T.C.
AKDENIZ UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF GENE AND CELL THERAPY

GENERATION OF PANCREATIC BETA CELL-LIKE INSULIN-PRODUCING CELLS FROM INDUCED PLURIPOTENT STEM CELLS

Büşra ÇETİN

MASTER OF SCIENCE THESIS

2021-ANTALYA

Büşra ÇETİN MASTER OF SCIENCE THESIS 2021-ANTALYA

T.C.
AKDENIZ UNIVERSITY
INSTITUTE OF HEALTH SCIENCES
DEPARTMENT OF GENE AND CELL THERAPY

GENERATION OF PANCREATIC BETA CELL-LIKE INSULIN-PRODUCING CELLS FROM INDUCED PLURIPOTENT STEM CELLS

Büşra ÇETİN

MASTER OF SCIENCE THESIS

ADVISOR
Prof. Dr. Ahter Dilşad ŞANLIOĞLU

This thesis was supported by Akdeniz University Scientific Research Projects Coordination Unit (BAP, Project No: 5773) and The Scientific and Technological Research Council of Turkey (TUBITAK, Project No: 218S617).

“This thesis may be consulted provided that due acknowledgement is made”

2021-ANTALYA

To the Institute of Health Sciences;

This study has been approved by our jury as a Master of Science Thesis in the Department of Gene and Cell Therapy, Gene and Cell Therapy Master of Science program. 29/12/2021

Signatures

Thesis Advisor	: Prof. Dr. Ahter Dilşad ŞANLIOĞLU Akdeniz University
Member	: Prof. Dr. Mehtap KILIÇ EREN Aydın Adnan Menderes University
Member	: Asst.Prof.Dr. Devrim DEMİR DORA Akdeniz University

This thesis has been deemed by the jury appointed by the Administrative Board of the Institute and accepted by the Administrative Board of the Institute with decision dated ……/……./…..…. and numbered ………/………..

Institute Director

DECLARATION

I hereby declare that this thesis is my own work; that it does not include any unethical act from the planning to the writing of the thesis; that I have acquired all the information included in this thesis in accordance with the ethical principles; that I have made due acknowledgements to all the information and comments that were not acquired by this thesis work; and that I have included all these references in the references list.

Büşra ÇETİN

Thesis Advisor
Prof. Dr. Ahter Dilşad ŞANLIOĞLU

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to;

My advisor, my guide and mentor, Prof.Dr. Ahter Dilşad ŞANLIOĞLU, who has always encouraged me to carry the torch forward in academic life and guided me with her knowledge,

Prof. Dr. Salih ŞANLIOĞLU, who did not spare his support since the beginning of my academic life till this day,

Esteemed Committee members of my thesis defense exam, Prof. Dr. Mehtap KILIÇ EREN, Asst. Prof. Dr. Devrim DEMİR DORA, Prof.Dr. Türker BİLGEN and Asst. Prof.Dr. Reha Onur AZİZOĞLU,

My dear friends, Gizem ŞEKER ERSAN, Bahar AKKAYA, Elif Özgecan ŞAHİN, Fulya ERENDOR, Özlem YILMAZ and Zeynep Hilal DURKAYA, who have been with me in every aspect of my life since the first day of my graduate education, beyond being a colleague, with their guidance, support, and constant help throughout my thesis studies,

All esteemed Faculty Members of our Gene and Cell Therapy Department, and the employees of the Institute of Health Sciences of Akdeniz University for their patience and support throughout the official procedures,

My mother Fatma ÇETİN, who is the greatest architect of my life, who didn't spare her unparalleled support, and to my first teacher, my dear father Ömer ÇETİN, who never lost faith and determination in my work,

My dear sisters Ebru AYDIN, Esra ÇETİN and my brother Mehmet AYDIN, whom I chose as my mentors and guides in life and academia,

And lastly, to my precious nieces, the purest joys of my life Ceylin Azra and Deniz Arya.

May the force be with you.

ABSTRACT

Objective: Studies on generation of pancreatic beta cell-like insulin-producing cells (IPCs) from induced pluripotent stem cells (iPSCs) aim to compensate for beta cell destruction/dysfunction in diabetes. Pancreas or islet transplantations are frequently associated with disadvantages such as insufficient number of donors and requirement for immunosuppression. Although exogenous insulin administration is life-saving, it is still not considered optimal, with possible long-term complications. IPCs generated from iPSCs originating from individuals' own cells have a great potential to provide an infinite beta cell source and eliminate/reduce requirement for immunosuppression; however, optimally efficient and safer strategies are still needed. In our study, we aimed to generate beta cell-like IPCs from IPSCs via an improved approach.

Method: We used small molecules activating key signaling pathways throughout beta cell development and the TNF-Related Apoptosis-Inducing Ligand (TRAIL) molecule, the protective effect of which is frequently reported in diabetes. iPSC characterization was done via confirmation of morphological criteria and pluripotency, along with mycoplasma analysis. We identified three different stages (definitive endoderm, pancreatic differentiation, and differentiation into beta cell-like IPCs) via immunocytochemical stainings for specific markers and insulin ELISA assay for detection of the insulin release.

Results: We obtained IPCs from human iPSCs via use of specific small molecules and sTRAIL. Differentiation into three distinct stages of beta cell-like IPCs were confirmed by expression of Sox17 and FoxA2 (definitive endoderm); PDX-1 and insulin (pancreatic differentiation); and PDX-1, insulin, NeuroD1, Pax6, and Islet-1 (differentiation into IPCs). Significantly increased insulin secretion was evident by sTRAIL application.

Conclusion: The combined approach of IPC generation used in our study may be useful as an improved strategy, with enhanced insulin secretion in cells in response to different doses of sTRAIL stimulation.

Keywords: Induced pluripotent stem cells, Insulin-producing cells, TRAIL

ÖZET

Amaç: Uyarılmış pluripotent kök hücrelerden (uPKHler) pankreatik beta hücresi benzeri insülin üreten hücre (İÜH) oluşturulmasını hedefleyen çalışmalar, diyabette beta hücre yıkımının/disfonksiyonunun telafisini amaçlamaktadır. Pankreas veya adacık transplantasyonları, yetersiz donör sayısı ve immun baskılama gerekliliği gibi dezavantajlarla ilişkilendirilmektedir. Ekzojen insülin uygulamasının da, hayat kurtarıcı nitelikte olmasına rağmen optimal düzelme sağlayamadığı, ve muhtemel uzun dönem komplikasyonları engelleyemediği bilinmektedir. Bireylerin kendi hücrelerinden elde edilen uPKHlerden oluşturulan İÜHlerden sınırsız beta hücre kaynağı elde edilebilme olasılığı ve immun baskılama gerekliliğinin ortadan kalkma/azalma potansiyeli heyecan yaratmaktadır; ancak optimum düzeyde etkin ve daha güvenli yaklaşımların gerekliliği de bilinmektedir. Çalışmamızda, iyileştirilmiş bir protokolle uPKHlerden beta hücre-benzeri İÜHler elde edebilmeyi hedefledik.

Yöntem: İÜH eldesinde, beta hücre oluşum sürecinde anahtar sinyal yollarını aktive ettiği bilinen spesifik küçük moleküller ve diyabette koruyucu etkisi gösterilmiş olan TNF-Related Apoptosis-Inducing Ligand (TRAIL) molekülünü kullandık. uPKHlerin karakterizasyonları, morfolojik kriterlerin ve pluripotensinin doğrulanması ve mikoplazma analizi ile gerçekleştirildi. İÜH eldesindeki üç farklı aşama (definitif endoderm, pankreatik farklılaşma, ve beta hücresi benzeri İÜHlere farklılaşma), spesifik belirteçler için immunositokimyasal boyamalar ve insülin salınımının belirlenmesi için insülin ELISA analizi ile doğrulandı.

Bulgular: Spesifik küçük moleküllerin ve sTRAIL'ın kullanıldığı bir protokolle insan uPKHlerinden İÜHler elde ettik. İÜH oluşumundaki farklı aşamalar, Sox17 ve FoxA2 (definitif endoderm); PDX-1 ve insülin (pankreatik farklılaşma); ve PDX-1, insülin, NeuroD1, Pax6 ve Islet-1 (İÜHlere farklılaşma) ekspresyonları ile doğrulandı. sTRAIL uygulaması ile insülin salınımında önemli artış gözlendi.

Sonuç: Çalışmamızda kullandığımız kombine protokol, insülin salınımının sTRAIL ile artırıldığı güçlendirilmiş bir yaklaşım olarak yararlı olabilir.

