Original Article

γ-Aminobutyric Acid (GABA) Induced *in Vitro* Differentiation of Rat Pancreatic Ductal Stem Cells into Insulin-Secreting Islet-Like Cell Clusters

(γ-aminobutyric acid / pancreatic ductal stem cells / differentiation / insulin-secreting cells)

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Abstract. *In vitro* produced β-like cells can provide promising cell therapy for curing the epidemic of diabetes. In this context, we aimed to investigate the effects of different concentrations of γ-aminobutyric acid (GABA) on the differentiation of rat pancreatic ductal epithelial-like stem cells (PDESCs) into β-like cells. The PDESC line cells were cultured in the basal media (DMEM/F12 + 10% FBS + 1% penicillin-streptomycin) supplemented with 0 μM, 5 μM, 50 μM, 500 μM, and 5 mM of GABA for 28 days to induce their differentiation. The differentiated cells were detected by cell morphology, dithizone (DTZ) staining, immunofluorescence staining, real-time polymerase chain reaction (qPCR), and glucose-stimulated insulin secretion (GSIS) assay to validate their identity. At the end of 28 days, compared with the control group, enrichment of induced cells was high among the 5 μM, 50 μM, 500 μM, and 5 mM GABA induction groups. The formation of islet-like cell clusters (ICCs) began at 14 days, and the cell clusters showed a growth trend with the culture time. The induced ICCs were positive for DTZ staining, while the control group showed negative results for DTZ staining and the differentiated cells were also positive for β-cell-specific markers (Ins1 and Pdx1). GSIS assay of 50 μM induction group cells at 28 days showed significantly higher levels of C-peptide and insulin secretion than the control, 5 μM, 500 μM, and 5 mM GABA-treated groups ($P < 0.01$). At the same time, the 50 μM induction group cells also showed significantly higher levels of Ins1, Pdx1 and Nkx6.1 mRNA as compared to the 5 μM, 500 μM and 5 mM GABA groups ($P < 0.01$). Thus, the addition of GABA to the basal medium effectively induced differentiation of adult rat PDESCs into insulin-secreting β-like cells, and 50 μM was the most effective concentration for the induction.

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Abbreviations: Akt – protein kinase B; ANOVA – analysis of variance; BSA – bovine serum albumin; DMEM – Dulbecco's Modified Eagle's Medium; DMSO – dimethyl sulphoxide; DTZ – dithizone; EGF – epidermal growth factor; ELISA – enzyme-linked immunosorbent assay; FACS – fluorescence-activated cell sorting; GABA, γ-aminobutyric acid; FBS – foetal bovine serum; GSIS – glucose-stimulated insulin secretion; ICCs – islet-like cell clusters; Ins1 – insulin 1; Nkx6.1 – homeobox protein Nkx-6.1; PI3K – phosphatidylinositol-3-kinase; PBS – phosphate-buffered saline; PDESCs – pancreatic ductal epithelial-like stem cells; Pdx1 – pancreatic and duodenal homeobox 1; qPCR – real-time polymerase chain reaction; RPMI – Roswell Park Memorial Institute medium; sc-RNA-seq – single-cell RNA sequencing; SD – Sprague Dawley; VDCCs – voltage-dependent calcium channels.

Introduction

The pancreas is made up of two compartments; exocrine (acinar, centroacinar and ductal cells) and endocrine (islet of Langerhans) (Zhou and Melton, 2018; Qadir et al., 2018). The endocrine pancreas contains numerous islets of different hormone-secreting cells, which are β cells (insulin), α cells (glucagon), δ cells (somatostatin), PP cells (pancreatic polypeptide), and ε cells (ghrelin) (Shih et al., 2013). Islet β cells hold the task of maintaining normoglycaemia through their insulin secretion, and their dysfunction or lack in number leads to diabetes, a condition in which patients cannot maintain normoglycaemia (Ravindranath Aathira, 2014; Piero, 2015; Zimmet et al., 2016; Afelik and Rovira, 2017a). For the treatment of diabetes, exogenous insulin

is mainly used, which can only fix the problem temporarily without adjusting the debilitative penalties of diabetes and lifestyle problems of its patients (Rogers, 2019). Although islet transplantation can cure diabetes (Kuise and Noguchi, 2011), it has issues related to the donor shortage and life-long need of immunosuppression (Jacobson and Tzanakakis, 2017; Asghar and Zhu, 2018). On the other hand, *in vitro* derived β-like cells can provide a scalable supply of insulin-secreting cells to cure the epidemic of diabetes (Benthuysen et al., 2016; Peng et al., 2018; Cañibano-Hernández et al., 2019). In this regard, various kinds of inducing factors have been evaluated for regeneration or replacement of functional β cells from stem, precursor, or differentiated cell types (Pagliuca et al., 2014; Rezania et al., 2014; Corritore et al., 2016; Aguayo-Mazzucato and Bonner-Weir, 2018; Zhou and Melton,, 2018). Keeping in mind the dependency of the pancreas on facultative progenitors, which are most widely believed to be present in ductal branches of the pancreas (Criscimanna et al., 2011; Inada et al., 2008; El-Gohary et al., 2016; Ghani et al., 2019), most of the researchers have used PDESCs for *in vitro* development of β-like cells (Corritore et al., 2016; Afelik and Rovira, 2017b; Zhou and Melton, 2018).

