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In Vitro Induction of Human Dental Pulp Stem Cells Toward Pancreatic Lineages

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TITLE:

In Vitro Induction of Human Dental Pulp Stem Cells Toward Pancreatic Lineages

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KEYWORDS:

insulin-producing cells, IPCs, pancreatic lineages, human dental pulp stem cells, hDPSCs, diabetes mellitus

SUMMARY:

This protocol presents a comparison between two different induction protocols for differentiating human dental pulp stem cells (hDPSCs) toward pancreatic lineages *in vitro*: the integrative protocol and the non-integrative protocol. The integrative protocol generates more insulin producing cells (IPCs).

ABSTRACT:

As of 2000, the success of pancreatic islet transplantation using the Edmonton protocol to treat type I diabetes mellitus still faced some obstacles. These include the limited number of cadaveric pancreas donors and the long-term use of immunosuppressants. Mesenchymal stem cells (MSCs) have been considered to be a potential candidate as an alternative source

of islet-like cell generation. Our previous reports have successfully illustrated the establishment of induction protocols for differentiating human dental pulp stem cells (hDPSCs) to insulin-producing cells (IPCs). However, the induction efficiency varied greatly. In this paper, we demonstrate the comparison of hDPSCs pancreatic induction efficiency via integrative (microenvironmental and genetic manipulation) and non-integrative (microenvironmental manipulation) induction protocols for delivering hDPSC-derived IPCs (hDPSC-IPCs). The results suggest distinct induction efficiency for both the induction approaches in terms of 3-dimensional colony structure, yield, pancreatic mRNA markers, and functional property upon multi-dosage glucose challenge. These findings will support the future establishment of a clinically applicable IPCs and pancreatic lineage production platform.

INTRODUCTION:

Diabetes mellitus is an ongoing global concern. An International Diabetes Federation (IDF) report estimated that the global prevalence of diabetes would increase from 151 million in 2000 to 415 million in 2015[1,2]. The latest epidemiology-based study has predicted that the estimated worldwide diabetes prevalence will increase from 451 million in 2017 to 693 million in 2045[1]. The success of pancreatic islet transplantation using the Edmonton protocol was first demonstrated in 2000, when it was shown to maintain endogenous insulin production and stabilize the normoglycemic condition in type I diabetic patients[3]. However, the application of the Edmonton protocol still faces a bottleneck problem. The limited number of cadaveric pancreas donors is the main issue since each patient with type I diabetes requires at least 2–4 islet donors. Furthermore, the long-term use of immunosuppressive agents may cause life-threatening side effects[4,5]. To address this, the development of a potential therapy for diabetes in the past decade has mainly focused on the generation of effective insulin-producing cells (IPCs) from various sources of stem cells[6].

Stem cells became an alternative treatment in many diseases, including diabetes type I, which is caused by the loss of beta-cells. Transplantation of IPCs is the new promising method for controlling blood glucose in these patients[7]. Two approaches for generating IPCs, integrative and non-integrative induction protocols, are presented in this article. The induction protocol mimicked the natural pancreatic developmental process to get the matured and functional IPCs[8,9].

For this study, hDPSCs were characterized by flow cytometry for MSC surface marker detection, multilineage differentiation potential, and RT-qPCR to determine the expression of stemness property and proliferative gene markers (data not shown)[8–10]. hDPSCs were induced toward definitive endoderm, pancreatic endoderm, pancreatic endocrine, and pancreatic beta-cells or IPCs (**Figure 1**), respectively[7]. To induce the cells, a three-step induction approach was used as a backbone protocol. This protocol was called a non-integrative protocol. In the case of integrative protocol, the essential pancreatic transcription factor, *PDX1*, was overexpressed in hDPSCs followed by the induction of overexpressed *PDX1* in hDPSCs using a three-step differentiation protocol. The difference between non-integrative and integrative protocol is the overexpression of *PDX1* in integrative protocol and not in the non-integrative protocol. The pancreatic differentiation was compared between the integrative and non-integrative protocols in this study.

PROTOCOL:

This work was performed in accordance with the Declaration of Helsinki and approved by the Human Research Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Human DPSCs (hDPSCs) were isolated from human dental pulp tissues extracted from both premolars and molars due to wisdom teeth issues. Informed consent was obtained from the patients under an approved protocol (HREC-DCU 2018/054).

1. Integrative induction protocol

1.1. Preparation of lentiviral vector carrying *PDX1*

1.1.1. Use human embryonic kidney (HEK) 293FT cells for viral packaging. Culture and maintain these cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% Antibiotic-Antimycotic.

1.1.2. Generate *PDX1*-lentivirus vectors by co-transfection of 10 µg each of the packaging and envelope plasmids and 20 µg of the targeted gene plasmid into HEK293FT cells using calcium phosphate transfection system, e.g., *psPAX2*, *pMD2.*G, and human *pWPT-PDX1*[11]. Ensure that the cells are 80%–90% confluent during the time of infection.