Anahtar Kelimeler: Uyarılmış pluripotent kök hücreler, İnsülin üreten hücreler, TRAIL

TABLE OF CONTENTS

ÖZET i

ABSTRACT ii

TABLE OF CONTENTS iii

INDEX OF FIGURES vi

ICONS and ABBREVIATIONS vii

1. INTRODUCTION 1

2. LITERATURE REVIEW 2

2.1 The Human Pancreas: A General Look 2

2.2 Embryonic Development of the Pancreas: A Brief Summary 3

2.3 Defining the Problem 5

2.4 Generation of Beta Cell-Like Insulin-Producing Cells from Pluripotent Stem Cells: A Brief History 7

2.5 TNF-Related Apoptosis-Inducing Ligand and Its Protective Potential in Diabetes 10

3. MATERIALS and METHODS 12

3.1 Characterization of the Induced Pluripotent Stem Cells (iPSCs) to be Used in Generation of Insulin-Producing Cells (IPCs) 12

3.1.1. Morphological Characterization 12

3.1.2 Confirmation of iPSC Pluripotency 12

3.1.2.1 Alkaline Phosphatase (AP) Live Staining 12

3.1.2.2 Tra-1-60 Live Staining 12

3.1.2 Testing for Mycoplasma Contamination 13

3.2 Culturing and Passaging of the iPSC Colonies 14

3.3 Dissociation of iPSC Colonies into Single Cells for Production of IPCs 14

3.4 Generation of IPCs from iPSCs 15

3.4.1 Differentiation of iPSCs into Definitive Endoderm 15

3.4.2 Pancreatic Differentiation from Definitive Endoderm: 16

3.4.3 Differentiation of Pancreatic Progenitor Cells into IPCs 16

3.5 Immunocytochemical Stainings for Conformation of the Definitive Endoderm Formation, Pancreatic Differentiation, and Generation of IPCs 17

3.6 Measurement of the Basal and sTRAIL-Induced Insulin and C-Peptide Secretion Levels 18

3.7 Statistical analysis 19

4. RESULTS 20

4.1 Induced Pluripotent Stem Cells (iPSCs) were cleaned off from the differentiated cells, morphologically characterized, and expanded 20

4.2 Pluripotency of iPSCs was confirmed via Alkaline Phosphatase (AP) and Tra-1-60 Live Stainings 21

4.3 iPSC colonies tested negative for Mycoplasma 21

4.4 iPSC colonies were dissociated into single cells for generation of IPCs 21

4.5 iPSCs were differentiated into definitive endoderm 22

4.6 Pancreatic differentiation was induced from definitive endoderm 23

4.7 IPCs were established from pancreatic progenitor cells 23

5. DISCUSSION 27
6. CONCLUSION AND SUGGESTIONS 30

REFERENCES 31

RESUME 37

INDEX OF FIGURES

Figure 2.1 Key anatomical properties of the human pancreas. 3

Figure 2.2 Differentiation of ES cells into pancreatic beta cells throughout the embryological development. 4

Figure 2.3 Transcription factors regulating the formation of the pancreatic cells. 5

Figure 2.4 Current strategies for IPC generation. 6

Figure 2.5 Comparison of a few different approaches used in differentiation of human pluripotent stem cells into IPCs 9

Figure 3.1 The reagents used at different stages of IPC generation from iPSCs 15

Figure 4.1 Passaging of iPSC colonies via ReLeSR solution 20

Figure 4.2 Alkaline Phosphatase (AP) and Tra-1-60 live stainings of iPSC colonies 21

Figure 4.3 Results of mycoplasma analysis 21

Figure 4.4 Dissociation of iPSC colonies using GCDR. 22

Figure 4.5 Confirmation of definitive endoderm formation 22

Figure 4.6 Immunoscytochemical stainings of pancreatic progenitors for insulin and PDX1 expressions 23

Figure 4.7 Immunocytochemical stainings of IPCs differentiated from pancreatic progenitors 24

Figure 4.8 Insulin positive cells resulting from experimental settings which included or did not include sTRAIL application 25

Figure 4.9 Insulin levels released to the culture medium following sTRAIL applications 26

Figure 4.10 C-Peptide levels released to the culture medium following sTRAIL applications 26

ICONS AND ABBREVIATIONS

BMPs : Bone Morphogenic Proteins

ESC : Embryonic Stem Cells

GSK3-β : Glycogen Synthase Kinase 3-β

HUVEC : Human Vascular Endothelial Cells

iPSC : Induced Pluripotent Stem Cell

IPC : Insulin-Producing Cell

MODY : Maturity-onset Diabetes of the Young

NKX6-1 : NK6 Homeobox 1

NGN3 : Neurogenin 3

PDX1 : Pancreatic and Duodenal Homeobox 1

RA : Retinoic Acid

SCG : Sodium Cromoglycate

T1D : Type 1 Diabetes

T2D : Type 2 Diabetes

TGF-ß : Transforming Growth Factor-β

TRAIL **:** TNF-Associated Apoptosis-Inducing Ligand

V-XF : Vitronectin-XF

1. INTRODUCTION

Discovery of the ability of induced pluripotent stem cells (iPSCs) to differentiate into various cell types in the human body has been a breakthrough for regenerative medicine studies and *in vitro* disease modeling. Studies aiming generation of new insulin-producing cell (IPC) sources using iPSCs gave birth to direct methodologies for differentiation of iPSCs into IPCs with addition of small molecules to the culture at certain intervals throughout beta cell development. In the early stages of embryogenesis, endoderm develops as one of the three germ layers during gastrulation, and endocrine, ductal, and exocrine portions of the pancreas originate from the definitive endoderm. Thus, formation of beta cells from iPSCs starts with definitive endoderm formation, followed by pancreatic differentiation, and finally generation of beta cell-like IPCs. Use of iPSCs for generation of IPCs has many advantages. For instance, unlike allogeneic beta cells obtained via pancreatic islet isolation from donated pancreases, IPCs generated from iPSCs originating from the patients' own cells are less likely to require immunosuppression. This option also surpasses the enzymatic and mechanical stress to beta cells during islet isolation which significantly affect graft survival. Yet although these processes have a great potential in providing new beta cell sources, generation of IPCs from iPSCs are in fact methodologies with low efficiency that are influenced by various factors, thus requiring improvement in many different aspects.

We aimed to generate beta cell-like IPCs via use of a combination of approaches involving specific small molecules and the TNF-Related Apoptosis-Inducing Ligand (TRAIL) in its soluble form. TRAIL has been discovered as a member of the TNF superfamily in 1995, and was associated in later studies with a protective role in diabetes among its other functions, by works of our group and other researchers. Use of soluble TRAIL (sTRAIL) in our study in the final stage of IPC differentiation increased the insulin secretion, as a significant finding.

2. LITERATURE REVIEW

2.1. The Human Pancreas: A General Look

Human pancreas is located on the posterior wall in the upper abdominal cavity, as an elongated-shaped organ with a length of 12-25 cm in the adult, in close relation with the duodenum, mesenteric vessels, stomach, and the spleen (Figure 2.1). It can be divided into four parts as the head, neck, body and tail regions, and as a complex gland it includes two morphologically and functionally distinct regions as the exocrine and endocrine pancreas. The exocrine portion makes up the major part of the pancreas. It is composed of acinar cells that produce digestive enzymes including trypsin, lipase, and amylase which are secreted into the duodenum via a complex ductal tree. The islets of Langerhans, constituting less than 2% of the pancreas as the endocrine portion, are scattered between the clusters of acinar cells. While around 40-60% of the endocrine cells are beta cells which produce insulin, the rest of the cells are composed of alpha, delta, pancreatic polypeptide and epsilon cells, which secrete glucagon, somatostatin, pancreatic polypeptide, and ghrelin, respectively. An average human pancreas is known to bear around one million of these microorgans. Different signals triggered by autonomic nerves, as well as circulating metabolites such as glucose, amino acids and ketone bodies, and circulating and local hormones regulate islet functions (Atkinson, Campbell-Thompson et al. 2020). Hormone release from the endocrine pancreas is regulated by the close relation between the islets and the vascular cells, maintaining the delicate control of the glucose homeostasis (Avolio, Pfeifer et al. 2013).

