15 cells. However, in Ly3 cells, ISL-1 and c-Myc could be recruited on the ISL-1 promoter region containing the conserved ISL-1 binding site (Fig. 4C). These results led us to suspect that there could be protein–protein interactions between ISL-1 and c-Myc in DLBCL but not in pancreatic β-cells.

To examine this hypothesis, we used co-immunoprecipitation assay to analyze whether ISL-1 could physically associate with c-Myc. As shown in Fig. 4D, the reciprocal coimmunoprecipitation of endogenous ISL-1 with c-Myc was found in Ly3 cells rather than in HIT-T15 cells. Further ChIP-re-IP assay also showed that ISL-1 and c-Myc could form a ISL-1/c-Myc transcriptional complex and bind to ISL-1 promoter in Ly3 cells (Fig. 4E). These results indicate that c-Myc may cooperate with ISL-1 and synergistically contribute to the aberrant ISL-1 up-regulation in DLBCL.

Luciferase assay in Ly3 cells confirmed the above conception that c-Myc could stimulate transcriptional activation of the ISL-1 promoter in coordination with ISL-1, and this effect was disappeared with ISL-1 knockdown (Fig. 4F), indicating that the transcriptional activity of c-Myc on ISL-1 promoter might be ISL-1-dependent. Consistently, a significant decrease of *ISL-1-luc*

