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Transcriptome Profiles and Gene Expression of Min6 Cells Are Altered by Pancreatic Stellate Cells

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Abstract

Aim: To identify the influence of pancreatic stellate cell (PSCs) secretions on gene expression profiles of Min6 cells by whole transcriptome sequencing. **Methods:** Pancreatic stellate cells (PSCs) were isolated from C57BL6J mice and propagated *in vitro* to acquire the activated phenotype. Total RNA was isolated from monocultured (MC) and PSC cocultured (CC) Min6 cells to prepare cDNA libraries, which were subjected to whole transcriptome sequencing for identifying differential expression of β-cell transcription factors (*Pdx-1, Rfx6* and *NeuroD1*) related to insulin gene transcription and GSIS related genes such as *Glut2, Gck, Abcc8, Kcnj11* and L-type Ca^{2+} channels (*Cacnb2, Cacna1c*). qRT-PCR was used to validate the gene expression. GSIS of Min6 cells was examined by estimating insulin levels in response to high glucose challenge. **Results:** Transcriptome analysis of discovery set revealed that coculture of Min6 cells with PSCs caused increased expression of β-cell specific genes (*Ins1, Rfx6* and *NeuroD1*) concomitant with decreased expression of *Pdx-1, MafA* and *Nkx2-2*. Expression of GSIS associated genes (*Glut2, Gck, Abcc8, Kcnj11* and *Cacnb2*) was decreased in such conditions. Validation by qRT-PCR in Min6 cells cocultured with PSCs revealed increased significant expression of *Ins1* (2.1 ± 0.22 folds; $p \leq 0.001$), *Rfx6* (1.68 ± 0.23 folds; $p \leq 0.002$) and *NeuroD1* (0.96 ± 0.11 folds; $p \leq 0.01$), accompanied by downregulation of *Cacnb2* (−0.93 ± 0.57 folds; $p \leq 0.05$). PSC secretions did not restore the GSIS from glucose unresponsive higher passage Min6 cells (MC: 1.33 ± 0.42; CC: 1.55 ± 0.72 pmol/mg protein; $p = ns$) upon high glucose stimulation. However, glucose responsive higher passage Min6 cells cocultured with PSCs presented increased insulin secretion (MC: 7.025 ± 0.64; CC:

14.84 ± 1.01 pmol/mg protein; $p \leq 0.04$) concomitant with marginal increase of insulin contents. **Conclusion:** PSC secretions increase *Ins1, Rfx6* and *NeuroD1* gene expression, GSIS from glucose responsive Min6 cells, but do not restore the GSIS from glucose unresponsive Min6 cells.

Keywords

β-Cells, Gene Expression, Islets, Pancreatic Stellate Cells, Transcriptome

1. Introduction

The functioning of pancreatic β-cells, which perform a pivotal role in maintaining glucose homeostasis, is influenced by various factors including nutritional [1] [2] [3], metabolic [4] [5] and hormonal factors [6] [7] [8], as well as pancreatic inflammatory microenvironment [9] [10] [11] [12]. While islet functions are largely unruffled in healthy individuals, alterations in one or more of these factors cause considerable morbidity experienced in pancreatic disease that may be associated with fibrosis [13] [14] [15], resulting from inflammatory cells and activation of pancreatic stellate cells (PSCs). Elevated levels of pancreatic proinflammatory cytokines such as IL-1β, TNF-α and IFN-γ encountered in pancreatic fibro-inflammatory disease conditions like chronic pancreatitis (CP) [16], and pancreatic cancer (PC) [17] [18] [19], and emanating from various sources including activated PSCs are known to contribute towards β-cell damage and impaired insulin secretion. Activated PSCs are also known to secrete IL-6 [20] [21], as well as growth factors such as Activin-A [22], and Hepatocyte Growth Factor (HGF) [23], which are known to promote Glucose Stimulated Insulin Secretion (GSIS) [24]-[29] from β-cells. In addition, the accumulation of PSCs in peri-islet regions and PSC infiltration [14] [15] [30] [31] into the islets of patients with CP and PC also suggest a probable role for PSCs in influencing β-cell function associated with pancreatic disease.

Recent studies with rodents involving coculture of activated PSCs with islets and with β cell lines indicated variable influence of PSCs on insulin secretory response of β-cells. Rat insulinoma cell lines (RINm-5F, INS-1) showed decreased insulin secretion [14] [32] when cocultured with PSCs or incubated with PSC conditioned medium. In yet another study, increased insulin secretory response associated with a decrease in total insulin content was reported when mouse islets were cocultured with PSCs [33]. Availability of adequate information regarding gene expression in β-cells in response to the presence of PSCs in coculture conditions, would resolve these observations and comprehensively elucidate the influence of PSCs on β-cell functions. In view of these considerations, the present study involving indirect coculture of Min6 cells with activated PSCs was aimed to 1) study the transcriptome profile alterations and the accompanying 2) expression of β-cell specific transcription factors and GSIS related genes in Min6 cells.

2. Materials and Methods

2.1. Chemical

All the chemicals used in the experiments are obtained from Sigma unless otherwise mentioned.

2.2. Cells and Culture Conditions

Mouse insulinoma cells

Min6 cells were obtained from National Centre for Cell Sciences, Pune, India as well as from AddexBio, San Diego, USA. Min6 cells were routinely cultured in DMEM containing 25 mM glucose supplemented with 15% FBS in T25 flasks and maintained in a humidified chamber with 5% CO_2 at 37˚C. Where required, Min6 cells at lower passage (P11) were cultured to higher passages (P53-64) under the above conditions.