1.1.3. Collect the medium containing lentiviral particles at 48 and 72 h after transfection.

1.1.4. Filter the collected medium through a 0.45 µm filter; concentrate the viruses with a centrifugal filter at 100 kDa nominal molecular weight cut-off (NMWCO).

1.2. *PDX1* overexpression

1.2.1. Use passage 3-5 hDPSC that are 70-80% confluent. Trypsinize and count the cells using a cell counter. Seed 1 x 10^6 hDPSCs onto a 60 mm tissue culture-treated dish and incubate overnight.

1.2.2. 24 h later, add fresh virus particles obtained from step 1.1.4 to transduce polybrene pre-treated cells (4 µg/mL polybrene, 30 min under 37 °C and 5% CO_2) at the desired multiplicity of infection (MOI). For example, in this case, MOI used is 20. Maintain the cells in the cell culture incubator for 24 h at 37 °C with 5% CO_2.

1.2.3. After the 24 h infection period, discard the medium containing viral particles. Add fresh culture media and continue growing the cells for 48 h. All cultures are maintained at 37 °C and 5% CO_2.

1.2.4. Check the aggregated morphology of the transfected cells before proceeding to the induction step. *PDX1* transduction is confirmed by gene expression analysis (**Supplemental Figure 1**).

NOTE: Check the cell morphology under a microscope before proceeding to the next steps.

1.2.5. Harvest and proceed to a three-step induction protocol (step 1.3) as a microenvironmental induction approach.

1.3. Three-step induction protocol

NOTE: The three-step induction protocol resulted in the series of pancreatic differentiation of hDPSCs to definitive endoderm, pancreatic endoderm/endocrine, and pancreatic beta-cells/IPCs using serum-free medium (SFM)-A, SFM-B, and SFM-C, respectively.

1.3.1. Discard the culture medium, and then wash the transduced-hDPSCs with 1x phosphate-buffered solution (PBS).

1.3.2. Add 0.25% trypsin-EDTA solution to the cells and incubate for 1 min at 37 °C, 5% CO_2.

1.3.3. Add the culture medium to stop the trypsin activity and gently flush the cells to get the single cell suspension. Count the cells and aliquot 1 x 10^6 cells into each collecting tube.

1.3.4. Centrifuge the cell suspension at 468 x *g* (Relative Centrifugal Force: RCF), 4 °C, for 5 min. Discard the supernatant and save the cell pellet.

1.3.5. Resuspend the pellet in 3 mL of first pancreatic induction medium, i.e., serum-free medium (SFM)-A. Seed the cells on a low attachment culture plate (60 mm). Maintain the cells at 37 °C and 5% CO_2 for 3 days.

NOTE: Observe cells morphology under an inverted microscope before proceeding to the next step.

1.3.6. Remove SFM-A and add 3 mL of the second induction medium, i.e., SFM-B. Maintain the cells for the next 2 days at 37 °C, 5% CO_2.

NOTE: Observe cells morphology under an inverted microscope before proceeding to the next step.

1.3.7. Remove SFM-B and add 3 mL of the third induction medium, i.e., SFM-C. Maintain the cells for the next 5 days in a cell culture incubator maintained at 37 °C with 5% CO_2. Change the medium every 48 h. Observe their morphology after 5 days.

NOTE: Each induction medium is supplemented with different reagents as described; SFM-A: 1% bovine serum albumin (BSA), 1x insulin-transferrin-selenium (ITS), 4 nM of activin A, 1 nM of sodium butyrate, and 50 µM of beta-mercaptoethanol; SFM-B: 1% BSA, 1x ITS, and 0.3 mM taurine; and SFM C: 1.5% BSA, 1x ITS, 3 mM taurine, 100 nM of glucagon-like peptide (GLP)-1, 1 mM nicotinamide, and 1x non-essential amino acids (NEAAs).

1.3.8. Make sure to check the cell morphology under an inverted microscope after each step. The cells will become more aggregated and float as colonies.

1.3.9. Collect cell colonies for further analysis. Check for colony morphology and size. Perform pancreatic gene marker expression analysis and functional analysis (Glucose stimulated C-peptide secretion assay).

2. Non-integrative induction protocol

NOTE: The non-integrative protocol is the backbone protocol for delivering the IPCs with the three-step induction process as a microenvironmental induction approach[8,9].

2.1. Use passage 3–5 hDPSCs at 70%–80% confluency.

2.2. Discard the culture medium and wash hDPSCs with 1x PBS.

2.3. Add 0.25% trypsin-EDTA solution onto the cells and incubate for 1 min at 37 °C, 5% CO_2.

2.4. Add the culture medium to stop the trypsin activity and gently pipette the cells to obtain the single cell suspension. Count cells and aliquot 1 x 10^6 cells into each collection tube.

2.4.1. Centrifuge the cell suspension at 468 x *g* (RCF), 4 °C, 5 min. Discard the supernatant.

2.4.2. Resuspend the pellet in SFM-A and seed onto a low attachment culture plate (60 mm). Culture the cells at 37 °C and 5% CO_2 for 3 days. Check the cell morphology under an inverted microscope.