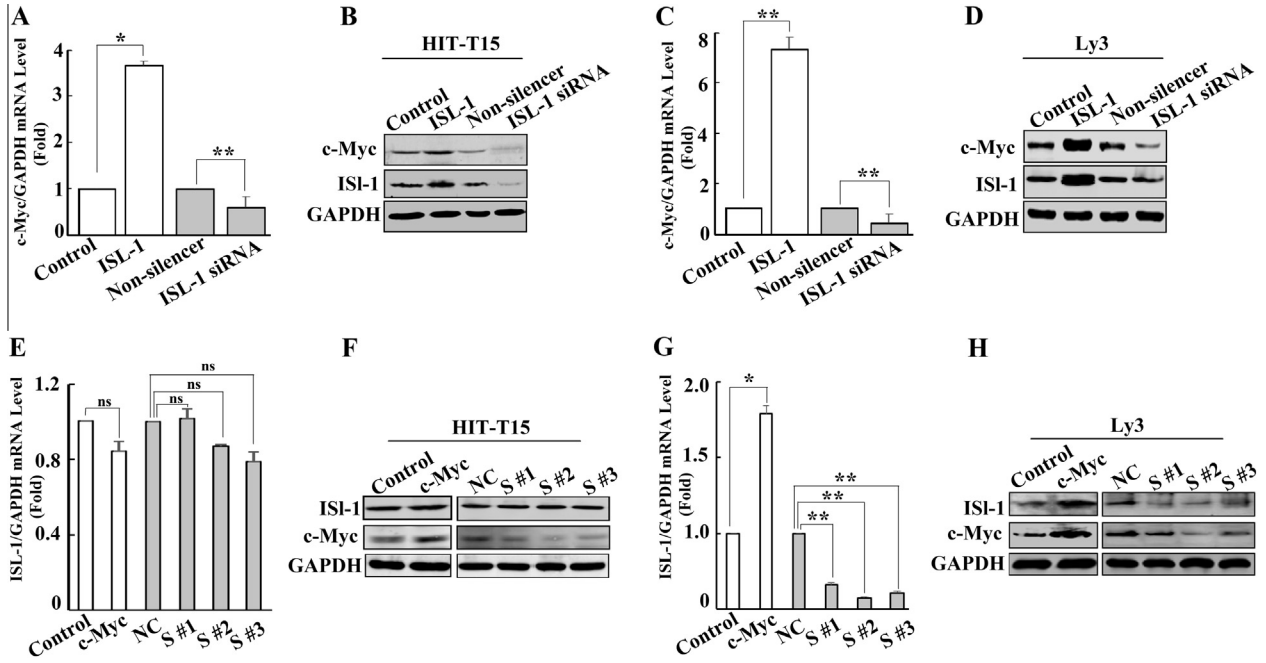

Fig. 3. Regulatory relationship between ISL-1 and c-Myc was analyzed in pancreatic β-cells and DLBCL. (A)–(D) The expression of c-Myc was measured by real-time RT-PCR (A and C) and Western blot (B and D) in HIT-T15 cells (A and B) and Ly3 cells (C and D) with ISL-1-overexpressing or knockdown. (E)–(H) The expression of ISL-1 was measured by real-time RT-PCR (E and G) and Western blot (F and H) in HIT-T15 cells (E and F) and Ly3 cells (G and H) with c-Myc-overexpressing or knockdown. Statistical analysis was carried out using a Student's *t*-test. (*$p < 0.05$; **$p < 0.01$; vs each control; ns, not significant.)

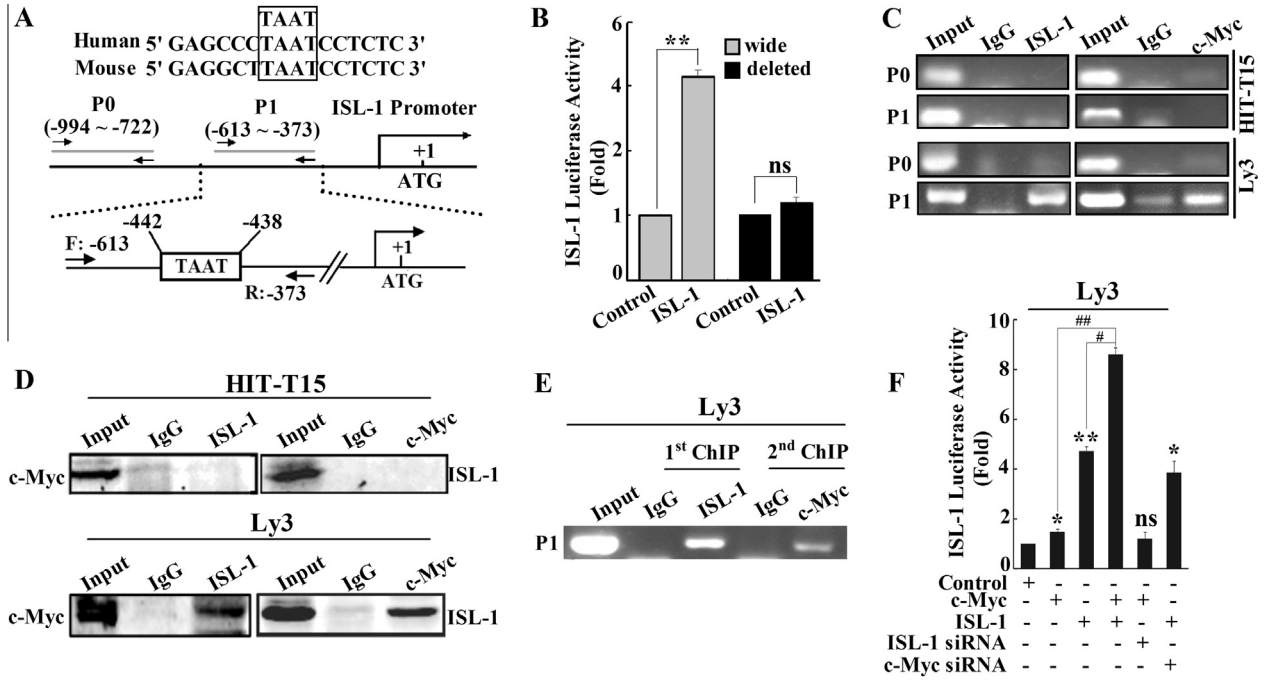

Fig. 4. ISL-1 establishes a positive feedback regulatory loop through interacting with c-Myc in DLBCL rather than in pancreatic β-cells. (A) Consensus binding site (TAAT box) for ISL-1 on the ISL-1 promoter was analyzed by MatInspector software. (B) The transcriptional activity of *ISL-1-luc* was analyzed by luciferase reporter assay in 293T cells. (C) ChIP assay was performed with ISL-1 or c-Myc antibody for immunoprecipitation using chromatin harvested from HIT-T15 (top panel) and Ly3 cells (bottom panel), respectively. (D) Endogenous Co-IP assay between ISL-1 and c-Myc was performed in HIT-T15 (top panel) or Ly3 cells (bottom panel), respectively. (E) ChIP-re-IP assay was performed using chromatin harvested from Ly3 cells. (F) The luciferase activity of *ISL-1-luc* (wide type) was analyzed by luciferase reporter assay in Ly3 cells after transfected with indicated plasmids. Statistical analysis was carried out using a Student's *t*-test. (*$p < 0.05$; **$p < 0.01$; vs each control; ns, not significant.)