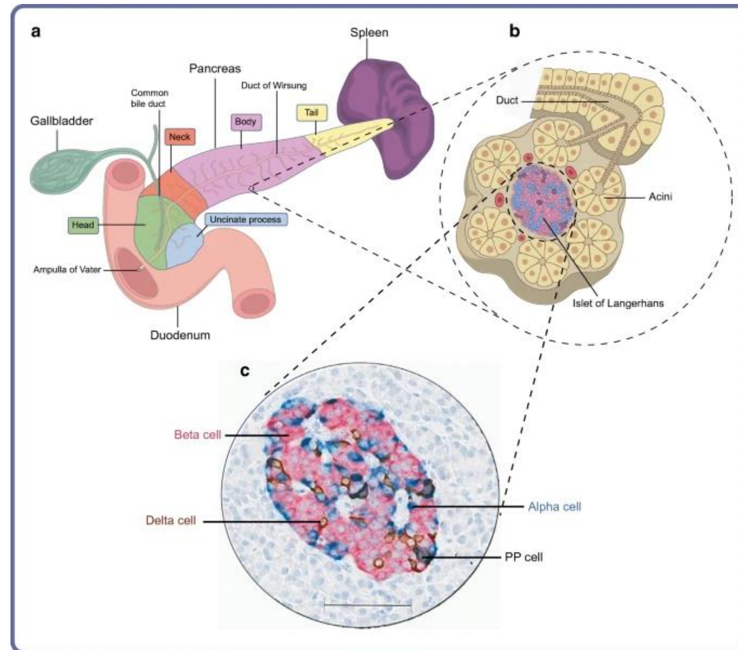

Figure 2.1. Key anatomical properties of the human pancreas. (a) Gross anatomical image of the pancreas; (b) Organisation of the endocrine and exocrine portions; (c) Four endocrine cell types in the human endocrine pancreas (Atkinson, Campbell-Thompson et al. 2020).

2.2 Embryonic Development of the Pancreas: A Brief Summary

It is well known that all the cell types, tissues, and organs are derived from a single cell, the fertilized egg. Throughout early development, following multiple cell divisions, morphogen gradient interpretation takes place where cells respond to various signaling molecules in a concentration-dependent manner (J.B Gurdon 1998). Gastrulation stage appears as a key step in early embryonic development. As cells move inward at or near the embryo surface, the single layered blastula reorganizes into three layers as the ectoderm, mesoderm, and the endoderm, which form specific tissues and organs. The pancreas, along with the other gastrointestinal organs, originate from the endoderm, and is formed from the fusion of the dorsal and ventral pancreatic ducts (Figure 2.2). One common progenitor cell is believed to give rise to all the cell types in the islets. Acini and ductal cells appear as prominent structures before the islets of Langerhans become apparent, after which the endocrine cell numbers rapidly start to increase, mainly via ductal epithelial neogenesis. Endocrine cells in the human fetus start budding from the ducts approximately at 10 weeks of gestation, where they contribute to the formation of

the functional endocrine pancreas throughout pregnancy, after which islet remodeling takes place for around 4 years at least (O'Dowd and Stocker 2013).

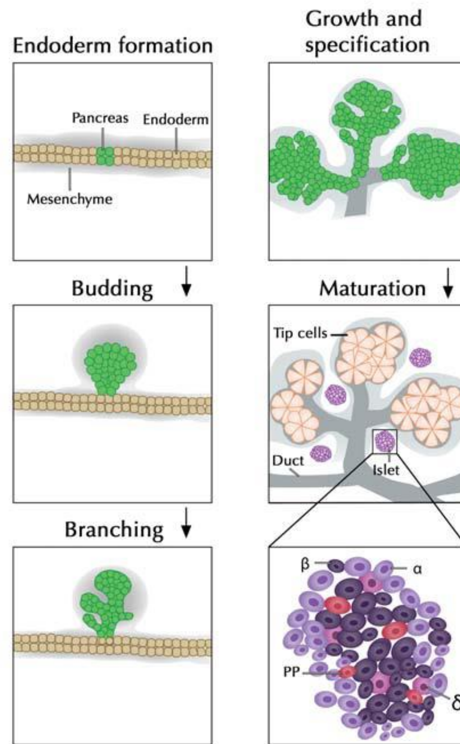

Figure 2.2. Differentiation of ES cells into pancreatic beta cells throughout the embryological development. Pancreas originates from the definitive endoderm pattern which generates the gut tube. The dorsal and ventral pancreatic buds arise from the endoderm, followed by induction and expansion of the pancreatic epithelium, which gives rise to the endocrine progenitors (M 2010).

Genes that control cell differentation and cell fates throughout the embryonic development of the pancreas are regulated by a network of specific transcription factors, some of which are specific for certain stages whereas others are necessary in more than one stage throughout endocrine cell development (Figure 2.3).

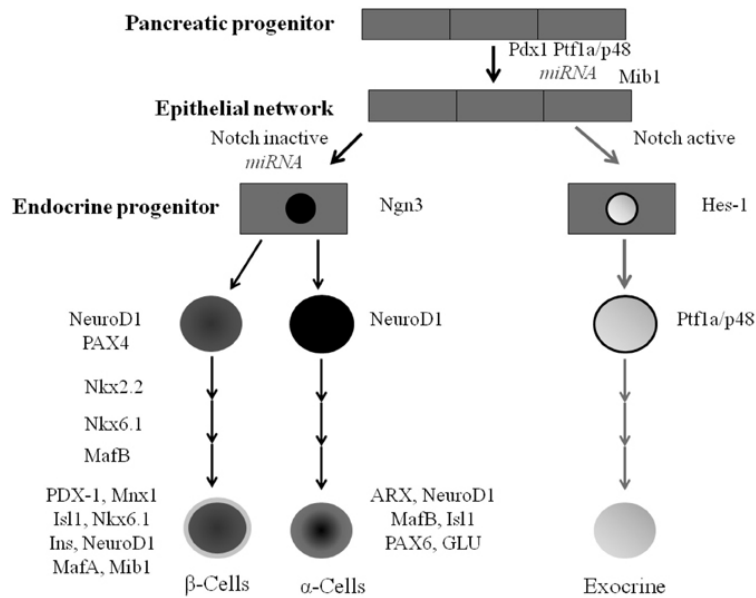

Figure 2.3. Transcription factors regulating the formation of the pancreatic cells. Pancreatic progenitor cells that give rise to all pancreatic cell types express PDX1 and/or Ptf1a (O'Dowd and Stocker 2013).

2.3 Defining the Problem

Pancreatic beta cells are essential cells that make up 65-80% of the human pancreatic islets (Thomsen and Gloyn 2014). They synthesize insulin and amylin, and take part in glucose homeostasis in the body. Insufficiency of beta cells in mass and/or function is characterized in the two main forms of diabetes, namely type 1 and type 2 diabetes (T1D and T2D). In T1D, beta cells are destroyed by an autoimmune mechanism, while in T2D, varying degrees of beta cell destruction are defined in proportion to the severity of insulin resistance, which occurs due to factors such as genetic predisposition and obesity. The pancreas in T1D is associated with insulitis, beta cell loss, and reduced pancreas size, while increased amyloid deposition, reduced beta cell mass, and increased pancreatic fat content are the characteristics of the pancreases of individuals with T2D (Atkinson, Campbell-Thompson et al. 2020). It is emphasized that in both types of diabetes, increased effectiveness of current treatment modalities, as well as development of novel therapeutic approaches are necessary.

Exogenous insulin administration against insulin insufficiency developing as a consequence of excessive beta cell destruction is known to be life-saving, yet does not completely prevent complications related to diabetes (Shapiro, Lakey et al. 2000, Kondo,

Toyoda et al. 2018). Compensating for the progressive loss of beta cell mass with pancreatic beta cell transplantation is currently defined as one of the most effective methods for eliminating beta cell deficiency, but factors such as donor insufficiency and necessity for immunosuppressive agent use are important problems that need to be overcome in this treatment approach. Thus novel treatment modalities are needed.

Many different strategies for generating new beta cell sources have been developed (Figure 2.4). Besides direct differentiation from pluripotent stem cells, beta cell-like insulin-producing cells (IPCs) can be generated via various different approaches, such as conversion of terminally differentiated non-pancreatic cells into IPCs which gave successful results particularly with developmentally-related cells; conversion of non-beta cells within the pancreas to IPCs which has been efficient with acinar, ductal, and alpha cells; and also induction of inherent functional beta cell proliferation (Jing Shena 2013). Of these, direct differentiation from pluripotent stem cells, namely embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are among the most promising perspectives, though still with aspects that require improvement.