γ-Aminobutyric acid (GABA) is a neurotransmitter and one of the factors that have been widely studied for finding treatment or cure of diabetes. It is widely present in the nervous and non-nervous tissues (Tillakaratne et al., 1995), including endocrine pancreas (Adeghate and Ponery, 2002; Franklin and Wollheim, 2004). The co-existence of GABA, GABA receptor and its anabolic enzymes in pancreatic islet cells (Reetz et al., 1991; Franklin and Wollheim, 2004) is an indication for the involvement of GABA in pancreatic endocrine cell function (Ligon et al., 2007; Braun et al., 2010). After its release, GABA acts as an autocrine signalling molecule for regulating β-cell function, and on the islet cell types, through paracrine signalling (Franklin and Wollheim, 2004). Additionally, GABA has also been found to protect β cells from apoptosis *in vitro* (Soltania et al., 2011) and to convert the α cells into β cells *in vivo* (Ben-Othman et al., 2017).

Using the aforementioned reports as a foundation, here we report the *in vitro* differentiation of PDESCs into islet-like cell clusters through the treatment with GABA. The induced ICCs were positive for Pdx1 and Ins1 and also secreted C-peptide and insulin upon glucose stimulation.

Material and Methods

Cell line

In this study, we used cells from the adult Sprague Dawley (SD) rat pancreatic ductal epithelial-like stem cell line that was isolated and cultivated by our laboratory. Briefly, after isolation of the pancreas from an adult rat, enzymatic digestion with collagen and trypsin was used to separate the pancreatic ductal epithelial stem cells (PDESCs) through a dextrin discontinuous density gradient. Further, sorting of these PDSECs was carried out through FACS to confirm their purity, and protein expression of stem cell markers, CK19, NeuroD2, Oct4, PCNA, and Nanog, was verified through immunofluorescence staining, which showed positive results. After isolation and characterization of their identity and both proliferation and differentiation abilities, the cells were preserved in the China Typical Culture Collection Centre under the accession number C201457.

Differentiation protocol and experimental groups

In the first stage, undifferentiated rat PDESCs were cultured in cell culture petri plates using RPMI-1640 media (Gibco, Lot no#72400120, Gaithersburgh, MD), supplemented with 10% foetal bovine serum (FBS) (Gibco), 1% penicillin-streptomycin (100 U penicillin, 100 µg streptomycin; Gibco), 10 ng/ml epidermal growth factor (EGF) (Gibco) for three days (Fig.1 A). After their successful proliferative training, the cells were further divided into four differentiation protocols according to the different concentration gradients of GABA (0 µM, 5 µM, 50 µM, 500 µM, and 5mM) added to the basic culture medium (DMEM/F12 + 10% FBS + 1% penicillin-streptomycin (all from Gibco)) in the second stage, and were cultured in 12-well cell culture plates (Fig.1B). Each treatment group consisted of 36 replicates that were used for performing different assays. The cells cultured in basal media having no GABA supplementation were considered as control.

Cell morphology

The morphological changes were observed using an inverted microscope and pictures were taken every three days.

Dithizone (DTZ) staining

DTZ staining of the induced cells was performed by using the method described previously (Shiroi et al., 2005). Briefly, 50 mg of DTZ (Sigma-Aldrich, Lot no#D5130, St. Louis, MO) was dissolved in 5 ml of dimethyl sulphoxide (DMSO) to prepare its stock solution, which was filtered through a 0.22 µm filter and stored at –20 °C until used. About 10 µl of the stock solution was diluted in 1 ml of Hank's buffered salt saline solution that was used as the working solution. After removing the old culture media, cells were washed carefully with PBS for three times. One ml of DTZ stain working solution was added and the cells were incubated at 37 °C for 15 min. The inverted microscope was used to observe the results of DTZ staining, which stained cells crimson red. After examination, cells were washed with PBS and new culture media was added. In the course of 4 h, the DTZ stain had faded. The cells that were not treated with DTZ were used for subsequent real-time polymerase chain reaction (qPCR) and insulin secretion assays to avoid any possibility of affecting the results of subsequent experiments.