Isolation and activation of pancreatic stellate cells

Male C57BL6J mice (approximately three months old) were purchased from National Centre for Laboratory Animal Sciences, Hyderabad and were used to isolate the quiescent stellate cells from mouse pancreas as per the method described earlier [34], with minor modifications. Briefly, surgically resected mouse pancreatic tissue was inflated by injecting Hank's balanced salt solution (HBSS) containing Collagenase P (Roche, Germany), Protease type IV and DNase I (Roche, Germany). The distended tissue was incubated in a shaking water bath at 37˚C for 5 - 7 minutes. Finely minced tissue was further incubated at 37˚C for 5 minutes in a shaking water bath. The digested tissue was then centrifuged and pelleted at 450 ×g for 10 minutes. The resultant supernatant was discarded and the pellet was resuspended in HBSS containing 0.3% BSA and centrifuged. The obtained pellet was resuspended in 10 mL of 0.3% BSA containing HBSS and 8 mL of Nycodenz® (Axis Shield, Norway) gradient solution. The cell suspension was carefully layered under HBSS buffer containing 0.3% BSA and centrifuged at 1400 ×g for 20 minutes at 4˚C. The fuzzy band formed at the interphase was collected and centrifuged at 450 ×g for 10 minutes. The obtained pellet with primary cells was suspended in IMDM (Himedia, India) containing 20% FBS (Himedia, India) along with 1% penicillin and streptomycin (Himedia, India). The quiescent phenotype of isolated PSCs was ascertained by appearance of lipid droplets within 6 - 8 hours. These primary cells are subsequently passaged to obtain the activated phenotype of PSCs under *in vitro* conditions. All the indirect coculture experiments are conducted using activated PSCs of passage 3.

2.3. Indirect Coculture of PSCs with Min6 Cells

Min6 cells were subjected to indirect coculture with activated PSCs using transwell inserts with polycarbonate membrane (Himedia, India) having a pore size of 0.4 μm as per the method described earlier [14] [35]. PSCs (25×10^3cells) suspended in complete IMDM were seeded on a transwell insert followed by seeding of Min6 cells (0.25×10^6 cells) suspended in complete DMEM into the

culture well plate. After separate incubation of cells for 24 - 36 hours, the spent media were replaced with fresh media and culture inserts with PSCs were placed in culture wells containing Min6 cells. Incubation was continued for the next 72 hours followed by harvesting Min6 cells for further studies involving RNA isolation, transcriptome profiling and qRT-PCR validation of the target gene expression as well as GSIS assays.

2.4. Whole Transcriptome Sequencing

Total RNA was isolated from Min6 cells employing RNeasy kit (Qiagen, Germany) as per the manufacturer's protocol. Quantity and quality of the isolated RNA was assessed using Bioanalyzer 2100 (Agilent, Germany) and samples which showed RNA integrity number > 8 were used in this study. Whole transcriptome sequencing was performed on monocultured and PSC cocultured Min6 cells samples employing Ion Total RNA-Seq kit V2, including Ion RNA-Seq Core kit v2, Ion RNA-Seq Primer Set (Life technologies, USA). Briefly, 5 µg of total RNA was purified using RiboMinus™ Eukaryote System v2 Kit to obtain rRNA-depleted total RNA. RNA thus obtained was fragmented using RNase III for 10 minutes and purified using magnetic beads. Purified RNA was quantitated on Agilent Bioanalyzer using RNA 6000 Pico Kit which yielded fragments in the range of 100 - 200 nucleotides. Whole transcriptome libraries were constructed by hybridizing and ligating the RNA and subjected to reverse transcription. The obtained cDNA was purified and amplified by polymerase chain reaction. The yield and size distribution of the amplified cDNA was assessed; barcoded pooled transcriptome libraries were sequenced on Proton Semiconductor Sequencer (Ion torrent, Life Technologies, USA). Whole transcriptome sequencing was performed using a single set of total RNA samples isolated from monocultured and PSC cocultured Min6 cells. Sequencing reads were assessed for quality and single-end mRNA reads were mapped onto Mouse genome (mm10) and analyzed using Partek Genomics Suite v6.6. BAM files were imported into the software; samples were assigned the respective attributes using categorical (Coculture Vs Monoculture) variables.

2.5. Gene Expression Studies

Validation of target gene expression by qRT-PCR

Total RNA was isolated from both monocultured and PSC cocultured Min6 cells using TRIzol® reagent (Ambion®, Life Technologies, USA) as per the manufacturer's protocol. 1 µg of total RNA was used to synthesize the cDNA reaction using Superscript IV First-Strand Synthesis System (Invitrogen, Lithuania) in a total volume of 20 µL reaction setup. The prepared cDNA was used to validate the differential expression of β-cell specific and GSIS related genes that were identified in the discovery study, using the Power Sybr® green PCR Master Mix (Applied Biosystems, United Kingdom) on Step One® Real Time PCR System (Applied Biosystems, Singapore). Forward and reverse primer sequences for the target genes used in this study are listed in **Table 1**. Relative target gene

Table 1. Primer sequences used in qRT-PCR study.