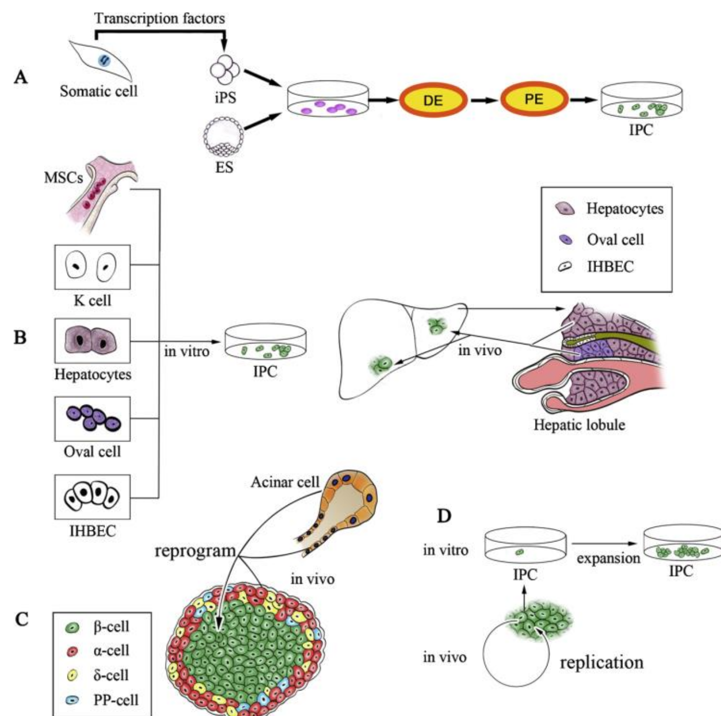

Figure 2.4. Current strategies for IPC generation. (A) Direct differentiation of ESCs or iPSCs into IPCs. (B) *In vitro* and *in vivo* reprogramming of MSCs, K cells, hepatocytes, oval cell and intrahepatic biliary epithelial cells (IHBECs) into IPCs. (C) Reprograming of non-beta cells to beta cell-like cells in the pancreas. (D) Proliferation of the existing functional beta cells (Jing Shena 2013).

In this context, ESCs, which were first isolated from human embryos in 1989, and iPSCs, that were obtained by reprogramming of differentiated cells by Yamanaka S. et al in 2006, have created excitement with their pluripotent nature, thus potential to differentitate into any cell type in human tissues. Due to ethical problems and the necessity of immune suppression, which constitute important obstacles in studies/potential treatment approaches related to ESCs, more studies in this field progressively started to focus on iPSCs. Stem cells, which are responsible for the development and regeneration of tissues and organs, are classified as unipotent, multipotent, pluripotent or totipotent, according to their differentiation capacity. The ability of the pluripotent stem cells to regenerate and transform into any cell type can be used to mimic the natural development of various tissues constituting the human body. Studies aiming production of IPCs as new beta cell sources accordingly utilize combinations of specific stimulators that activate or inhibit key signaling pathways in pancreas development (Pagliuca, Millman et al. 2014). However, the necessity to develop new/complementary approaches is still emphasized, by elimination of deficiencies in the relevant experimental processes in particular to efficiently generate cells that possess potent glucose-induced insulin secretion abilities.

2.4. Generation of Beta Cell-Like Insulin-Producing Cells from Pluripotent Stem Cells: A Brief History

Development of efficient experimental procedures for IPC generation from pluripotent stem cells involved many studies contributing to each critical stage throughout the process. In a pioneering study, the stimuli required for the formation of the definitive endoderm, followed by establishment of pancreatic progenitor cells, and finally the IPCs were determined using human ESCs, and IPCs were created with 7% efficiency (D'Amour, Bang et al. 2006). Another important development in this field was differentiation of ESCs into the pancreatic endoderm, followed by conversion into glucose-induced insulin-secreting cells in the *in vivo* setting, after transplantation into mice (Kroon, Martinson et al. 2008). Upon this development, production of proliferative and multipotent pancreatic progenitor cells could be achieved through combined expression of PDX1 and NKX6-1 (Schulz, Young et al. 2012). Relevant studies continued with various different strategies, and a significant work reported that IPCs established

from human ESCs were able to treat diabetes in diabetic mice within 40 days (Rezania, Bruin et al. 2014).

Rezania et al. published a differentiation protocol in which they added vitamin C, protein kinase C pathway activators, TGF-beta receptor inhibitors, and thyroid hormones (Rezania, Bruin et al. 2014) (Figure 2.5). Focusing specifically on an AXL inhibitor, the researchers reported that the use of this molecule increased the amount of MafA mRNA and gave cells the ability to release glucose-sensitive insulin, and with this method, the percentage of IPC yield was 50%. In another study, a 30% yield of IPCs with high similarity to adult beta cells following a 5-week differentiation period was reported (Pagliuca, Millman et al. 2014) (Figure 2.5). These cells were then implanted under the renal capsule of immunodeficient mice and within 2 weeks began to release glucose-sensitive insulin. The importance of this study was that the duration of the maturation process following transfer of the cells to the *in vivo* setting, and initiation of their insulin release afterwards was much shorter compared to other similar work. The use of indolactam V for PDX1+ cells and vesicular monoamine transporter 2 inhibitors for insulin+ cells, induced pancreatic lineage formation (Chen, Borowiak et al. 2009, Sakano, Shiraki et al. 2014). Studies have also been conducted in which PDX1, Ngn3, and MafA molecules, which are critical for beta cell development and differentiation, were transferred to different cell types via Adenoviral vectors, to directly reprogram the cells into beta-like cells (Akinci, Banga et al. 2012, Akinci, Banga et al. 2013, Yang, Akinci et al. 2013). Results from all these studies have led to the emergence of direct methodologies that can mediate differentiation of iPSCs into IPCs via addition of small molecules to the culture at certain times, to induce the expression of critical molecules at critical stages for beta cell formation (Kunisada, Tsubooka-Yamazoe et al. 2012).

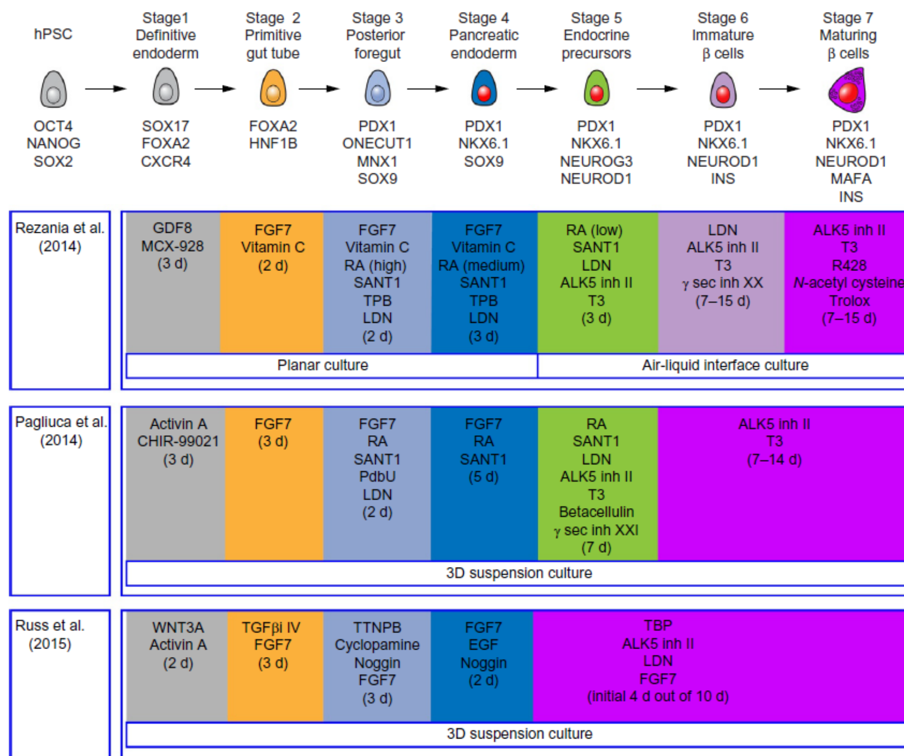

Figure 2.5. Comparison of a few different approaches used in differentiation of human pluripotent stem cells into IPCs (Maja B.K. Petersen 2018).

Overall, the process of IPC generation from iPSCs can be divided in a more simple pattern into three main parts: establishment of the definitive endoderm; pancreatic differentiation; and generation of beta cell-like IPCs. Critical information obtained from various studies revealed that particularly the formation of the PDX1+ pancreatic progenitors, which direct formation of the pancreas from definitive endoderm, and the differentiation of PDX1+ cells into IPCs are critical steps in the differentiation process of iPSCs into IPCs (Kunisada, Tsubooka-Yamazoe et al. 2012). Small molecules added at defined times throughout the process differentiated iPSCs first into Sox17 and FoxA2+ definitive endoderm, then into PDX1+ pancreatic progenitor cells, and finally into IPCs. In this study, we have used a combined approach utilizing activin A and a GSK3beta inhibitor for differentiation of iPSCs into definitive endoderm, followed by dorsomophin, retinoic acid, and TGF-beta type 1 receptor inhibitor to induce pancreatic differentiation; and forskolin, dexamethasone, Alk5 inhibitor, nicotinamide, and sodium cromoglycate for

generation of IPCs, also supported at this stage via TNF-Related Apoptosis-Inducing Ligand application (Figure 3.1).