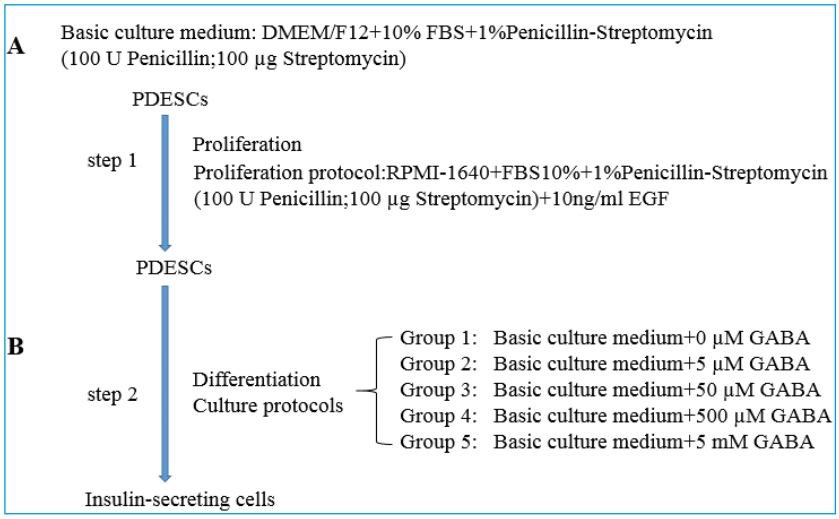

Fig. 1. Different protocols for inducing differentiation of PDESCs. (**A**) Composition of basic culture media and the protocol for proliferation of cells; (**B**) differentiation protocols and groups of cell cultures.

Immunofluorescence staining of induced ICCs

The following steps were taken for the immunofluorescent staining of cells: rinsing of cells with PBS; fixation of cells with 4% paraformaldehyde in PBS for 5 min; three washes with cold PBS for 5 min each; permeabilizing membrane with 0.2% Triton X-100 (Sangon Biotech, China) for 30 min; washing twice with cold PBS for 5 min each time; blocking with 1% BSA for 30 min; incubation of cells in a wet box for 1 h at room temperature with primary antibody (diluted in 1% BSA); removal of liquid and washing the cells three times with PBS for 5 min each; incubation with secondary antibody (diluted in 1% BSA) and keeping in the dark for 1 h at room temperature; removal of the secondary antibody and washing the cells three times with PBS for 5 min each time. All nuclei were stained with Hoechst 33342 (Solarbio, Beijing, China).

The primary and secondary antibodies used in this experiment were primary antibodies: anti-rabbit PDX1 antibody (Abcam, ab47267, UK), anti-mouse Insulin + Proinsulin antibody (Abcam, ab8304); secondary antibodies: goat anti-rabbit IgG (green, Abcam, ab15007), goat anti-mouse IgG (Alexa Fluor® 647, red, Abcam, ab150115).

RNA extraction and real-time RT-PCR

Total RNA of cells subjected to differentiation was extracted using a RaPure Total RNA kit (Magen, China, R4011-02) at 0 days (d) and 28 d according to instructions of the supplier. The extracted total RNA was reverse transcribed into cDNA using the PrimeScriptTM RT reagent kit with gDNA Eraser (TaKaRa, RR047A, Beijing, China). RT PCR was performed in an Applied Biosystems 7300 Real-Time PCR System using TB Green Premix Ex Taq II (Takara, RR 820A/B, Kusatsu, Japan). Primers used in the experiment are shown in Table 1, and the expression level of the target genes was calibrated with β-actin.

Insulin and C-peptide detection by enzyme-linked immunosorbent assay (ELISA)

After culturing the cells in differentiation protocols for 28 d, the induced cells were subjected to ELISA for detection of insulin and C-peptide. ELISA was performed using a rat ins ELISA kit (MLBIO, Shanghai, China) and a rat C-peptide ELISA kit (MLBIO, China) after the exposure of induced cells to glucose. Before performing the ELISA, cells were washed three times with PBS to remove the previously present insulin. Six wells were randomly selected from each treatment group and cells in three wells were given low-glucose stimulation (5 mM glucose added) and high-glucose stimulation (25 mM glucose added). After 30 min of exposure to glucose, the culture solution was collected and centrifuged at **2,000 rpm (for authors: please cite this entry at *g* units**) for 10 min. After that, the supernatant was removed and the following procedures were performed for both insulin and C-peptide measurements: all the solutions were prepared according to the instructions of

Table 1. Real-time PCR primer sequences

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
Ins1	CCCGGCAGAAGCGTGGCATT	CATTGCAGAGGGGTGGGCGG
Pdx1	CCGAGCTTCTGAAAACTTTGAGG	TGGGAGCCTGATTCTCTAAATTGG
Nkx6.1	AGGCTCGGTCCTTTGCTT	CCTTTCTCCCTTGGTGGC
β-actin	GGTCATCACTATCGGCAAT	GTGTTGGCATAGAGGTCTT

the supplier followed by addition of 50 µl of standard and sample solution to the appropriate wells except the blank well, then 100 µl of enzyme conjugate was added to the standard and sample wells except the blank well, and the microtitre plate was covered with an adhesive strip and incubated for 60 min at 37 °C, then the microtitre plate was washed four times and 50 µl of substrate A and B was added to each well, and after gentle mixing again incubated for 15 min at 37 °C; in the next step, 50 µl of stop solution was added to each well and in the last step, optical density was read at 450 nm using a microtitre plate reader and results were calculated.

Statistical analysis

Experimental data was analysed by SPSS 19.0 using the mean ± standard deviation, the significance analysis was performed using one-way ANOVA (comparison between three or more treatments) and Student's *t*-test (comparison between two groups). The mean values were calculated from three or more biological replicates and plotted using GraphPad Prism 6 software.