2.4.3. Remove SFM-A, add SFM-B (Day 3) and then maintain the cells for the next 2 days under 37 °C, 5% CO_2.

2.4.4. Remove SFM-B, add SFM-C (Day 5) and then maintain the cells for the next 5 days under 37 °C, 5% CO_2. SFM-C is changed every 48 h.

NOTE: Each induction medium is supplemented with different reagents as described; SFM-A: 1% BSA, 1x ITS, 4 nM of activin A, 1 nM of sodium butyrate, and 50 µM of beta-mercaptoethanol; SFM-B: 1% BSA, 1x ITS, and 0.3 mM taurine; and SFM C: 1.5% BSA, 1x ITS, 3 mM taurine, 100 nM of GLP-1, 1 mM nicotinamide, and 1x NEAAs.

2.4.5. Make sure to check the cell morphology under the inverted microscope after each step and at Day 10.

NOTE: The cells will become more aggregated and will float as colonies.

2.4.6. Collect cell colonies for further analysis. Check for colony morphology and size. Perform pancreatic gene marker expression analysis and functional analysis (Glucose stimulated C-peptide secretion assay).

REPRESENTATIVE RESULTS:

In this article, the outcomes of both the induction protocols were compared. The diagrams of both induction protocols are illustrated in **Figure 2A,C**. In both the protocols, the evaluation was performed under a light microscope, and images were analyzed with ImageJ. hDPSCs were able to form colony-like structures from the first day of induction in both induction protocols. The colony's morphology was round and dense, and all colonies floated in the culture vessels throughout the induction period (**Figures 2B,D**). The total colony count of both protocols was also determined; the result showed that the total colony count in the case of integrative induction protocol was slightly higher compared to the non-integrative induction protocol (**Figure 2E**). However, the difference was not statistically significant. Furthermore, the colony size distribution in both induction protocols was also evaluated (**Figure 2F**). According to our results, small- to medium-size colonies were formed upon the integrative induction, which was important for reducing the necrotic core inside the colony.

Pancreatic gene marker analysis was conducted using RT-qPCR according to our recent publication[10]. Information about the list of primers used in this study is included in **Table 1**. The mRNA value was presented as a relative mRNA expression by normalized to 18S ribosomal RNA and the control using the formula of $2^{\Delta\Delta Ct}$. The results of this study revealed that the integrative induction protocol yielded colonies with high expression of late pancreatic markers, including *ISL-1*, *MAF-A*, *GLUT-2*, *INSULIN*, and *GLP-1R* (**Figure 3**), suggesting the differentiation of hDPSCs toward IPCs. The functional evaluation of hDPSC-IPCs was also described (**Figure 4**) in this study. A glucose-stimulating C-peptide secretion[8,9] assay was employed using an ELISA kit. The results showed that the integrative and non-integrative induction protocols yielded colonies that were able to secrete C-peptide.

FIGURE AND TABLE LEGENDS:

Figure 1: Diagram of MSC differentiation toward IPCs.

Figure 2: Morphology, total colony count, and colony size distribution of IPCs using two different protocols. Diagram of the integrative protocol by overexpression of essential transcription factor, *PDX1,* followed by three-steep induction protocol (**A**). Morphology of transfected hDPSCs in each step of induction (**B**). Diagram of non-integrative as a backbone protocol (**C**). Morphology of hDPSC-IPCs using the non-integrative protocol (**D**). Total colony (**E**) and colony size distribution (**F**) of two different protocols.

Figure 3: Pancreatic gene expression analysis of hDPSC-IPCs obtained from two different protocols. The mRNA expression of pancreatic endoderm (*PDX1* and *NGN-3*) (**A**), pancreatic islet markers (*ISL-1*, *MAF-A*, *GLUT-2*, and *INSULIN*) (**B**), and pancreatic-related marker (*GLP-1R*) (**C**) was analyzed.

Figure 4: Functional analysis of hDPSC-IPCs obtained from two different protocols. C-peptide secretion in each induction protocol, integrative (**A**) and non-integrative (**B**) protocols, were determined by a glucose-stimulated C-peptide secretion (GSCS) assay.

Supplemental Figure 1: *PDX1* mRNA analysis after *PDX1* transduction. *PDX1* mRNA expression analysis after 48 h of *PDX1* transduction at MOI 20 in hDPSCs is shown.

Table 1: Primer Information.

DISCUSSION:

Achieving higher IPCs production from MSCs plays an essential role in diabetes therapy. The critical steps of the integrative protocol rely on the quality of cells to be used for the transduction and the quality of transduced cells. Some cell requirements that should be checked for successful transduction are ensuring cell healthiness, cell banking management, and cells are in a mitotically active state. Further, monitoring the viability of transduced cells also plays an important role. Less successful transduction is caused by the poor viability of the stimulated cells[12]. For the non-integrative protocols, the morphological appearance and floating colonies should be achieved because they are related to the maturation of pancreatic differentiation[9].