Gene	Transcript ID	Forward and Reverse Primer Sequences	Amplicon Size (bp)
Ins1	NM_008386	5-GCCCTTAGTGACCAGCTATAATC-3	154
		5-GGACCACAAAGATGCTGTTTG-3	
Pdx-1	NM_008814	5-GAAATCCACCAAAGCTCACG-3	190
		5-CAAGTTCAACATCACTGCCAG-3	
Rfx6	NM_001159389	5-CGGTGCATTCTTTATGCTCA-3	219
		5-TGTCAAGCCCTTTCCAGAAT-3	
MafA	NM_194350	5-GAGGTCATCCGACTGAAACAG-3	203
		5-GCCAACTTCTCGTATTTCTCCT-3	
NeuroD1	NM_010894	5-CCAGGGTTATGAGATCGTCAC-3	171
		5-TTCTTGTCTGCCTCGTGTTC-5	
Nkx2-2	NM_001077632	5-TTCCATAACCATCGCTACAAG-3	236
		5-TTGGCATTGTGGTCCTACTG-3	
Glut2	NM_031197	5-CTTGGCTTTCACTGTCTTCAC-3	220
		5-GTGAGCAGATCCTTCAGTCTC-3	
Gck	NM_010292	5-GGATGACAGAGCCAGGATG -3	219
		5-TGGGCAACATCTTTACACTGG -3	
Abcc8	NM_011510.3	5-TGCTCTTTGTCCTGGTGTG-3	140
		5-GTCCAGTAGATAAGCAGAGCG-3	
Kcnj11	NM_001204411	5-GAGGACGGGCTCACAGAC-3	155
		5-CACCAGACCATGGCAAAG-3	
Cacnb2	NM_023116	5-CAGCCTTGGAGTCGACTTTTT -3	205
		5-CTATTTTTCCTCCTGGCTCCTT -3	
Cacna1c	NM_001256001	5-TTCTTCCTCTTTGTGGCTTCT-3	233
		5-CAGCTGCATTGGCATTCAT-3	
Mtpn	NM_008098	AAAACGGAGACTTGGATGAGG	247
		TCAGCACCCTTTGACAGAAG	
β-Actin	NM_007393	5-CATCCGTAAAGACCTCTATGCC-3	231
		5-GACTCATCGTACTCCTGCTTG-3	

expression was normalized to the expression levels of *β-actin*. The fold difference between the monoculture and coculture samples was calculated by using Pfaffl's $2^{-\Delta\Delta Ct}$ method and the obtained values were log2 transformed.

2.6. Glucose Stimulated Insulin Secretion

Glucose Stimulated Insulin Secretion (GSIS) from Min6 cells was examined as per the method described earlier [24]. Min6 cells were incubated in Krebs-Ringer bicarbonate with Hepes (KRBH) buffer containing (mmol/L): NaCl, 120; KCl, 5; $CaCl_2$, 2.56; $MgCl_2$, 1.1; $NaHCO_3$, 25; Hepes (Himedia), 10; along with 0.2% Bovine Serum Albumin, pH 7.4. After incubation at 37˚C for 72

hours, the cells were washed twice in KRBH buffer and pre-incubated in KRBH containing 2.5 mM glucose for 30 minutes. The cells were incubated for one hour in presence of 2.5 mM (basal) glucose, followed by stimulation with 25 mM (high) glucose, for another hour. The cell supernatants collected after basal and high glucose stimulation were stored at −20˚C until further use.

2.7. Measurement of Insulin Contents in Min6 Cells

Min6 cells were lysed in Radio Immuno Precipitation Assay (RIPA) buffer (Cell Signaling Technologies, USA) and incubated on ice for 5 minutes. The cell lysates were then briefly sonicated and centrifuged at 14,000 ×g for 10 minutes at 4˚C. The concentration of the total protein in these samples was measured using Bradford's method. The supernatants were collected and stored at −20˚C until further use to measure the total insulin content. Insulin was estimated using mouse insulin ELISA (Mercodia, Sweden) kit and OD was recorded at 450 nm on a microplate reader (BioRad Model 680, Japan). Insulin contents were normalized to total protein and expressed in terms of pmoles of insulin per mg protein.

2.8. Statistical Analysis

Probability (p) values between the MC and CC groups were calculated using Student's t-test using Microsoft Excel program. Data are represented as mean +/− SEM. $p \leq 0.05$ is considered to be statistically significant.

3. Results

3.1. Whole Transcriptome Analysis Identifies Differentially Expressed Genes in Higher Passage Min6 Cells Cocultured with PSCs

The discovery set of cDNA libraries, with an average size of 248 and 269 bp length respectively, were prepared from Min6 cells cultured in presence or in absence of PSCs and subjected to whole transcriptome sequencing. These cDNA libraries generated ≈ 67 million reads with a mean length of 107 bp and 54% usable reads for MC higher passage Min6 cells and ≈58 million reads with 92 bp as mean length and 46% usable reads for CC samples. The usable reads obtained from the whole transcriptome sequencing were aligned separately on to the mouse mm10 reference genome, which identified 29610 transcripts during the analysis. Expression levels of the transcripts were normalized as reads per kilobase per million mapped reads (RPKM) to identify the differentially expressed genes. Of all the genes subjected to transcriptome analysis in the discovery set, important changes could be noted with regard to increased expression of β-cell specific genes such *Ins1* (1.68 folds), *Rfx6* (2.33 folds) and *NeuroD1* (1.31 folds) accompanied with decreased expression of *Pdx1* (−1.92 folds), *MafA* (−2.32 folds), *Nkx2-2* (−1.46 folds) and GSIS associated genes such as *Glut2* (−3.28 folds), *Gck* (−1.10), *Abcc8* (−1.61 folds), *Kcnj11* (−2.36 folds), *Cacnb2* (−2.97 folds) and *Cacna1c* (−1.30 folds). These results are depicted in Table 2 with their respective reads and RPKM values.