Results

GABA induces differentiation of pancreatic ductal stem cells into islet-like cell clusters

Undifferentiated pancreatic ductal epithelial-like stem cells grow as gravel stones and have a polygonal shape (Fig.1 A, D, G, J, M). At 14 d of induction, the control group and the inducing group with 5 µM, 50 µM and 500 µM GABA in the culture medium showed significant cell enrichment (Fig.2 B, E, H, K). At 28 d of culture, enrichment of the control group was enhanced, but no islet-like cell clusters were present, as it was further confirmed by DTZ staining (Fig.2 C), while in the induction group to which different concentrations of GABA were added, the cells showed obvious islet-like cell clusters (Fig.2 F, I, L, O). Taken together, these results show that the addition of GABA in basal culture media can induce differentiation of PDESCs into islet-like cell clusters.

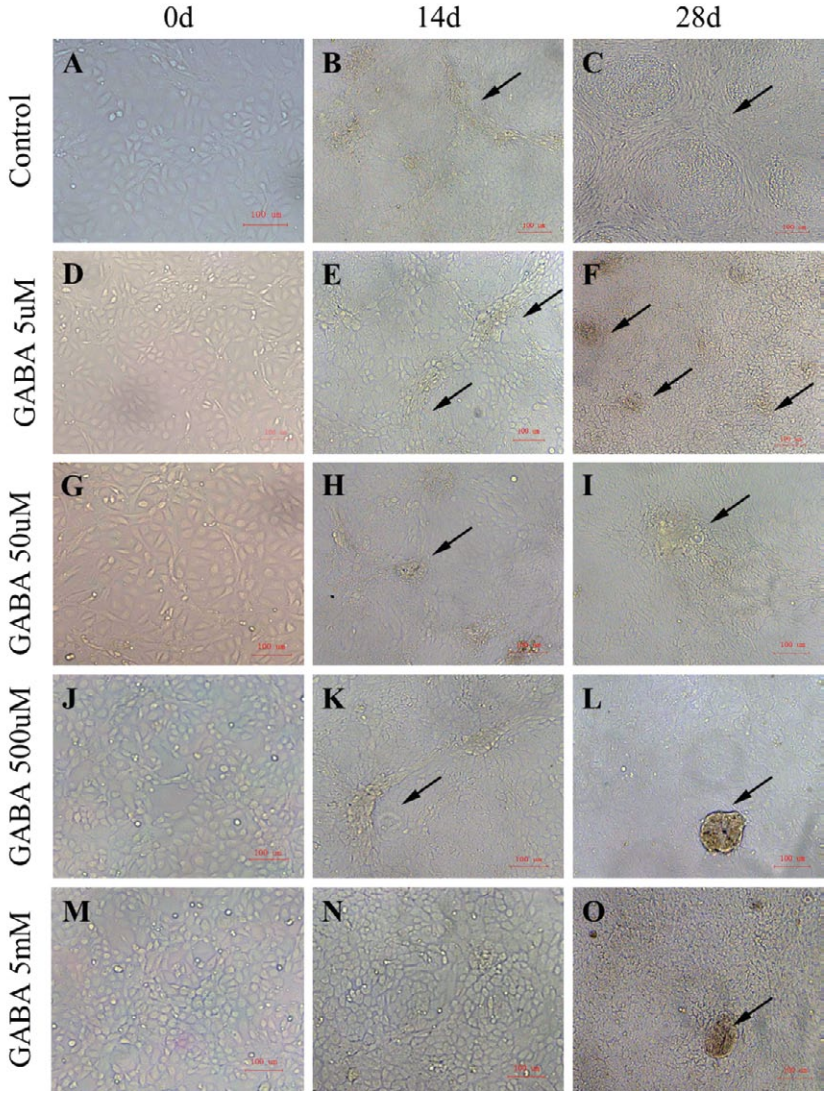

Fig. 2. Cell morphology during induction at different time points. (**A**, **D**, **G**, **J**, **M**) indicates the cell morphology on day 0. (**B**, **E**, **H**, **K**, **N**), cell morphology on day 14. (**C**, **F**, **I**, **L**, **O**), cell morphology of induced cells at the 28th day. Scale bar = 100 µm

The induced islet-like cell clusters were positive for DTZ staining

PDESCs were treated with different concentrations of GABA for their 28-day culture duration and after completion of the 28th day, induced cells were stained with DTZ staining solution. The results showed that the induced cell mass was stained crimson red (Fig. 3), indicating that the cytoplasm of the induced cells was rich in zinc ions, which is in line with the characteristics of islet-like cell clusters. Cells in the control group were negative for the DTZ staining (Fig. 3 A), while clusters of cells in the groups supplemented with 5 μM GABA (Fig. 3 B), 50 μM (Fig. 3 C), 500 μM (Fig. 3 D), and 5 mM (Fig. 3 E) showed positive results for DTZ staining. These results indicate that the addition of GABA as a supplement in basal media can induce differentiation of PDESCs into islet-like cell clusters, which attain the DTZ staining.