In terms of modification and troubleshooting of the technique for the integrative induction protocol, healthy and good quality cells can be obtained by using cells in passages 3–5 and 70%–80% confluence, while the quality of transduced cells should be monitored by routinely checking the quality of the stimulated cells before moving on to the next step. This finding is in correlation with a previous study that mentioned that several factors influencing the efficiency of the transduction rely on cell health, cell confluence, and the number of passages[13]. In this study, the non-integrative protocol with a three-step induction process still faces limitations, mainly concerning the colony size and colony number formation. This result is consistent with our previous study[8,9]. To overcome this problem, we suggest checking the quality of the cells as well as the quality of the low attachment culture vessels.

The limitation of this technique is the complexity of the integrative induction protocol, which was due to the use of two different consecutive platforms. Furthermore, the higher MOI does not imply higher efficiency of transduction. In a similar study using lentivirus transduction, a higher MOI could not achieve better transduction efficiency. The authors suggest using an adjuvant such as protamine sulfate[14]. The limitations of using the non-integrative protocols are related to technical issues such as the handling techniques for the production and harvesting of the colony and varying sizes and morphological structures of the produced IPCs.

The essential transcription factor, *PDX1*, was significant, thereby having a potential role in the commitment of MSC induction toward the mature IPCs[15–18]. The modification of the backbone protocol by using *PDX1*-overexpressed hDPSCs followed by a three-step induction protocol was mainly aimed at the successful high-yield production of mature and functional IPCs[19–21]. The findings of this protocol reflected that the MSC induction toward mature IPCs required pre-endodermic expression of *PDX1*[9]. The morphological evaluation showed that the non-attached 3D structure of colonies could be obtained in both protocols by using low attachment vessels. This structure was important for achieving the maturation of pancreatic differentiation[7,9,22,23]. In addition, according to colony size evaluation, the integrative protocol resulted in a positive trend of total colony number and small- to medium-size colony production, which can prevent a necrotic core of the colony. Therefore, it will be beneficial for further transplantation[7,19,21,24,25]. The upregulation of late-stage pancreatic gene markers (*ISL-1, MAF-A*, *GLUT-2*, *INSULIN*, and *GLP-1R*) was observed in this protocol. The late-stage pancreatic gene markers in the integrative induction protocol were higher compared to the backbone protocol. It was revealed that the overexpression of *PDX1* increased the number of

pancreatic progenitors, i.e., a better result in terms of the progression of mid- to late-stage pancreatic development could be achieved[19,26–28]. Furthermore, to clarify the function of IPCs, a GSCS assay was performed. hDPSC-IPCs produced from both protocols secreted C-peptide, suggesting the functionally active colonies.

To summarize the future applications of the technique used in this study, both pancreatic induction protocols were able to generate the IPCs *in vitro*. In terms of the total colony count, small- to medium-size colony production, and pancreatic marker expression, the integrative protocol showed a beneficial trend for IPC formation, supporting further MSC application in stem cell-based diabetes treatments for human and veterinary practices[7,29–32].

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DISCLOSURES
The authors have nothing to disclose.

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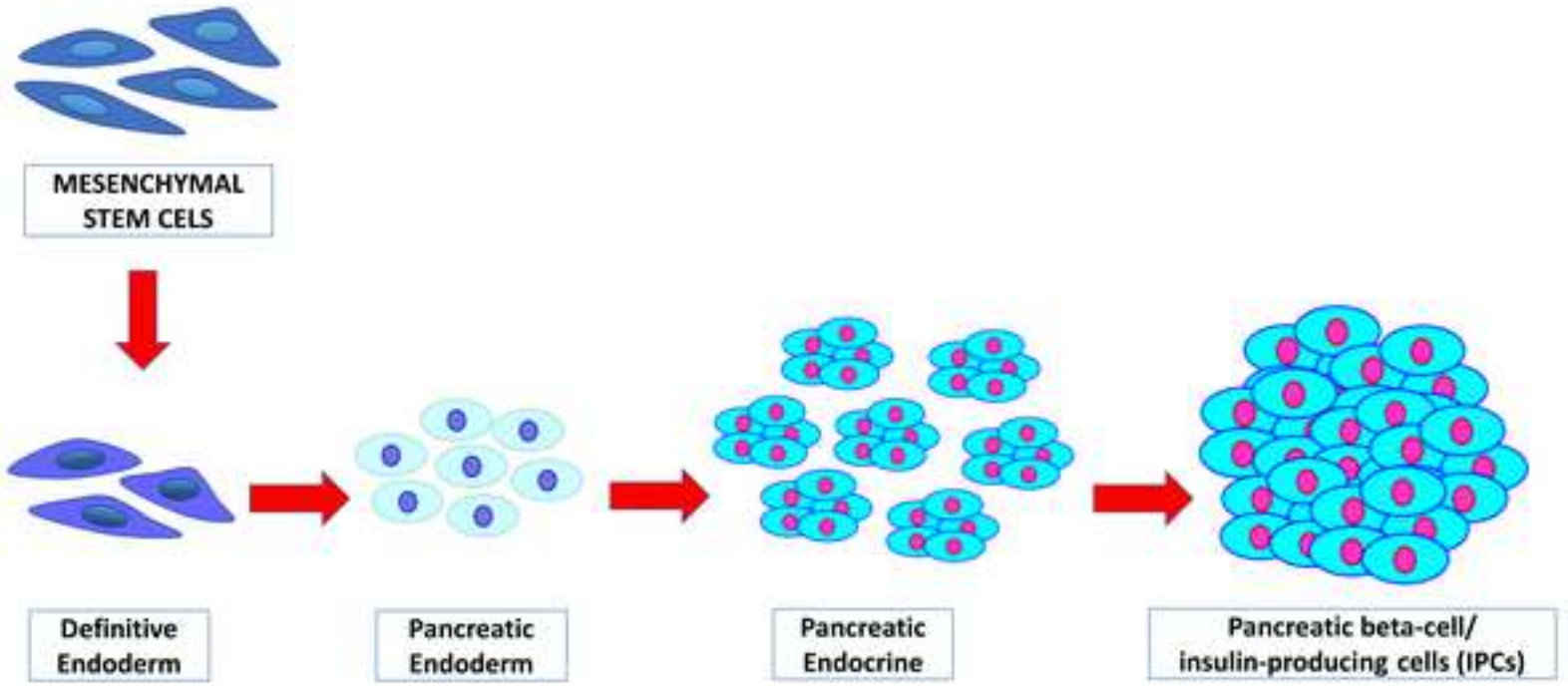
Chenphop Sawangmake, Figure 1