Table 2. List of differentially expressed β-cell specific and GSIS related genes in PSC co-cultured higher passage Min6 cells identified in whole transcriptome analysis from the discovery set. Reads, Reads Per Kilobase Million (RPKM) values and the respective fold changes for the mentioned genes were obtained after analysis of the data using Partek Genomics Suite v6.6.

Transcript ID	Gene	Reads (MC)	Reads (CC)	RPKM (MC)	RPKM (CC)	Fold Change (in CC)
NM_008386	*Ins1*	215373	361863	5659.18	11267.6	1.68017
NM_001185083	Ins2	3793.6	5540.4	126.461	218.863	1.46046
NM_001185084	Ins2	91252	36758.9	2447.11	1168.15	−2.48245
NM_008387	Ins2	42757.4	42740.5	1389.78	1646.26	−1.0004
NM_008814	*Pdx1*	16627	8647	201.983	124.478	−1.92286
NM_001159389	*Rfx6*	272	633.999	1.23012	3.39777	2.33088
NM_177306	*Rfx6*	0.000466417	0.00266188	2.36E−06	1.60E−05	5.70708
NM_194350	*MafA*	9398	4038	136.048	69.2703	−2.32739
NM_010894	*NeuroD1*	3514	4638	22.0285	34.4539	1.31986
NM_001077632	*Nkx2-2*	336.332	166.843	3.88355	2.28293	−2.01587
NM_010919	*Nkx2-2*	1095.67	747.992	8.36017	6.76331	−1.46481
NM_144955	Nkx6-1	3717	1550	32.3748	15.9982	−2.39806
NM_001159925	Pax4	0	2.96899	0	0.0417347	NC
NM_001159926	Pax4	0	3.31972	0	0.0467713	NC
NM_011038	Pax4	0	0.711296	0	0.00971125	NC
NM_001244198	Pax6	302.777	224.771	1.29655	1.1406	−1.34705
NM_001244201	Pax6	510.772	367.411	2.21269	1.88612	−1.39019
NM_001244200	Pax6	1512.37	787.479	6.34593	3.91562	−1.92052
NM_001310144	Pax6	2529.64	1276.08	10.7354	6.41744	−1.98236
NM_001244202	Pax6	320.591	256.054	1.20341	1.13899	−1.25204
NM_013627	Pax6	203.656	167.305	0.756839	0.736784	−1.21727
NM_001310145	Pax6	40.8918	18.1927	0.181263	0.0955644	−2.2477
NM_001310146	Pax6	70.2081	30.1526	0.314966	0.160297	−2.32842
NM_031197	Slc2a2 (*Glut2*)	82	25	0.498838	0.180223	−3.28
NM_001287386	*Gck*	2.07337	0.300956	0.0136717	0.00235167	−6.88927
NM_010292	*Gck*	614.928	556.699	3.52548	3.78216	−1.1046
NM_011510	*Abcc8*	20209	12511	63.8033	46.8075	−1.6153
NM_001204411	*Kcnj11*	295.043	330.939	1.72958	2.29895	1.12166
NM_010602	*Kcnj11*	7004.72	2959.99	35.157	17.605	−2.36647
NM_001252533	*Cacnb2*	33.2901	11.1948	0.136463	0.0543803	−2.9737
NM_001309519	*Cacnb2*	12.8203	0.358676	0.0515395	0.00170871	−35.7434
NM_023116	*Cacnb2*	962.894	512.447	3.81602	2.40661	−1.87901
NM_001159533	*Cacna1c*	478.524	389.614	0.550427	0.531074	−1.2282
NM_001159534	*Cacna1c*	564.212	431.145	0.64899	0.587685	−1.30864
NM_001159535	*Cacna1c*	450.751	375.024	0.51871	0.511413	−1.20192

Continued

NM_001255997	*Cacna1c*	33.1207	19.3569	0.0388171	0.0268834	−1.71106
NM_001255998	*Cacna1c*	26.2488	18.2617	0.0305914	0.0252205	−1.43737
NM_001255999	*Cacna1c*	27.7333	17.35	0.0325031	0.0240962	−1.59846
NM_001256000	*Cacna1c*	498.247	599.278	0.571348	0.814346	1.20277
NM_001256001	*Cacna1c*	907.42	1101.3	1.03987	1.49554	1.21366
NM_001256002	*Cacna1c*	381.026	383.143	0.430394	0.512859	1.00556
NM_001290335	*Cacna1c*	26.1866	16.2691	0.0323491	0.0238161	−1.60959
NM_009781	*Cacna1c*	27.5241	17.269	0.032258	0.0239837	−1.59384
NM_001083616	Cacna1d	360.529	251.773	0.622008	0.514742	−1.43196
NM_001302637	Cacna1d	249.841	186.019	0.426104	0.375953	−1.34309
NM_028981	Cacna1d	90.4243	111.495	0.162721	0.237761	1.23302
NM_001081023	Cacna1s	1.1352	1.892	0.00283924	0.00560758	1.66667
NM_014193	Cacna1s	1.8648	3.108	0.00471152	0.0093054	1.66667