Induced cells in islet-like clusters co-expressed insulin and Pdx1

Immunofluorescence evaluation of the islet-like cell cluster differentiated from PDECs through GABA treatment showed that these cells were immunoreactive to β-cell marker genes, *Ins1* (Fig. 4 E, I, M, Q) and *Pdx1* (Fig. 4 F, J, N, R). Compared with the control group in which cells only expressed a small amount of Pdx1, the cell clusters induced by GABA treatment showed co-expression of insulin and Pdx1. The inhomogeneous distribution of nuclei is because of the clustered steric structures of induced cells. The induced cells were gathered in groups (clusters) from the single layer cells at the start of the differentiation protocol, which might be the reason for inhomogeneous distribution of nuclei. The results of insulin and Pdx1 in simple immunostaining shows clear positive results. However, we cannot rule out the possibility of non-specific false positive results in merged samples.

Quantitative real-time PCR analysis of gene expression

On days 0 and 28 of culture, qPCR was performed to check expression of the *Pdx1*, *Ins1*, and *Nkx6.1* genes. The expression of insulin mRNA was up-regulated in the control group and 5 mM GABA-induced group compared with PDESCs, but the difference was not significant ($P > 0.05$), while in 5 μM and 500 μM GABA-induced group, the expression level of insulin mRNA was significantly up-regulated ($P < 0.05$). The 50 μM GABA-induced group showed the highest expression of insulin mRNA as compared to the control, 5 μM, 500 μM, and 5 mM GABA-induced groups ($P < 0.01$) (Fig. 5 A). Compared with PDESCs, the expression of *Pdx1* mRNA was up-regulated in the control group and 500 μM GABA-induced group, but the difference was not significant ($P > 0.05$). The expression of *Pdx1* mRNA was significantly up-regulated in 5 μM, 50 μM and 5mM GABA-induced cells ($P < 0.05$), while the expression of *Pdx1* mRNA was most significantly up-regulated in 50 μM GABA-induced cells ($P < 0.01$) (Fig. 5 B). At 28 days of induction, the relative expression of *Nkx6.1* mRNA in the control group and GABA-induced group was significantly up-regulated ($P < 0.01$) and the expression of *Nkx6.1* mRNA in the 50 μM GABA-induced group was significantly higher than that in the

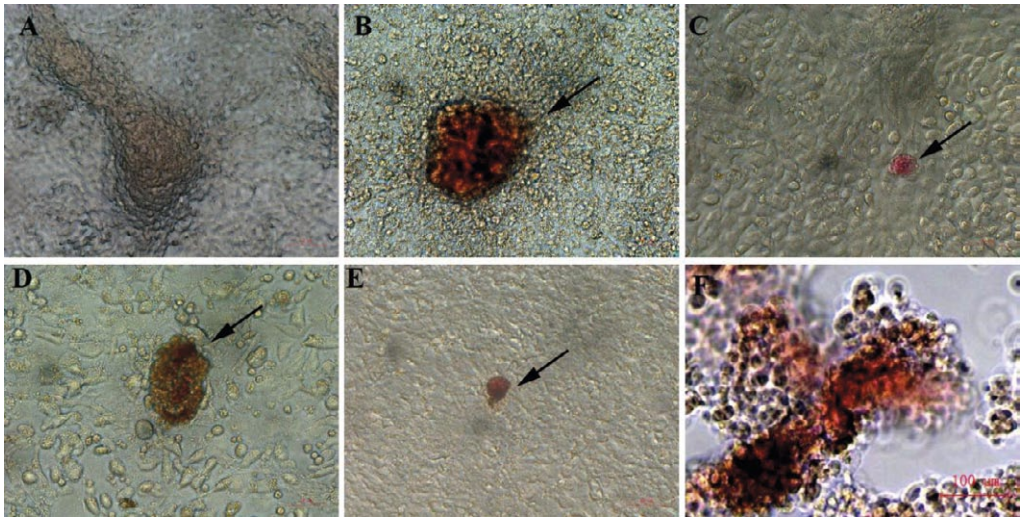

Fig. 3. DTZ staining of induced cells derived from rat PDESCs. (**A**) Control group, showing cell aggregation with negative DTZ staining. (**B**) Pancreatic islet-like structure formed in the group treated with 5 μM GABA showing crimson red colour after DTZ staining. (**C**) GABA 50 μM treated group showed positive results of DTZ staining. (**D**) 500 μM GABA-supplemented induced cells were also positive for DTZ staining. (**E**) 5 mM GABA supplementation group of induced cells showed positive results for DTZ staining. (**F**) DTZ staining of rat pancreatic islets as a positive control. Scale bars: A, B, C, D, E bar = 50 μm, F bar = 100 μm