2.5. TNF-Related Apoptosis-Inducing Ligand and Its Protective Potential in Diabetes

TNF-Related Apoptosis-Inducing Ligand (TRAIL) is a member of the TNF superfamily, discovered by two separate research groups in 1995 (Wiley, Schooley et al. 1995, Pitti, Marsters et al. 1996). It is a transmembrane protein known to play an important role in regulation of mechanisms associated with selective apoptosis in tumor cells, as well as anti-inflammatory pathways. Unlike other TNF family members, it is widely expressed in human tissues and has 5 different receptors to which it can bind. While four of these are transmembrane receptors, one is a soluble receptor (OPG). Two of the transmembrane receptors (TR-1/DR4 and TR-2/DR5) are death receptors; they contain death domains in their cytoplasmic portion, and when TRAIL binds to these receptors, caspase-dependent apoptosis is activated. TR-3/DcR1 and TR-4/DcR2 receptors are termed as decoy receptors, which do not mediate apoptosis upon TRAIL binding (Griffith and Lynch 1998). All 4 transmembrane receptors also have the potential to trigger cell survival and proliferation by activating intracellular anti-apoptotic pathways.

TRAIL's widespread expression in human tissues and the fact that it has 5 different receptors to which it can bind to indicated multiple roles to be attributed to this molecule (LeBlanc and Ashkenazi 2003). TRAIL was shown to exert a proangiogenic effect on primary human vascular endothelial cells (HUVECs) (Secchiero, Gonelli et al. 2004), and induced survival and proliferation by activating the Akt and ERK pathways (Sheridan, Marsters et al. 1997, Secchiero, Gonelli et al. 2003). Besides its apparent role in endothelial cell physiology, this molecule also induced survival against proinflammatory cytokine-mediated apoptosis, as well as triggering migration and proliferation pathways in vascular smooth muscle cells (Secchiero, Zerbinati et al. 2004). In addition, when used at low doses (1 ng/ml), soluble TRAIL molecule induced NF-kB activation, IGF1R expression, and proliferation in primary human vascular smooth muscle cells (Kavurma, Schoppet et al. 2008). Importantly, the proliferation-inducing effect of TRAIL in endothelial and smooth muscle cells was accompanied by its protective effect on the same cells.

A protective role is attributed to TRAIL in diabetes. T1D developed much earlier and more severely than in normal mice after multiple low dose STZ administration in C57BL/6 mice that did not express TRAIL (Lamhamedi-Cherradi, Zheng et al. 2003). Di Bartolo et al. reported that $TRAIL^{-/-}$ mice displayed many complications associated with diabetes (Di Bartolo, Chan et al. 2011). Mi et al. treated islets isolated from 3-week-old NOD mice with varying concentrations of recombinant soluble TRAIL, and reported no apoptosis induction, unlike TNF-alpha and IFN-gamma that have a prominent apoptotic effect on pancreatic beta cells (Mi, Ly et al. 2003). In a study conducted by our group, we first provided high TRAIL expression in rat pancreatic islets with Adenoviral vectors. Later, upon transplantation of the aforementioned islets to diabetic recipient rats, we observed reduction in severity of insulitis, along with extended survival and enhanced function in allografts (Dirice, Sanlioglu et al. 2009). These findings also indicate that TRAIL may be part of a defense mechanism activated against the cytotoxic T lymphocytes. Accordingly, TRAIL most likely prevented autoimmune inflammation in the mentioned setting via cell cycle inhibition of the islet-infiltrating lymphocytes (Song, Chen et al. 2000). In another study by our group, Cyclophosphamide (CY), used as a diabetes-accelerating agent in NOD mice due to its suppressive effect on the immune-suppressive Treg cells, also strongly suppressed the TRAIL ligand throughout the disease course (Dirice, Kahraman et al. 2011). This provided additional proof supporting the protective role of TRAIL on pancreatic islets. We have also recently reported a proliferative effect of TRAIL on rodent pancreatic beta cells via Akt activation (Kahraman, Yilmaz et al. 2021). Thus based on all these findings, we questioned whether TRAIL would provide a beneficial effect in the course of IPC generation from iPSCs.

3. MATERIALS AND METHODS

3.1 Characterization of the Induced Pluripotent Stem Cells (iPSCs) to be Used in Generation of Insulin-Producing Cells (IPCs)

3.1.1. Morphological Characterization

The iPSC colonies were first morphologically characterized. Colonies with optimal morphology consisted of rounded, packed cells with well-defined borders.

3.1.2 Confirmation of iPSC Pluripotency

3.1.2.1 Alkaline Phosphatase (AP) Live Staining

Reagents used:

-Alkaline Phosphatase Live Staining Kit (Thermo Fisher A14353)

-DMEM/F12 Medium (Stem Cell Technologies)

AP is an early phenotypic marker of pluripotent stem cells, and live staining is a preferred method for confirmation of pluripotency as it does not perturb cell viability and allows maintenance of pluripotency and proliferation. To start AP live staining, we first removed the media from the wells and washed the cells twice with DMEM/F12 for 2-3 minutes. Then media in the wells were aspirated and 1X AP live staining solution (containing 3 ul of 500X AP live stain stock solution and 1.5 ul DMEM/F12) was added into each well, followed by incubation at room temperature (RT) for 20-30 minutes. After the aspiration of 1X AP live stain solution, the wells were gently washed with DMEM/F12 for 5 minutes. This step was carried out carefully not to disturb the cells. Following visualization, we replaced DMEM/F-12 medium with mTeSR™ Plus medium and continued the culture.

3.1.2.2 Tra-1-60 Live Staining

Reagents used:

- TRA-1-60 Alexa Fluor™ 594 Conjugate Kit for Live Cell Imaging (A24882)

TRA-1-60 is a cell surface antigen that is expressed in human pluripotent stem cells. This kit was used in order to confirm Tra-1-60 expression in iPSCs as a method to test pluripotency. The antibody solution was centrifuged for 2 min at 10.000 g and the supernatant was collected. Following aspiration of the medium, cells were covered with

the antibody solution at 1:50 dilution and incubated at 37°C for 30 min. Antibody solution was then removed and cells were washed gently with FluoroBrite^{TM} DMEM. Cells were visualized within 30 min following this step.

3.1.2 Testing for Mycoplasma Contamination

Reagents used:

-MycoFlour Mycoplasma Detection Kit (Thermo, M-7006)

For mycoplasma detection from the culture media, 1 mL of cell culture medium sample was collected and centrifuged at 1300 g for 10 minutes. The supernatant was transferred into microfuge tubes and centrifuged at 12,500 g for 15 minutes. About 0.5 mL of the medium was left in the tube and rest of the supernant was discarded. 26 µL of the 20X concentrated MycoFluor reagent was then added into the microfuge tube. Lastly, 10 µL of the stained medium was placed onto a clean microscope slide and covered with a clean coverslip, followed by sealing with the coverslip sealant via a cotton swab and left for drying for 10 seconds. The outcome was monitored under a fluorescent microscope.

3.2 Culturing and Passaging of the iPSC Colonies

Reagents and materials used:

-ReLeSR Solution (Stem Cell Technologies, 5873)
-mTeSR Plus Medium (Stem Cell Technologies, 5825)
-Rock Inhibitor (Y-27632) (Sigma, SCM075)
-Vitronectin XF (Stem Cell Technologies, 7180)
-D-PBS (Stem Cell Technologies, 37354)
-Non-tissue culture-treated 6-well culture plates

iPSC colonies were cultured in mTeSR medium on Vitronectin XF (V-XF), supplied with fresh medium every two days, in the presence of Rock Inhibitor. Vitronectin is a suitable matrix for feeder-free culture of pluripotent stem cells, compatible with various different stem cell media such as mTeSR, E7, and E8. Rock Inhibitor (Y-27632) is the selective inhibitor of Rho-associated coiled-coil containing protein kinase (ROCK). It helps with the maintenance of iPSCs in culture (Davies, Reddy et al. 2000). For coating of each well of a 6-well culture plate, V-XF was thawed at room temperature (RT) and diluted in Cell

Adhere Dilution Buffer at a final concentration of 10 ug/mL. Following gentle mixing, 1 ml of the solution (40 uL V-XF/ml) was poured on a single well to cover the whole surface, and incubated at RT for at least an hour. Following incubation, excess V-XF solution was removed with the culture plate slightly tilted on one side. Before addition of mTeSR medium for transfer of cells on to the matrix, each well was washed once with 1 ml Cell Adhere Dilution Buffer.