Chenphop Sawangmake, Figure 2

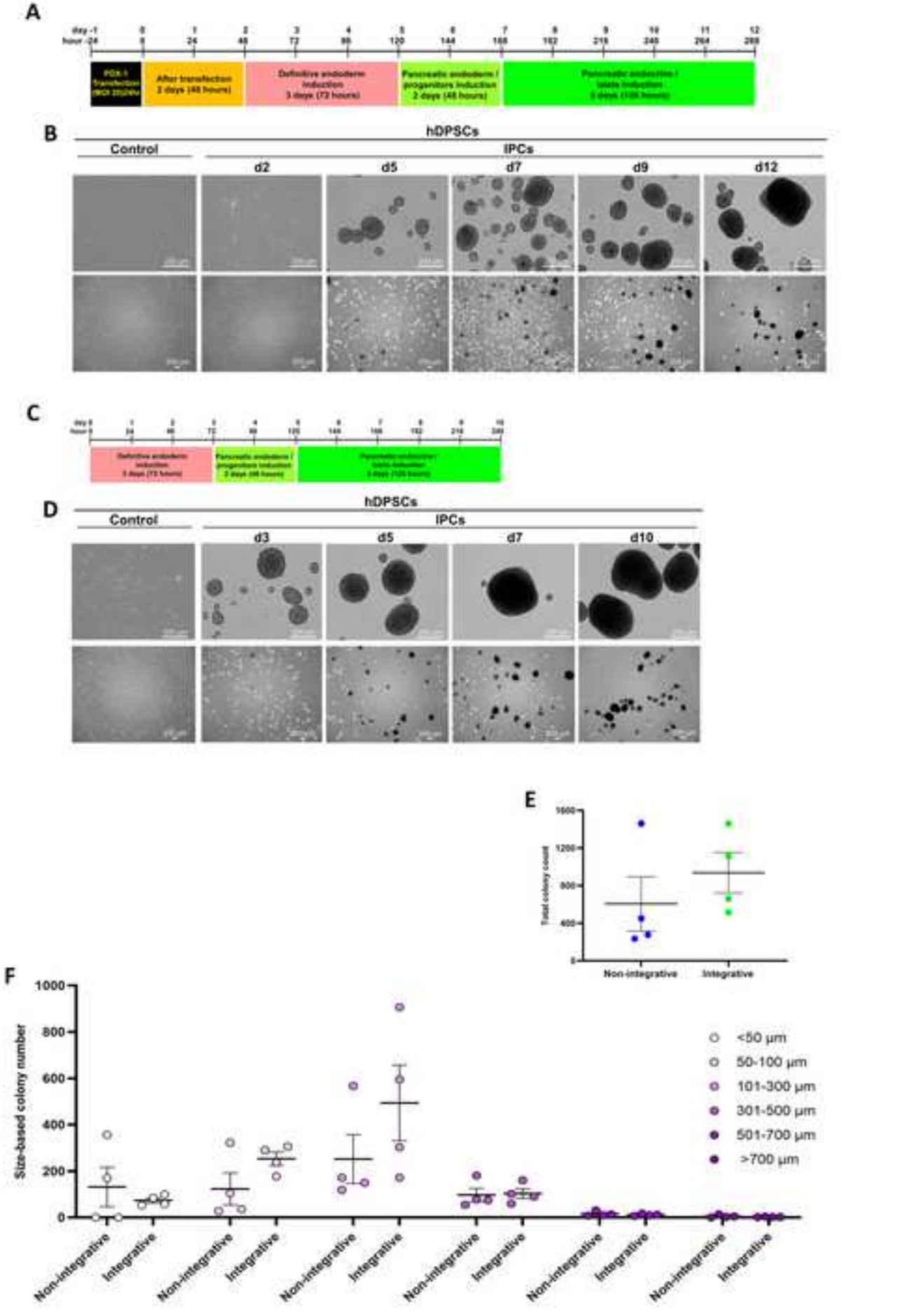


Figure3