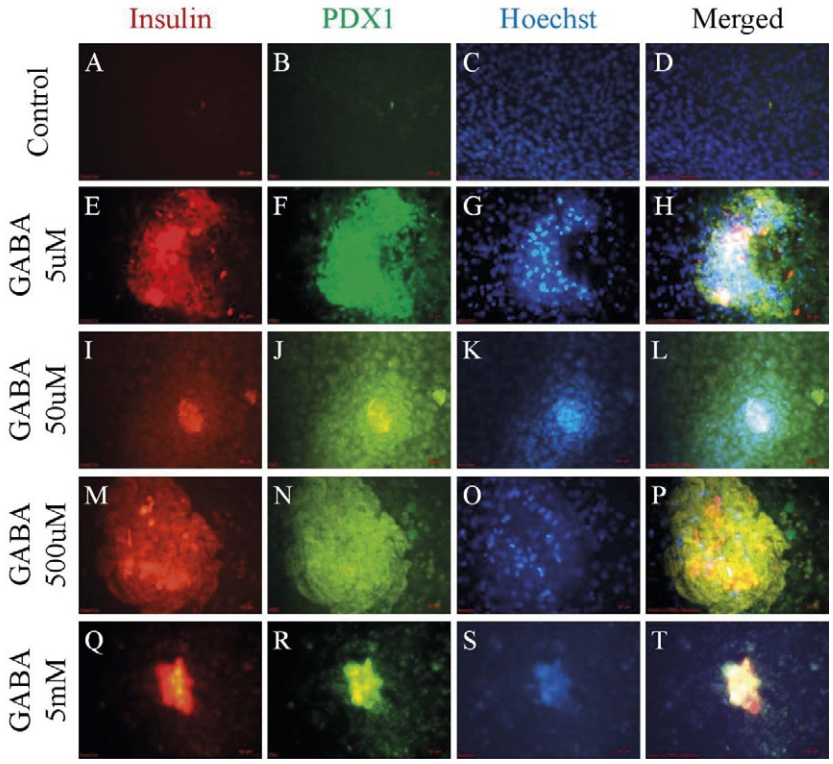

Fig. 4. Immunostaining of *in vitro* produced cells. Insulin-positive cells are shown in red (**A**, **E**, **I**, **M**, **Q**). PDX1-expressing cells are in green (**B**, **F**, **J**, **N**, **R**) and nuclei stained with Hoechst are shown in blue (**C**, **G**, **K**, **O**, **S**). The merged results are shown in (**D**, **H**, **L**, **P**, **T**). Scale bars = 50 µm

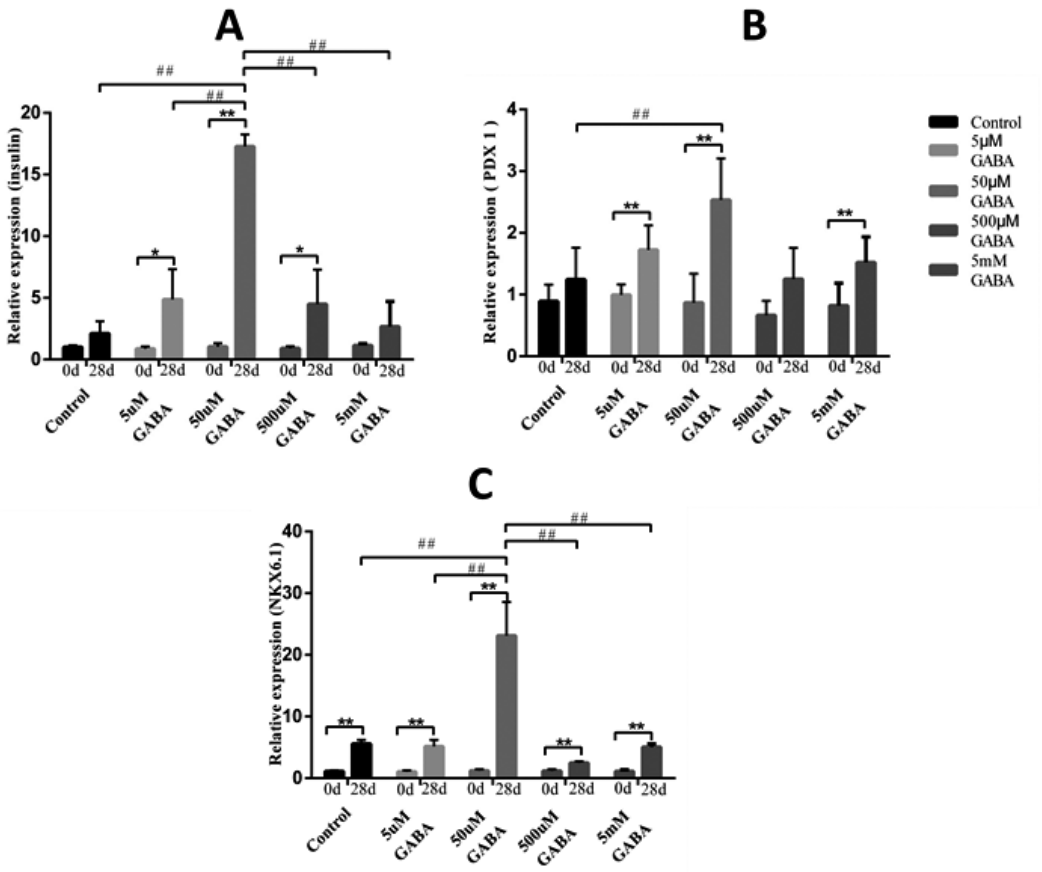

Fig. 5. Real-time PCR results. (**A**) The qPCR results of insulin show significantly increased expression in 50 µM supplemented group after 28 days of induction as compared to the control group. (**B**) Results of *Pdx1* gene expression show a similar pattern as insulin. (**C**) Results of *Nkx6.1* show its significantly increased expression in 50 µM supplemented group as compared to the control group.

control group, 5 μM, 500 μM, and 5 mM GABA-induced group ($P < 0.01$) (Fig. 5 C).