When the majority of the iPSC colonies become large, compact, and tend to merge with each other in culture, they are assumed to be at the most favorable time for passage. iPSCs require passaging every 4-5 days for large aggregates and 6-7 days for smaller aggregates. Non-tissue culture-treated 6-well culture plates were coated with V-XF and incubated at RT for at least an hour before use. mTeSR Plus medium was brought to RT. The mTeSR medium was aspirated from the wells and cells were washed with 1 mL of D-PBS and aspirated. 1 mL ReLeSR solution was added to each well and aspirated within 1 min, followed by incubation at 37ºC for 6-8 min. Following incubation, 1 mL mTeSR Plus medium was added to each well and detached aggregates were collected with a 5 mL serological pipette, followed by dissociation via pipetting up and down and transfer to Vitronectin-coated wells. Plates were then placed into 37°C incubators and not disturbed for 24 hours.

3.3 Dissociation of iPSC Colonies into Single Cells for Production of IPCs

Reagents used:

-Gentle Cell Dissociation Reagent (GCDR) (Stem Cell Technologies, 7174)

After incubation of the iPSC colonies in mTeSR™ Plus medium in the presence of 10 µm Rock Inhibitor (Y-27632) for 2 days, medium was aspirated from wells and 1 mL of GCDR was added, followed by incubation at room temperature (RT) for 12 min. After aspiration of the GCDR, 1 mL of mTeSR™ Plus medium was added and colonies were detached by gentle scraping with a serological pipette. Cell aggregates were then transferred into a 15 mL conical tube. The wells were rinsed with an additional 1 mL of mTeSR™ Plus to collect the remaining cell aggregates. Colonies were dissociated by pipetting up and down 5 or 6 times in a 15 ml conical tube with a 2 ml serological pipette. Lastly, broken cell aggregates were counted and plated at a density of $6x10^4$ per well into

cell culture plates and 10 µm Rock Inhibitor (Y-27632) was added. After 24 h incubation at 37°C, mediums were replaced with mTeSR™ Plus containing 1% Penicillin-Streptomycin.

3.4 Generation of IPCs from iPSCs

iPSCs generated from HEK293T cells were induced for differentiation into definitive endoderm, pancreatic progenitor cells, and beta cell-like IPCs via a 3-stage protocol including differentiation into definitive endoderm, pancreatic differentiation, and generation of beta cell-like IPCs (Figure 3.1)

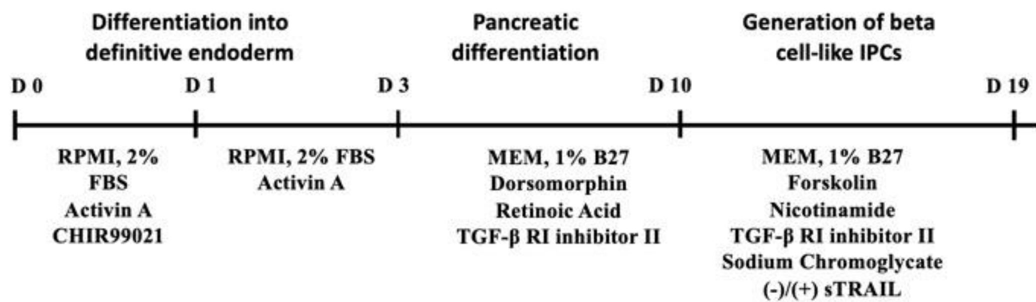

Figure 3.1 The reagents used at different stages of IPC generation from iPSCs.

3.4.1 Differentiation of iPSCs into Definitive Endoderm

Reagents used:

- RPMI 1640 Medium (Gibco, 11875093)
- FBS (Phosphate-Buffered Saline)
- Human Recombinant Activin A (ABM, Z101665)
- GSK3beta inhibitor (CHIR99021) (BioVision, 1677-5)

Following reagents were prepared in 5 ml RPMI-1640 medium:

- 0.1 ml FBS (2%)
- 5 µl of 0.1 mg/ml stock Activin A solution (100 ng/ml)
- 1.5 µl of 10 mM stock CHIR99021 solution (3 µM)

Cells were cultured for 24 hours in RPMI 1640 medium containing 2% FBS, 100 ng/ml Activin A and 3µM CHIR99021. The following day cells were incubated with RPMI medium containing 2% FBS in the presence of 100 ng/ml Activin A for 48 hours.

3.4.2 Pancreatic Differentiation from Definitive Endoderm:

Reagents used:

- MEM Zinc Option Medium (Thermo, A1048901)
- B27 (Thermo 0080085SA)
- Dorsomorphin (Sigma, P5499-5MG)
- Retinoic Acid (Sigma, R2625-50MG)
- TGF-beta RI inhibitor II (Alk5 inhibitor) (SB431542) (Sigma, 616452-2MG)

Following reagents were prepared in 5 ml iMEM Zinc Option Medium:

- 50 µl B27 (1%)
- 0.5 µl of 10 mM stock Dorsomorphin (1 µM)
- 1 µl of 10 mM stock Retinoic Acid (2 µM)
- 5 µl of 10 mM stock TGF-beta RI inhibitor II (SB431542) (10 µM)

RMPI medium was replaced with the improved MEM Zinc Option medium containing 1% B27, 1 µM dorsomorphin, 2 µM retinoic acid, and 10 µM SB431542. Cells were cultured for 7 days and the medium were renewed every 2 days. Lastly, cells were kept in Improved MEM Zinc Option medium containing 1% B27, for 1 day.

3.4.3 Differentiation of Pancreatic Progenitor Cells into Beta Cell-Like IPCs

Reagents used:

- Forskolin (Sigma, F3917-10MG)
- TGF-beta RI inhibitor II (Alk5 inhibitor) (Sigma, 616452-2MG)
- Nicotinamide (10 mM) (Stem Cell Technologies, 7154)
- SCG (Sodium Cromoglycate) (Sigma, S0750000)
- Soluble TRAIL (sTRAIL)

Following reagents were prepared in 5 ml iMEM Zinc Option Medium:

- 5 µl each of 10 mM stock Forskolin, TGF-beta RI inhibitor, Nicotinamide solutions (10 µM)
- 5 µl of 1 M SCG stock solution (10 mM)
- sTRAIL at specific concentration (1, 5, and 10 ng/ml)

Four different media were prepared and cells were cultured in these media for 8 days. sTRAIL concentrations of 1, 5, and 10 ng/ml were used:

1) 10 µM forskolin, 10 µM dexamethasone, 5 µM Alk5 inhibitor II, 10 µM nicotinamide and 10 mmol/l SCG.

2) 10 µM forskolin, 10 µM dexamethasone, 5 µM Alk5 inhibitor II, 10 µM nicotinamide and 10 mmol/l SCG + 1 ng/ml sTRAIL.

3) 10 µM forskolin, 10 µM dexamethasone, 5 µM Alk5 inhibitor II, 10 µM nicotinamide and 10 mmol/l SCG + 5 ng/ml sTRAIL.

4) 10 µM forskolin, 10 µM dexamethasone, 5 µM Alk5 inhibitor II, 10 µM nicotinamide and 10 mmol/l SCG + 10 ng/ml sTRAIL.

3.5 Immunocytochemical Stainings for Conformation of the Definitive Endoderm Formation, Pancreatic Differentiation, and Generation of IPCs

Solutions and Antibodies Used:

- **Fixative Solution**

  4% Paraformaldehyde (PFA) was prepared. For this, 2 g of PFA was dissolved in 50 ml of PBS. 200 µl NaOH was added.

- **Permabilization Solution (PBS-T)**

  For preparation of PBS-T, 20 µl of Tween 20 was dissolved in 20 ml PBS.

- **Blocking Solution (1% BSA)**

  This solution was prepared by dissolving 500 mg of BSA in 50 ml PBS-T.