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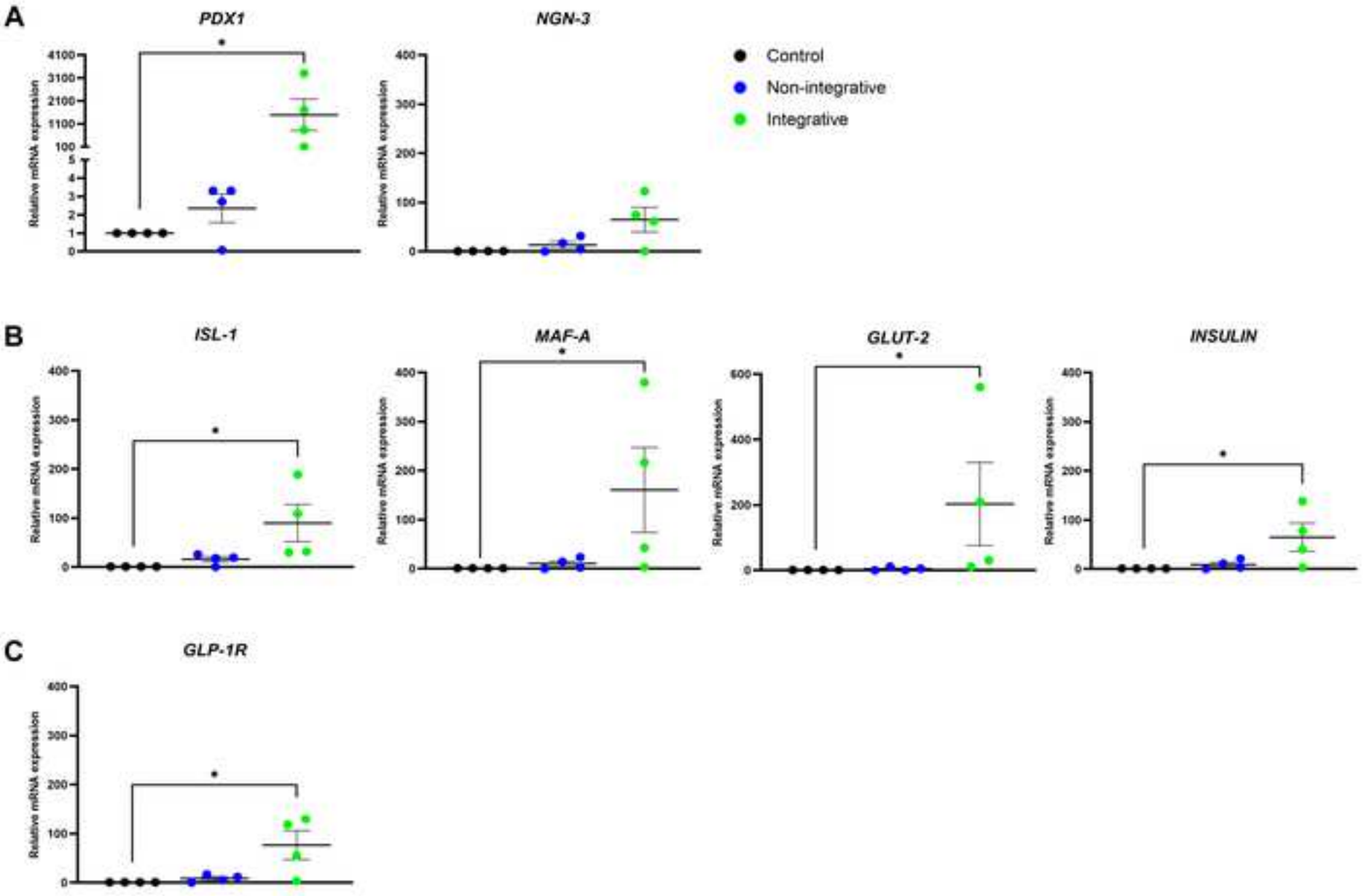