Overall, these results suggest that, at 28th day, the expression of *Pdx1*, insulin, and *Nkx6.1* were higher in 50 μM GABA-supplemented group as compared to the control group and all other treatment groups.

Induced islet-like cell clusters released insulin and C-peptide

At the 28th day, the induced islet-like cell clusters in all groups were stimulated by two different concentrations of glucose (5 mM, 25 mM), and the amount of insulin, as well as C-peptide, was detected through ELISA. In the low glucose stimulation group (5 mM), the insulin secretion levels of 5 μM GABA, 50 μM GABA, and 500 μM GABA groups were significantly higher than in the control group ($P < 0.01$). However, the insulin secretion of 5 mM GABA group was insignificantly higher than in the control group ($P > 0.05$) (Fig. 6 A). In the high glucose-stimulated group, the insulin secretion in 5 μM GABA and 50 μM GABA groups was significantly higher ($P < 0.01$) than in the control group. Still, the insulin secretion in the 500 μM GABA and 5 mM GABA groups was higher than that in the control group, but without any significant difference ($P > 0.05$) (Fig. 6 B). The amount of insulin released by islet-like cell clusters was highest in the 50 μM GABA-supplemented group in both low and high glucose-stimulation conditions. However, the difference in the amount of insulin released by the low and high glucose-stimulated groups was not significant. Regarding the secretion of C-peptide, 5 μM, 50 μM, 500 μM, and 5 mM GABA-supplemented groups have shown significantly ($P < 0.01$) higher amounts than the control group upon exposure to low as well as high amounts of glucose (Fig. 6 C, D). Moreover, in accordance with the insulin release, the amount of C-peptide was also highest in the 50 μM GABA-supplemented group in both low and high glucose-stimulated conditions. However, the difference in the amount of C-peptide between the low and high glucose groups was higher than the difference in the amount of insulin. This possibly indicates that cells in ICCs possessed the insulin-synthesizing machinery but lacked its secretion machinery when given the high glucose stimulation, indicating their incompetency to properly respond to the high load of glucose. Thus, these findings in favour of the aforementioned results show that GABA supplementation induced differentiation of stem cells into islet-like cell clusters that contained insulin-secreting cells.

Discussion

Here we report that culturing PDESCs in basic culture media (DMEM/F12 + 10% FBS + 1% pencillin-streptomycin) supplemented with different concentrations of GABA resulted in differentiation of these cells into islet-like cell clusters. The induced islet-like cell

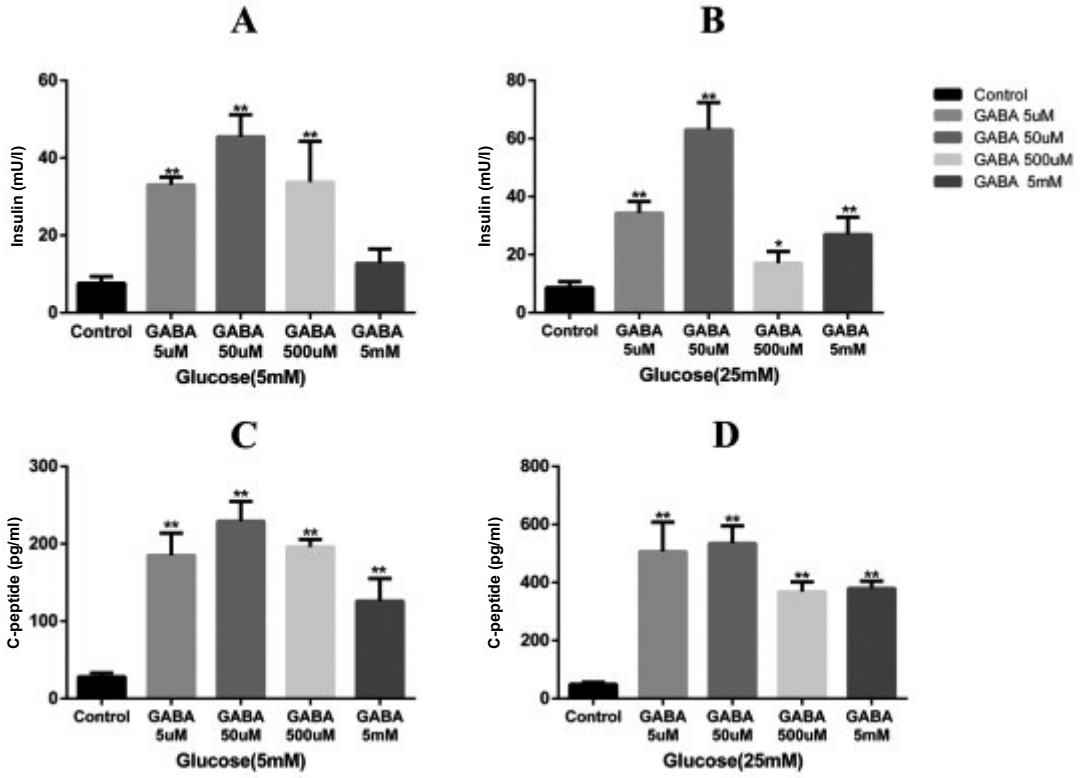

Fig. 6. Results of the ELISA test for insulin and C-peptide. (**A**, **C**) Low-glucose stimulation induces insulin and C-peptide secretion in produced cells. (**B**, **D**) High-glucose stimulation induces insulin and C-peptide secretion in produced cells.