- **Primary Antibodies:**

  PDX1 Rabbit polyclonal antibody, Abcam Ab47267

  Insulin Rabbit polyclonal antibody, Bioss BS0862R

  PAX6 Rabbit polyclonal antibody, Bioss BS11204R

  NeuroD1 Mouse monoclonal antibody, Origene AM32710PU-N

  SOX17 Mouse monoclonal antibody, Origene TA500044

  FOXA2 Mouse monoclonal antibody, Origene TA500073

  Islet-1 Mouse monoclonal antibody, Origene AM06335SU-N

- **Secondary Antibodies:**

  Goat-anti mouse IgG alexa flour plus 555, Invitrogen A32727

Goat anti rabbit IgG alexa flour plus 488, Invitrogen A32731

Dissociated cells were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature (RT). Fixed cells were permeabilized in PBS containing 0.1% Triton X (PBS-T). After addition of the permeabilization solution and incubation for 15 minutes at RT, permeabilization solution was removed and wells were washed with PBS-T:20 (PBS + Tween 20). 1% BSA solution was used for blocking and the samples were incubated for 1 h at RT. In the meantime, primary antibody dilutions were prepared at 1/300 dilution in 1% BSA solution. 500 µl of diluted antibodies were added to the wells, wrapped with aluminum foil and left for incubation at +4°C overnight (O/N). Primary antibodies were then removed, and wells were washed three times for 5 minutes with PBS T:20. Secondary antibody dilutions were prepared at 1/500 dilution and 500 ul of the dilutions were added to the wells, followed by incubation at RT for 1 hour in the dark. Three 10-min PBS washes were performed, and the samples were treated with 2 drops of DAPI per well in 500 ml PBS for 15 min at RT. Finally, the wells were washed with 1 ml of PBS, and 500 ml PBS were added to each well to visualize the stained cells.

3.6 Measurement of the Basal and sTRAIL-Induced Insulin and C-Peptide Secretion Levels

Reagents used:

All solutions and well strips were equlibrated to room temperature (RT) before the initiation of the experiment. Supernatants of IPC cultures treated with no sTRAIL or 1, 5 or 10 ng/ml sTRAIL were collected prior to immunostanings.

- Insulin ELISA Kit (Abcam, ab100578)

By using assay diluent B, supernatants and the insulin standard were diluted according to the manufacturer’s instructions. 100 µl of each supernatant and the standard samples were added to wells and left to incubation for 2.5 hours. After the incubation, samples were discarded. Wells were washed 4 times with 1X wash buffer. 100 µl of biotinylated insulin detection solution were added to each well and incubated for 1 hour at RT on a rocker for gentle shaking. Solutions were discarded and wells were washed 4 times with 1X wash buffer. 100 µl of HRP-Streptavidin solution was added to each well and left for incubation

on a rocker for 45 minutes. After discarding of HRP-Streptavidin, wells were washed with 1X wash buffer and in a dark room, 100 µl TMB One-Step Substrate Reagent was added to each well and incubated on a rocker for 30 minutes. Following this step, 100 µl of stop solution was added to each well and results were read at 400 nm immediately. For accurate results, both standards and supernatants were added twice and average values were used. Standart curve was plotted with x axis presenting the applied concentrations and y axis presenting the absorbance values. An equation containing the dilution factor was established and samples were calculated accordingly. Results of three separate experiments were used for statistical analysis.

- C-Peptide ELISA Kit (Origene, EA101026)

100 µL of each standard and samples were dispersed into each well. 50 µL of the Antiserum and 100 µL of the Enzyme Conjugate solution were added into each well and mixed. The plate was left for incubation for 60 min in RT with gentle shaking. All the wells were washed with 400 µL of wash solution per well. 100 µL of the Enzyme Complex was added to each well and left for incubation with gentle shaking at RT. Wells were re-washed via 400 µL of wash solution per well. 100 µL of Substrate Solution was dispersed into each well and left for incubation for 20 min in a dark room at RT. Enzymatic reaction was stopped by addition of 100 µL Stop Solution into each well. Absorbance was determined by microtiter plate reader at 450 nm.

3.7 Statistical Analysis

One-Way Anova, Dunnett Multiple Comparison test was used in the Prism program from GraphPad Software (San Diego, CA) for statistical analysis. P value analysis for each experiment is given in the figure legends (Fig. 4.9 - 4.10)

4. RESULTS

4.1 Induced Pluripotent Stem Cells (iPSCs) were cleaned off from the differentiated cells, morphologically characterized, and expanded:

The emerging iPSC colonies may often have differentiated cells around the undifferentiated core colonies, which have to be cleared off to obtain pure masses as starting materials for acquisition of Insulin Producing Cells (IPCs) (Figure 4.1 A, B). This can be done via manual methods, enzymatic methods, or non-enzymatic methods. We used the non-enzymatic ReLeSR solution to clean the pluripotent colonies from the differentiated cells and passaged the pluripotent colonies into new culture wells several times as aggregates before dissociating them into single cells. Morphological characterization of the iPSC colonies were done at this stage on the basis of colonies that consisted of rounded, packed cells with well-defined borders (Figure 4.1. C, D).

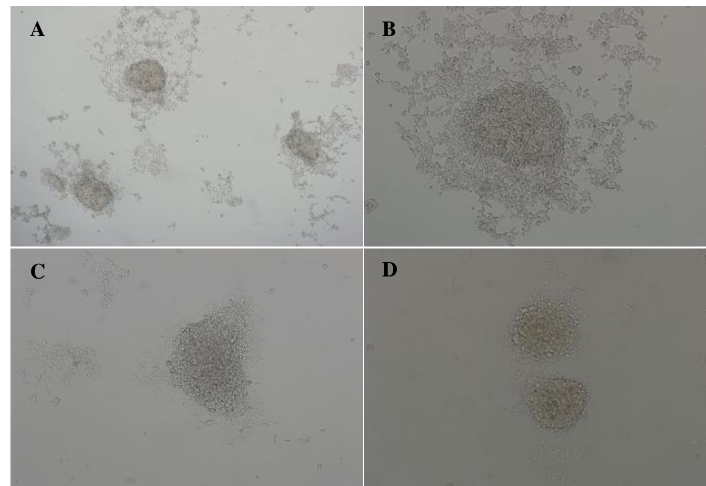

Figure 4.1. Passaging of iPSC colonies via ReLeSR solution. (A, B): Colonies before passaging with ReLeSR; (C, D): Colonies following ReLeSR treatment, with rounded and packed cells.

4.2 Pluripotency of the induced pluripotent stem cells (iPSCs) was confirmed via Alkaline Phosphatase (AP) and Tra-1-60 Live Stainings

To confirm the pluripotency of iPSCs to be used in IPC generation, we performed Alkaline Phosphatase (AP) and Tra-1-60 live stainings. Our colonies expressed substantial levels of AP and Tra-1-60, which are among the key pluripotency markers (Figure 4.2).

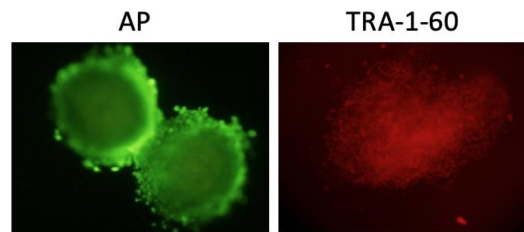

Figure 4.2. Alkaline Phosphatase (AP) and Tra-1-60 live stainings of iPSC colonies.

4.3 iPSC colonies tested negative for mycoplasma

Testing for possible mycoplasma contamination is one of the common characterization methods applied in iPSC colonies. We tested the iPSC colonies to be used in generation of IPCs for mycoplasma contamination and confirmed that our colonies did not contain mycoplasma (Figure 4.3).

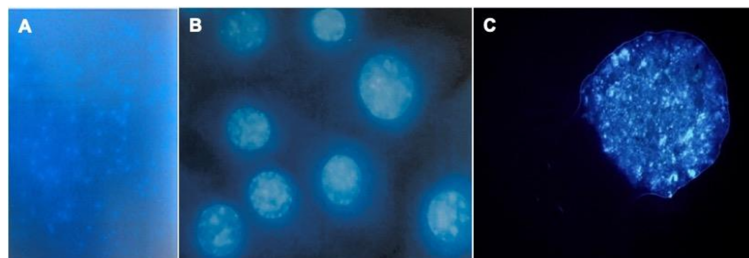

Figure 4.3. Results of mycoplasma analysis. (A) Mycoplasma-positive reference photomicrograph; (B) Mycoplasma-free reference photomicrograph (C) Images from mycoplasma analysis in our iPSC cultures.

4.4 iPSC colonies were dissociated into single cells for generation of IPCs

To start the differentiation, iPSC colonies were dissociated into single cells with the help of Gentle Cell Dissociation Reagent (GCDR) (Figure 4.4). Dissociated cells appeared either as single cells or two-three cell groups, which gave us the approppriate starting material for IPC generation.