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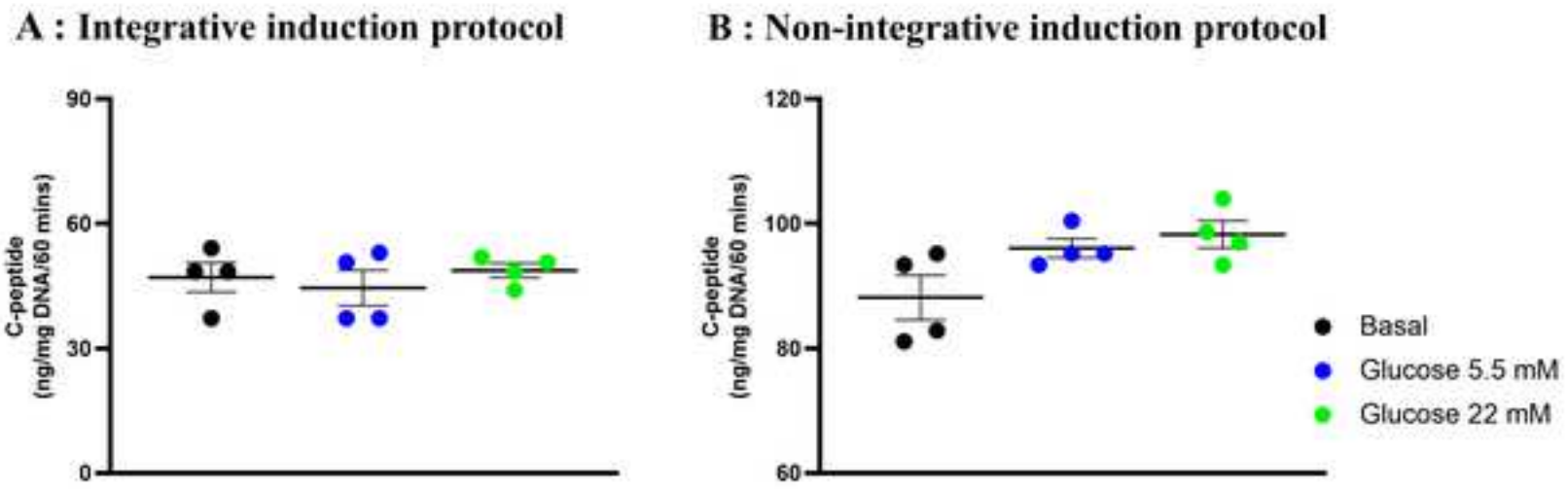


Table 1. Primer information

Genes	Accession number	Forward Primer Reverse Primer	Length (bp)	Tm (°C)
PDX-1	NM_000209.4	5' – AAGCTCACGCGTGGAAAGG – 3' 5' – GGCCGTGAGATGTACTTGTTG – 3'	145	57.89 52.38
NGN-3	NM_020999.3	5' – CGGTAGAAAGGATGACGCCT – 3' 5' – GGTCACTTCGTCTTCCGAGG – 3'	138	59.54 60.11
ISL-1	NM_002202.2	5' – TCCCTATGTGTTGGTTGCGG - 3' 5' – TTGGCGCATTTGATCCCGTA – 3'	200	60.32 60.39
MAF-A	NM_201589.3	5' – GCACATTCTGGAGAGCGAGA – 3' 5' – TTCTCCTTGTACAGGTCCCG – 3'	102	59.83 58.74
GLUT-2	NM_000340.1	5' – GGTTTGTAACTTATGCCTAAG – 3' 5' – GCCTAGTTATGCATTGCAG – 3'	211	52.25 54.24
INSULIN	NM_000207.2	5' – CCGCAGCCTTTGTGAACCAACA – 3' 5' – TTCCACAATGCCACGCTTCTGC – 3'	215	64.34 64.45
GLP-1R	NM_002062.4	5' – TCGCTGTGAAAATGAGGAGGA – 3' 5' – TCACTCCCGCTCTGTGTTTG – 3'	189	59.38 60.25
18S	NR_003286.2	5' – GTGATGCCCTTAGATGTCC – 3' 5' – CCATCCAATCGGTAGTAGC – 3'	233	55.04 54.86

Click here to access/download

Table of Materials

210707_62497_Table of Materials and Reagents (Final).xlsx

Response to reviewers' comments

Editorial comments:

Manuscript:

1. The editor has formatted the manuscript text as per the journal's style. Please retain and use the attached file for revision.

Response:

We retained and used the attached file for revision.

2. Please address all the specific comments marked in the manuscript.

Response:

We addressed all the specific comments marked in the manuscript by directly respond to each comment. All revisions were highlighted by blue font color.

3. The manuscript requires thorough copyediting. Please use professional copyediting services.

Response:

The manuscript was proofread by a professional copyediting service. We attached the invoices for your reference: Proofread invoice 1.pdf and Proofread invoice 2.pdf.

Video:

1. Please make a separate title card for the ethics statement.

Response:

The Ethic statement was separated in a new title card.

2. Please homogenize the narration and the text.

Response:

The narration and the text were homogenized.

3. Please un-italicize "In Vitro" in the title 6:29 –

Response:

The title used italicized "*In Vitro*" according to the previous editorial comments on June 25th, 2021.

4. The title "Conclusion" needs to fade out instead of cutting out. Please fix.

Response:

The conclusion card was fixed by fading out before the next scene.

5. There are three logos that appear at the end of the video. Please remove these as the ASV Criteria mentions not to use any branding.