activity was seen in ISL-1-overexpressing cells with c-Myc knockdown, supporting that c-Myc might be a transcriptional cofactor of ISL-1 in facilitating ISL-1 overexpression.

Collectively, our data show that ISL-1 can interact with c-Myc to form an ISL-1/c-Myc transcriptional complex, and enhance ISL-1 transcription through directly binding to ISL-1 promoter. Moreover, this cell-specific mechanism only exists in DLBCL rather than in pancreatic β-cells, which may result in the functional diversities of ISL-1 involved in different physiological and pathological processes.

4. Discussion

In this study, we demonstrate that ISL-1 is highly expressed in adult pancreatic β-cells and is specifically expressed in DLBCL specimens and cell lines. ISL-1 promotes the proliferation of pancreatic β-cells and DLBCL cells. SUDHL16 cells with ISL-1 overexpression exhibits much faster tumor growth in a xenograft mouse model, suggesting that its tumorigenesis effect is tissue specific. Further study reveals that ISL-1 can be conjunct with c-Myc to form an ISL-1/c-Myc complex that auto-regulates ISL-1 transcription only in DLBCL cells not in pancreatic β-cells. This "oncogenic" complex and the positive regulation feedback loop may be one of the major causes for the oncogenic function of ISL-1 in DLBCL.

We demonstrate that the ISL-1/c-Myc complex is only existing in DLBCL cells and is absolutely absent in normal pancreatic β-cells. This tissue specificity could be caused by many factors. One of them might be the existing of different cofactors that ISL-1 or c-Myc interact with. The potential cofactors should be rich in DLBCL, whereas, have relative low expression level in pancreatic islet β-cells, or the other way round. Meanwhile, it must be potential to interact with ISL-1 or c-Myc. Here, we propose STAT3 as one of the candidates in DLBCL. It has been reported that STAT3 is overexpressed in DLBCL [16], but is almost undetectable in normal pancreas [17]. Furthermore, STAT3 can cooperate with c-Myc [18] or ISL-1 [19] to promote different genes transcription. However, whether the cooperation between ISL-1 and c-Myc is a direct protein–protein interaction, or bridged by other cofactors are remained to be elucidated.

Additionally, another speculation leading to the regulatory difference of ISL-1 in pancreatic β-cells and DLBCL may be the occupation of ISL-1 binding sits by other pancreatic tissue-specific transcription factor. It has been reported that the initiator element (Inr) of the mouse Cox5b promoter contains overlapping binding sites for Sp1 and YY1 [20]. The expression of Sp1 could reduce the YY1-dependent activation of the Cox5b Inr by binding to the same Inr element on Cox5b promoter, which may help to determine the ultimate expression level of Cox5b in response to different physiological conditions [21]. The pancreatic and duodenal homeobox-1 (PDX-1) plays critical roles in embryologic pancreas development and postnatal islet homeostasis [22], and its target genes contain the consensus TAAT sequence [23], a same binding site of ISL-1[4]. As PDX-1 is specifically expressed in pancreas [22], it may participate in the regulation of the ISL-1 gene transcription by occupying the conserved TAAT binding site on the ISL-1 promoter, and therefore results in the absence of the positive feedback regulation formed by ISL-1/c-Myc complex in adult pancreatic β-cells. However, further investigations are waiting to be carried out for testifying this hypothesis.

Overall, our findings extend the knowledge about the functional diversity of ISL-1 in different conditions. We also show for the first time that ISL-1 plays an oncogenic role in DLBCL via a cell-specific feedback regulation. These results shed lights on the new roles of ISL-1 in different physiological and pathological processes and provide insights into the regulatory relationships between ISL-1 and other cofactors.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.021.

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