3.2. qRT-PCR Validation Identifies Increased Expression of *Ins1*, Rfx-6 and *NeuroD1* Genes in PSC Cocultured Higher Passage Min6 Cells

Changes in the expression levels of β-cell related genes observed in the discovery set were validated by qRT-PCR. In comparison to Min6 cells cultured in absence of PSCs, those cultured in presence of PSCs revealed increased expression levels of *Ins1* (2.1 ± 0.22 folds; $p \leq 0.001$), *Rfx6* (1.68 ± 0.23 folds; $p \leq 0.002$) and *NeuroD1* (0.96 ± 0.11 folds; $p \leq 0.01$), concomitant with decreased expression of *MafA* (−0.82 ± 0.29 folds; $p \leq 0.05$) and *Nkx2-2* (−0.43 ± 0.44 folds; $p \leq 0.39$) genes, without significant change in *Pdx-1* expression (0.4 ± 0.57 folds; $p \leq 0.57$) (**Figure 1(a)**).

3.3. PSCs Do Not Alter the Expression of GSIS Related Genes in Higher Passage Min6 Cells

As in case of β-cell related genes, changes in the expression levels of GSIS associated genes are also validated by qRT-PCR. Although the expression levels of *Glut2* (−1.12 ± 0.76 folds; $p \leq 0.76$), potassium channel subunit *Abcc8* (−0.61 ± 0.38 folds; $p \leq 0.18$) and L-type calcium channel subunit *Cacnb2* (−0.93 ± 0.57 folds; $p \leq 0.05$) were decreased as in the discovery set, results of the validation revealed no statistically significant change in the expression levels between mono and PSC cocultured Min6 cells. Although not significant, marginal increase in the expression of *Gck* (0.63 ± 0.81 folds; $p \leq 0.48$) and potassium channel subunit *Kcnj11* (0.53 ± 0.57 folds; $p \leq 0.40$) were noticed in PSC cocultured Min6 cells compared to monocultured Min6 cells. The L-type calcium channel, *Cacna1c* (0.60 ± 0.38 folds; $p \leq 0.38$) also did not show any significant increase in its expression (**Figure 1(b)**), which was noticed to be upregulated by 1.2-fold in the discovery study.