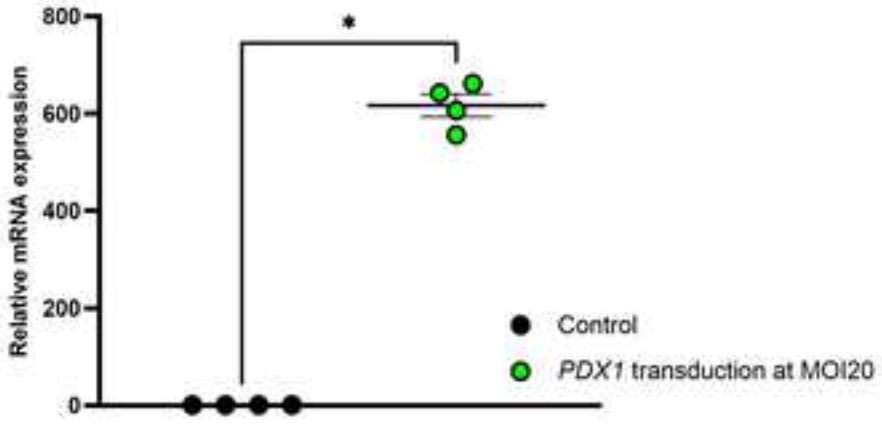
Response:

All logos which appear at the end of video were removed.

Supplemental figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental figure 1.tif

Chenphop Sawangmake, Supplemental figure 1



#36430 Paid Unfulfilled

May 23, 2021 at 6:24 am from Online Store

Unfulfilled (9)

Pages: 9 (1 Page = 250 Words)	Combined Proofreading and Editing 24 Hours	$9.49 × 9	$85.41

Shipping not required.

Paid

Subtotal	9 items	$85.41
Total		**$85.41**
Paid by customer		$85.41

Timeline

TODAY

$82.04 USD was added to your May 25, 2021 payout. — 9:26 PM

YESTERDAY

Order confirmation email was sent to Faculty of Veterinary Science, CU (watchareewan.r@gmail.com). — 6:24 AM

A $85.41 USD payment was processed on the Visa ending in 1045. — 6:24 AM

$82.04 USD will be added to your May 25, 2021 payout. — 6:24 AM

Faculty of Veterinary Science, CU placed this order on Online Store (checkout #20621269205015). — 6:24 AM

Notes

Edit

INVOICE

ADDITIONAL DETAILS

Edit

minimum_turnaround_phrase
5:19PM (your local time) on May 24, 2021

Customer

Faculty of Veterinary Science, CU
2 orders

CONTACT INFORMATION

Edit

watchareewan.r@gmail.com

SHIPPING ADDRESS

Edit

No shipping address provided

BILLING ADDRESS

Faculty of Veterinary Science
Chulalongkorn University
39 Henry Dunant Rd., Wangmai,
Pathumwan District, Bangkok 10330
Bangkok
Bangkok 10330
Thailand

Note saved

#37024 Paid Unfulfilled

July 5, 2021 at 10:59 pm from Online Store

Unfulfilled (11)

11 Pages 1 Page = 250 Words	Combined Proofreading and Editing 24 Hours	$9.49 × 11	$104.39

Shipping not required.

Paid

Subtotal	11 items	$104.39
Total		**$104.39**
Paid by customer		$104.39

Timeline

JULY 6

- $100.33 USD was added to your Jul 8, 2021 payout. — 9:57 PM
- You added a note to this order. — 6:36 PM

JULY 5

- Order confirmation email was sent to Bio ink Co.,Ltd. (Bioink.cu@gmail.com). — 10:59 PM
- A $104.39 USD payment was processed using a Mastercard ending in 7111. — 10:59 PM
- Unable to process a payment for $104.39 USD using a Mastercard ending in 7111. — 10:59 PM
- Unable to process a payment for $104.39 USD using a Mastercard ending in 7111. — 10:59 PM
- Unable to process a payment for $104.39 USD using a Mastercard ending in 7111. — 10:59 PM
- Unable to process a payment for $104.39 USD using a Mastercard ending in 7111. — 10:59 PM
- $100.33 USD will be added to your Jul 8, 2021 payout. — 10:59 PM
- Bio ink Co.,Ltd. placed this order on Online Store (checkout #21114255966231). — 10:59 PM

Notes

Edit

INVOICE

Service provider:
ProofreadingServices.com
14th Floor, 1 Broadway
Cambridge, MA 02142
SosId: 1902188

ADDITIONAL DETAILS

Edit

minimum_turnaround_phrase
9:05PM (your local time) on July 6, 2021

Customer

Bio ink Co.,Ltd.
1 order

CONTACT INFORMATION

Edit

Bioink.cu@gmail.com

SHIPPING ADDRESS

Edit

No shipping address provided

BILLING ADDRESS

Bio ink Co.,Ltd.
Bio ink Co.,Ltd.
254 Faculty of Veterinary science,
Chulalongkorn university
Henry Dunant road, Wangmai,
Pathumwan
Bangkok
Bangkok 10330
Thailand

Note saved