clusters were positive for DTZ staining and immunoreactive to Pdx1 and insulin genes. Further, the results of qPCR verified that the expression of Pdx1, Ins1, and Nkx6.1 was up-regulated in the induced cells. Importantly, the cells in induced clusters also secreted C-peptide and insulin when exposed to low (5 mM) and high (25 mM) glucose levels. Altogether the results of this study show that the induced cells in 50 μM GABA-supplemented group were positive for DTZ staining and immunoreactive to Pdx1 and Ins1. The expression of Pdx1, Ins1, and Nkx6.1 in this group was highest compared to the control and other GABA-supplemented groups. Further, the amount of C-peptide and insulin released was also significantly higher in this group than in all other groups. While the induced cells in 5 μM, 500 μM, and 5 mM GABA-supplemented groups were positive for DTZ staining and also immunoreactive to Pdx1 and Ins1, the expression of Pdx1, Ins1, and Nkx6.1 and the amount of C-peptide and insulin released was lower than that examined in the 50 μM GABA-supplemented group. Our results in line with previous studies show that *in vitro* differentiation of PDESCs into ICCs is possible (Li et al., 2008; Wang et al., 2008; Noguchi et al., 2010; Huch et al., 2013; Chen et al., 2016; Ma et al., 2017; Tan et al., 2019).

In many previous studies that have used PDESCs for *in vitro* development of β-like cells, to the best of our knowledge, GABA has not been used. GABA plays crucial roles in the regulation of pancreatic endocrine cell functions (Franklin and Wollheim, 2004) and its involvement was also found in neogenesis of β cells (Vieira et al., 2017) by transition from α cells (Ben-Othman et al., 2017; Rutter, 2017), or possibly through conversion of pancreatic ductal epithelial cells to β cells through mediation of glucagon-secreting α cell stage (Weir and Bonner-Weir, 2017). These shreds of evidence were used as a base for carrying out this study. However, the GABA-mediated conversion of α to β cells has become a controversy now as many groups have reported that GABA or its agonists neither convert α cells to β cells, nor reverse the induced diabetes (Ackermann et al., 2018; van der Meulen et al., 2018; Shin et al., 2019). The reason for the contrasting results was the use of different experimental model animals, use of different lineage-tracing techniques, and the difference in housing conditions (Eizirik and Gurzov, 2018).

The proposed mechanism that involves β-cell regeneration through GABA treatment consists in promotion of β-cell survival and growth through the activation of the PI3-k/Akt pathway (Aikin et al., 2000; Trümper et al., 2000; Ligon et al., 2007). The administration of GABA in mice and human has been reported to promote β-cell replication (Bansal et al., 2011; Wang et al., 2014). The exertion of this function of GABA involves its binding to the membrane $GABA_A$ receptors, which employ depolarizing effects and opening of voltage-dependent calcium channels (VDCCs) leading to the activation of the PI3K/Akt cell growth and survival signalling pathway (Ligon et al., 2007). We also consider this mechanism as a possible pathway that caused the differentiation of PDESCs into insulin-secreting cells.

In sum, we were the first to use GABA at different concentrations for inducing the *in vitro* differentiation of PDESCs into ICCs, and the overall results of the study suggest that 50 μM of GABA was the best concentration for efficiently converting PDESCs to ICCs. The expression of Pdx1 and Ins1, as well as the amount of C-peptide and insulin released, was higher in this group as compared to other groups. However, the amount of insulin released by the cells in this group was not as high as that released by the primary β cells. Thus, our data suggests that addition of GABA causes differentiation of rat PDESCs into ICCs. Hence, we suggest that GABA should be further investigated as a part of differentiating protocols for the differentiation of pancreatic progenitors into β-like cells.

In recent years, through use of scRNA-seq, researches have started to explore the much-complicated process of early pancreas development, which was previously not too well understood (Larqué et al., 2016; Byrnes et al., 2018; Scavuzzo et al., 2018; Yu et al., 2019). However, much is needed to be done to get the complete insight into the signalling pathways that play crucial roles during the early pancreas development. This will help researchers to mimic the *in vivo* developmental pathways of pancreas development in their *in vitro* ventures for developing *bona fide* β-like cells.

Authors' contribution

M. W. G. and Z. Y contributed equally. M. W. G and Z. Y. developed the idea, conducted experiments and wrote the main draft of the manuscript, W. J. and L. Y. helped in analysis of results, L. B., L. G. C., and M. W. B. helped in writing the manuscript, while X. M. revised the manuscript.

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