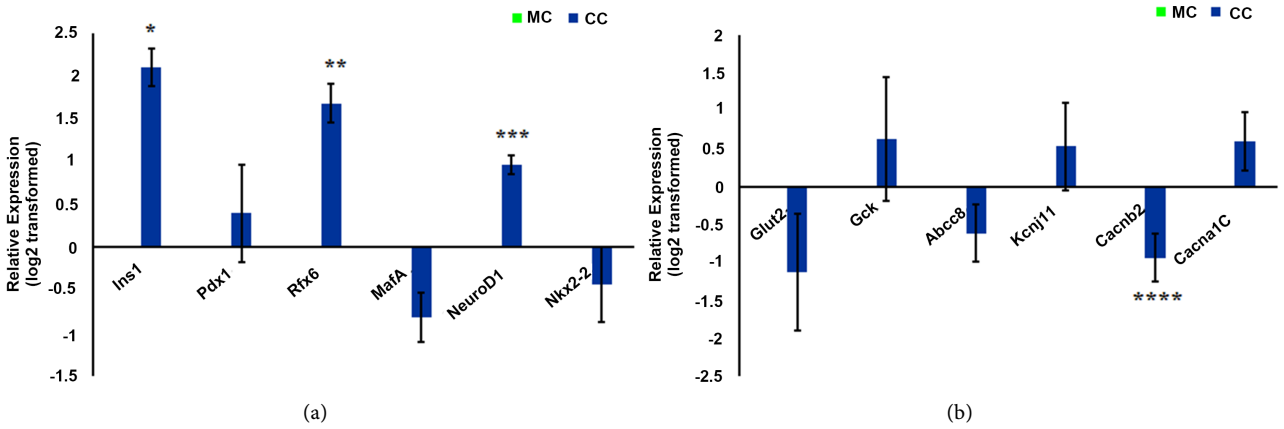


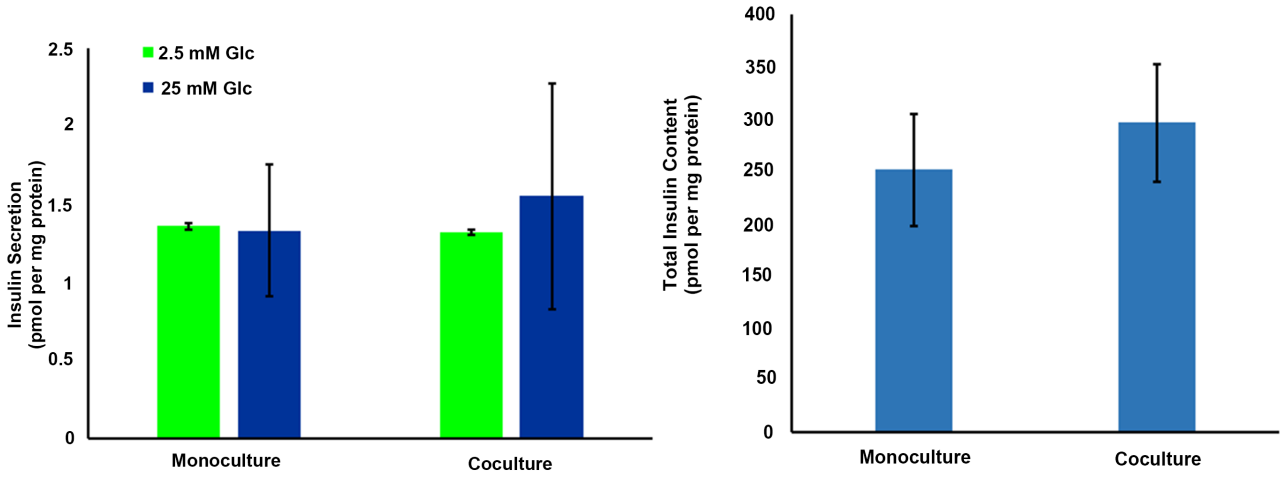
Figure 1. Relative expression of *β*-cell specific and GSIS related genes in PSC cocultured higher passage Min6 cells: (a) PSC cocultured Min6 cells showing a significant increase in the expression levels of *Ins1, Rfx6* and *NeuroD1*, while *Pdx1*, *MafA* and *Nkx2-2* expression levels were seen unaltered; (b) GSIS associated genes such as *Glut2*, *Gck, Abcc8, Kcnj11* and *Cacna1c* showing no significant change in their expression levels while *Cacnb2*, showed significant downregulation in PSC cocultured Min6 cells (n = 5). *β-actin* was used as an endogenous control to normalize the gene expression levels. Fold changes were calculated using $2^{-\Delta\Delta Ct}$ method and values were log transformed. Each target gene studied was analysed in duplicates. Data are represented as mean ± SEM. *$p \leq 0.001$, **$p \leq 0.002$, ***$p \leq 0.01$, ****$p \leq 0.05$. MC = Monoculture, CC = Coculture.

3.4. PSC Secretions Do Not Restore GSIS from Higher Passage Glucose Unresponsive Min6 Cells

GSIS response of Min6 cells at high passage (P53-64) was examined when they were cultured in presence or in absence of PSC secretions and the obtained results are depicted in Figure 2(a). It was noted that the high passage Min6 cells used in this study are unresponsive to high (25 mM) glucose stimulation and coculturing such cells with of PSC (MC: 1.33 ± 0.42; CC: 1.55 ± 0.72 pmol/mg protein; p = ns) did not reveal any statistically significant changes (Figure 2(a)). However, repeated subculturing of Min6 cells from passage 11 to passage 53-64 resulted in enhanced glucose responsiveness (MC: 7.025 ± 0.64; CC: 14.84 ± 1.01 pmol/mg protein; $p \leq 0.04$) of Min6 cells when cocultured with PSCs compared to monocultured Min6 cells (Figure 3(a)), suggesting that the influence of PSC secretions is dependent on the ability of Min6 cells to respond to high glucose stimulation. As shown in Figure 2(b), the total insulin contents of glucose unresponsive higher passage Min6 cells were found to be 251.31 ± 53.95 and 296.37 ± 56.75 pmol/mg protein ($p \leq 0.59$), whereas the glucose responsive higher passage Min6 cells showed 4871.94 ± 1271.23 and 6454.03 ± 474.21 pmol/mg protein ($p \leq 0.51$) (Figure 3(b)) in respective monocultured and PSC cocultured Min6 cells.

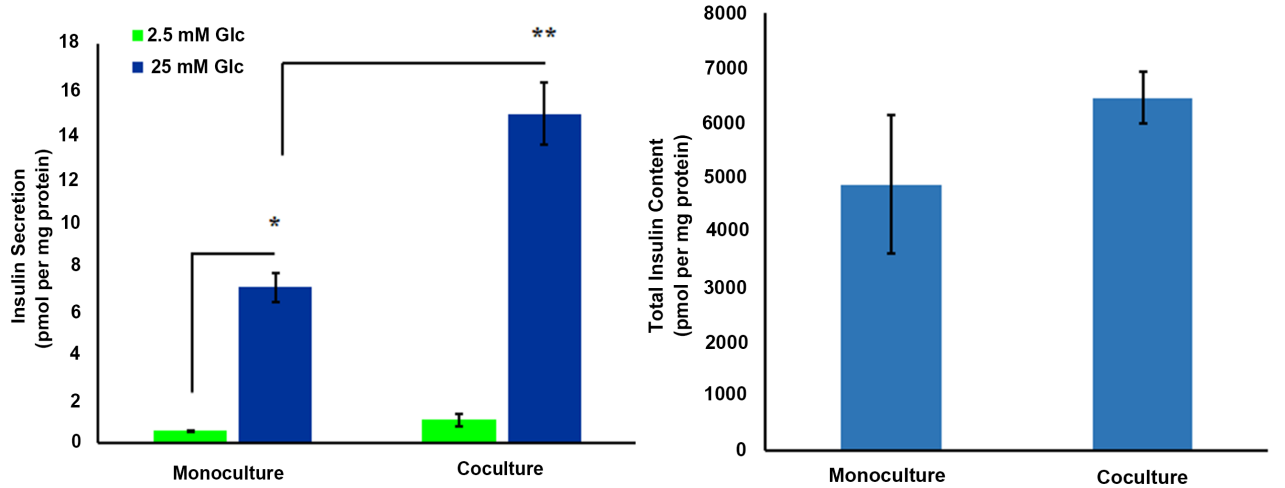
3.5. Increased Expression of *Ins1*, *Rfx6* and *NeuroD1* in PSC Cocultured Glucose Responsive Higher Passage Min6 Cells

Similar to glucose unresponsive higher passage Min6 cells, we found a significant upregulation of *Ins1* (0.82 ± 0.07 folds; $p \leq 0.008$), *Rfx6* (0.95 ± 0.18 folds; $p \leq 0.04$) and *NeuroD1* (0.93 ± 0.14 folds; $p \leq 0.03$), with a concomitant but not significant decrease in the expression of *Pdx-1* (−0.28 ± 0.54 folds; $p \leq 0.65$),



(a) (b)

Figure 2. Impaired GSIS response from higher passage Min6 cells: (a) Mono and PSC cocultured Min6 cells showing no significant change in the amount of insulin secreted when subjected to high (25 mM) glucose stimulation (n = 4), compared to basal (2.5 mM) glucose, suggesting the impaired glucose sensing ability of these higher passage Min6 cells; (b) PSC cocultured Min6 cells showing a marginal but not significant increase in the total insulin contents compared to monocultured Min6 cells (n = 3). Insulin secretion and insulin content was normalized to protein levels. Each sample was quantitated in duplicates and data are represented as mean ± SEM. p = ns. GSIS = Glucose Stimulated Insulin Secretion. Glc = Glucose.



(a) (b)

Figure 3. Pancreatic stellate cells potentiate the insulin secretion from glucose responsive higher passage Min6 cells: (a) Glucose responsive higher passage Min6 cells showing augmented insulin secretion from PSC cocultured Min6 cells compared to Min6 cells in monocultures in response to high (25 mM) glucose stimulation (n = 4); (b) PSC cocultured Min6 cells showing mild but not significant increase in total insulin content levels similar to PSC cocultured glucose unresponsive higher passage Min6 cells compared to monocultured Min6 cells (n = 4, p = ns). Insulin secretion and total insulin content levels were normalized to total protein. Each sample was analysed in duplicates and data are presented as mean ± SEM. *$p \leq 0.02$ Vs basal glucose stimulation in monocultured Min6 cells, **$p \leq 0.04$ Vs monocultured Min6 cells stimulated with high (25 mM) glucose.

MafA (1.15 ± 0.65 folds; $p \leq 0.22$) and *Nkx*2-2 (−1.5 ± 0.71 folds; $p \leq 0.61$) in glucose responsive higher passage Min6 cells. Unlike PSC cocultured glucose unresponsive higher passage Min6 cells, *Cacnb*2 (1.84 ± 0.38 folds; $p \leq 0.05$) along with *Cacna1c* (1.75 ± 0.123 folds; $p \leq 0.005$) showed increased gene ex-

pression in glucose responsive higher passage Min6 cells. *Glut2* (0.72 ± 0.22 folds; $p \leq 0.08$), *Gck* (1.42 ± 0.56 folds; $p \leq 0.12$), *ABCC8* (0.93 ± 1.0 folds; $p \leq 0.45$) were upregulated, while *Kcnj11* (−1.15 ± 2.33 folds; $p \leq 0.67$) expression was found to be decreased with no statistical significance (**Figure 4(a)** & **Figure 4(b)**).

4. Discussion

The present communication describes results obtained upon culturing Min6 cells exposed to secretions of pancreatic stellate cells. Although PSC infiltration into the islets was demonstrated earlier in pancreatic disease including chronic pancreatitis and pancreatic cancer as well as in diabetic rodent models [14] [15] [30] [31], their influence on β-cell function is not well understood. Hence the aim of the present study was to identify the influence of activated PSC secretions on global gene expression profile and study the insulin secretory responses in Min6 cells under *in vitro* conditions. The transwell inserts used in the present communication were advantageous since they permit interactions between secretions of PSCs kept in the upper chamber with Min6 cells present in the lower chamber. Data obtained using such an experimental setup indicated that PSC secretions do influence gene expression and functional ability of Min6 cells to secrete insulin in response to high glucose challenge.

Genes related to synthesis and secretion of insulin were differentially expressed in response to PSC secretions in the discovery study. While expression levels of *Ins1, Rfx6* and *NeuroD1* were increased, those of *MafA* and *Nkx2-2*

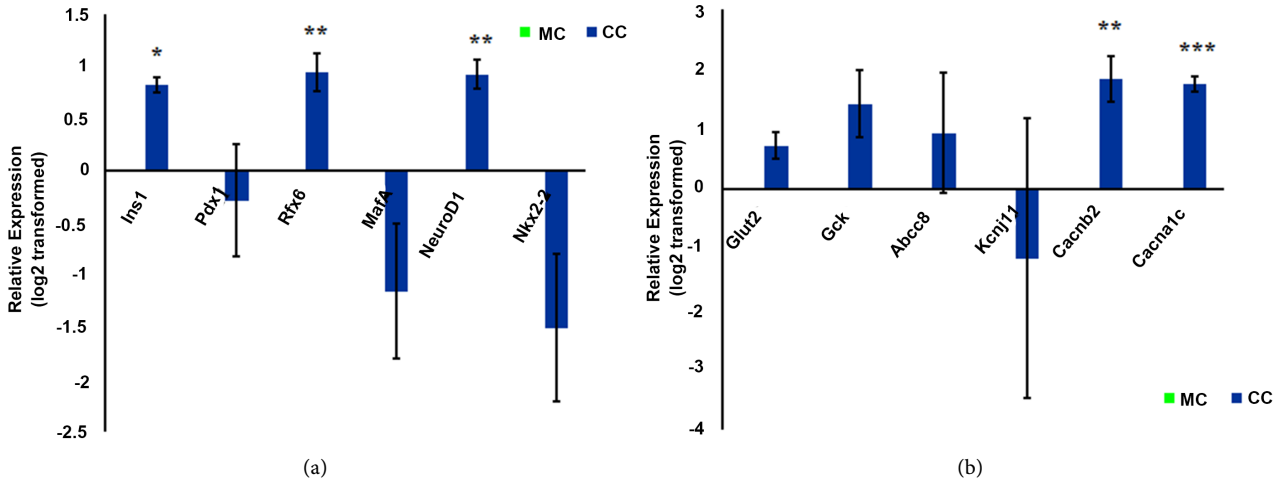


Figure 4. Relative expression of β-cell specific and GSIS related genes in PSC cocultured glucose responsive higher passage Min6 cells: (a) PSC cocultured glucose responsive higher passage Min6 cells showing increased expression of *Ins1, Rfx6* and *NeuroD1* with no significant changes in the expression of *Pdx1, MafA* and *Nkx2-2*, similar to PSC cocultured glucose unresponsive higher passage Min6 cells (n = 3); (b) Significant upregulation of *Cacnb2* and *Cacna1c* calcium channels with no alteration of other studied GSIS genes in cocultured glucose responsive higher passage Min6 cells, confirms that PSC secreted factors influence the expression of β-cell specific and GSIS associated genes in higher passage Min6 cells (n = 3). *β actin* was used as an internal control to normalize the target gene expression and obtained fold changes were log transformed. Each target gene studied was analyzed in duplicates. Data are represented as mean ± SEM. *$p \leq 0.008$, **$p \leq 0.05$, ***$p \leq 0.005$ Vs monocultured cells.

were decreased without apparent changes in *pdx-1* expression in Min6 cells exposed to PSC secretions in qRT-PCR validation study. Such changes related to *Ins1* gene expression were unaccompanied by changes in genes related to insulin secretory pathway. The enhanced expression of *Rfx6* and *NeuroD1* suggests that *Rfx6*, either on its own or in combination with *NeuroD1* [36], does influence expression of *Ins1* gene. These results gain credence from observations made in an earlier study suggesting the importance of *Rfx6* [37] [38], on β-cell insulin gene expression.

Despite the increased expression of *Rfx6*, which influences GSIS related changes [37] [38], our studies did not reveal altered expression of GSIS associated genes in response to PSC secretions on Min6 cells. Results obtained with indirect coculture did not reveal changes in GSIS of Min6 cells exposed to PSCs. Recent *in vitro* coculture studies presented varied results on influence of PSCs on GSIS response [13]-[18]. Studies involving indirect cocultue of PSCs and its secretions with RINm-5F and INS-1 cell lines reported decreased insulin secretion [14] [32], while direct coculture of mouse islets in presence of PSCs caused increased insulin secretion [33]. While considering the response of insulin secreting cells to PSCs under *in vitro* conditions, the innate ability of cells to respond to glucose challenges is of considerable importance. It is generally held that RINm-5F cells are not responsive to glucose challenges [39] [40], and the ability of Min6 cells to respond to glucose depends on the number of passages they undergo [41] [42].

In our study we employed Min6 cells of higher passage (P53-64) and examined their insulin secretory responses. These cells did not exhibit much change in their GSIS response either in monoculture or coculture. Such an observation may be ascribed to the reported inability of glucose unresponsive higher passage Min6 cells to high glucose challenge [41] [42]. This result also indicates the inability of PSC secretions to restore the lost ability of insulin secretion of higher passage Min6 cells to glucose challenge. In order to verify such an inference, we examined GSIS of lower passage Min6 cells (P11) that were cultured to higher passage (P53-64). Results of this experiment as shown in **Figure 3(a)** demonstrated the ability of such cells not only to respond to glucose challenge but also confirmed that PSC secretions further enhance insulin release by Min6 cells. The ability of higher passage Min6 cells to respond to glucose challenge in presence of PSC secretions also denotes that the glucose responsive nature of Min6 cells is of key relevance for the PSCs to influence insulin secretory abilities of Min6 cells. It is apparent that results obtained upon *in vitro* studies might not reflect *in vivo* situation, more so under disease conditions. Extrapolating the results reported herein to a physiological context, we may infer that even though PSCs can innately influence insulin secretory abilities of β-cells, it is possible that such an ability might be diminished in pancreatic disease.

The nature of secretions made by PSCs has not been characterized in the present study. It is known that PSC secretions, including inflammatory cytokines and different growth factors, can not only influence the pancreatic milieu but

also influence β-cell functions. Importantly, the nature of PSC secretions under *in vivo* disease conditions would largely determine their influence on β-cell functions. Despite these limitations, this is the first report on the influence of PSCs on transcriptome profiles as well as genes that are involved in the insulin synthesis and secretory pathway of higher passage Min6 cells. Further experiments including characterization of PSC secretome should yield important information about their influence on gene expression profiles and insulin secretory response from islet isolates obtained from diabetic conditions.

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