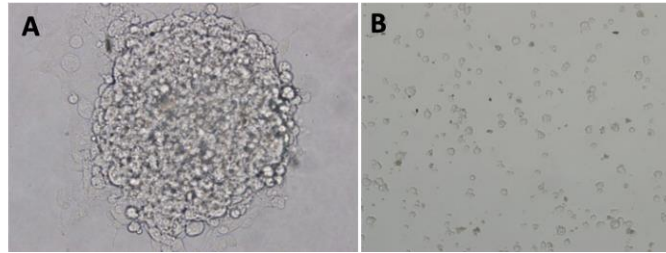

Figure 4.4. Dissociation of iPSC colonies using GCDR. (A) An iPSC colony before dissociation, (B) iPSC colony dissociated into single cells prior to initiation of the experimental procedures for generation of IPCs.

4.5 iPSCs were differentiated into definitive endoderm

Generation of cells positive for the endodermal markers Sox17 and FoxA2 is directive when determining the success of definitive endoderm differentiation. Activin A functions as a hormone, growth factor, and a cytokine, which has a high physiological importance in humans with an important role in cell differentiation. Activin/Nodal signaling is required for definitive endoderm generation from pluripotent stem cells (McLean, D'Amour et al. 2007). Inhibition of the Wnt/beta-catenin signaling pathway in the early embryonic period is also known to be required for pancreatic specification (Murtaugh 2008). Since aspects of this pathway are known to be mimicked by GSK3beta activity, GSK3beta inhibitor (CHIR99021) is recommended as also an effective molecule throughout this process (Doble and Woodgett 2003). Use of these two molecules together successfully generated definitive endoderm from human iPSCs, which accordingly stained positive for both Sox17 and FoxA2 definitive endoderm markers. We observed single cells at this stage, as well as cell groups (Figure 4.5).

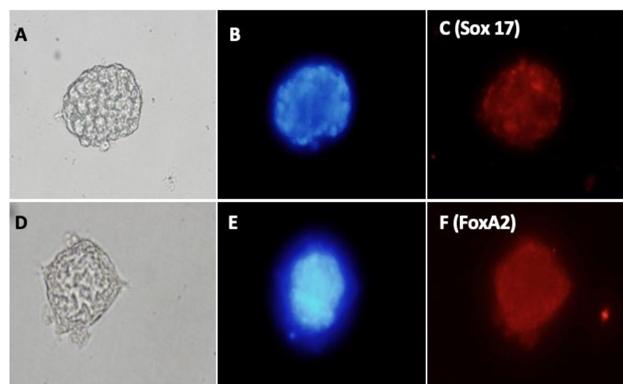

Figure 4.5. Confirmation of definitive endoderm formation**.** (A, D): Bright field images of the differentiated iPSCs at the definitive endoderm stage; (B, E): DAPI images of the differentiated cells. (C): Definitive endoderm cells stained positive for SOX17; (F): Definitive endoderm cells stained positive for FOXA2.

4.6 Pancreatic differentiation was induced from definitive endoderm

Pancreatic differentation constitutes a further step in generation of beta cell-like IPCs. We exposed cells at the definitive endoderm stage to retinoic acid, dorsomorphin, and TGF-beta type 1 receptor inhibitor (SB431542) to induce pancreatic differentiation. Retinoic acid, while inducing the formation of endocrine progenitor cells, also supports differentiation into beta cell-like cells (Ostrom, et al. 2008). Dorsomorphin contributes to pancreatic differentiation via blocking of the BMP signaling pathway, and is known to be more effective in the mentioned process compared to Noggin, also being a more stable molecule. TGF-beta type 1 receptor inhibition (SB431542), when administered together with dorsomorphin and retinoic acid, increased pancreatic differentiation (Kunisada et al. 2012). Cells at this stage stained positive for insulin and PDX1 (Figure 4.6).

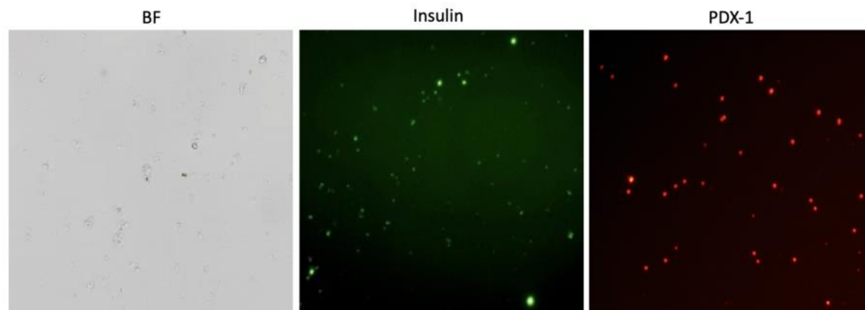

Figure 4.6. Immunocytochemical stainings of pancreatic progenitors for insulin and PDX-1 expressions. BF: Bright Field

4.7 IPCs were established from pancreatic progenitor cells

Different signaling pathways are thought to be active in efficient differentiation of PDX1+ cells into IPCs. Combined use of forskolin, dexamethasone, and Alk5 inhibitor II has been reported to increase insulin production. Forskolin is an adenylate cyclase activator that increases cAMP rates. High intracellular cAMP levels are thought to contribute to the differentiation of PDX1+ cells into IPCs (Kunisada, Tsubooka-Yamazoe et al. 2012). Inhibition of the endogenous TGF-beta pathway with Alk5 inhibitor II at this stage has also been reported to increase the yield of IPCs (Kunisada, Tsubooka-Yamazoe et al. 2012, Kondo, Toyoda et al. 2017). The effectiveness of this protocol was observed to increase a great deal with addition of sodium cromoglycate (SCG), which inhibits the bone morphogenetic protein 4 signal (Kondo, Toyoda et al. 2017). Cells at this stage stained positive for PDX1, NeuroD1, Pax 6, insulin, and Islet-1 (Figure 4.7).

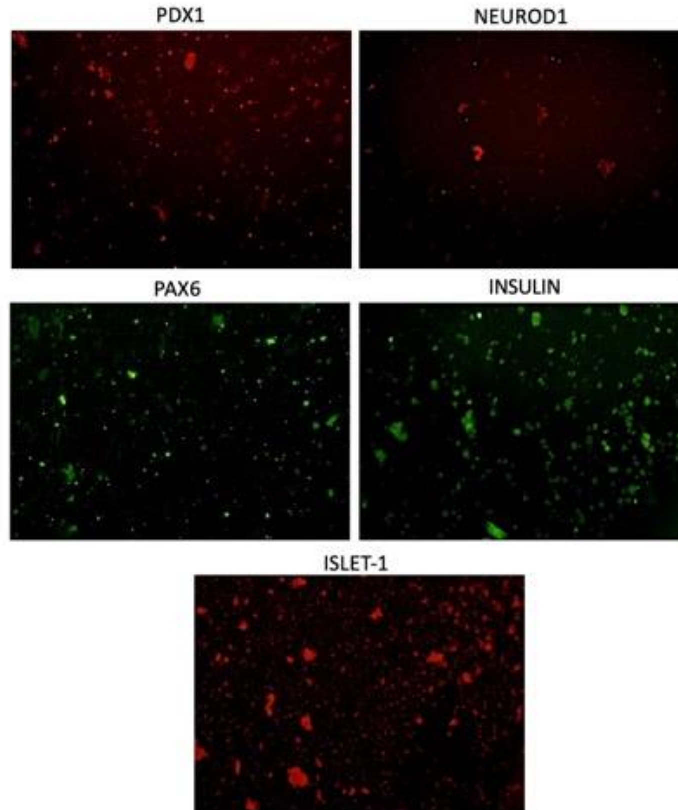

Figure 4.7. Immunocytochemical stainings of IPCs differentiated from pancreatic progenitors.

We have also tested the effect of TNF-Related Apoptosis-Inducing Ligand (TRAIL) at its soluble form on insulin and c-peptide secretion from the IPCs generated. TRAIL is known for its protective effect on pancreatic beta cells and an antidiabetic effect in development of both type 1 and type 2 diabetes in corresponding animal models. We have shown recently that TRAIL also stimulates beta cell proliferation (Kahraman, Yilmaz et al. 2021). TRAIL may be exerting its such protective/antidiabetic/proliferative effects in a variety of ways. To test if it affects insulin production and release, we applied increasing concentrations of sTRAIL at the final (third) stage of IPC generation, in addition to other small molecules specific for this stage (Figure 4.8). The amounts of insulin and c-peptide synthesized to the culture medium following sTRAIL applications as well as the basal insulin and c-peptide levels were measured by ELISA analysis. Production/release levels of insulin and c-peptide from IPCs increased with increasing concentrations of sTRAIL applied (Figure 4.9 and figure 4.10).

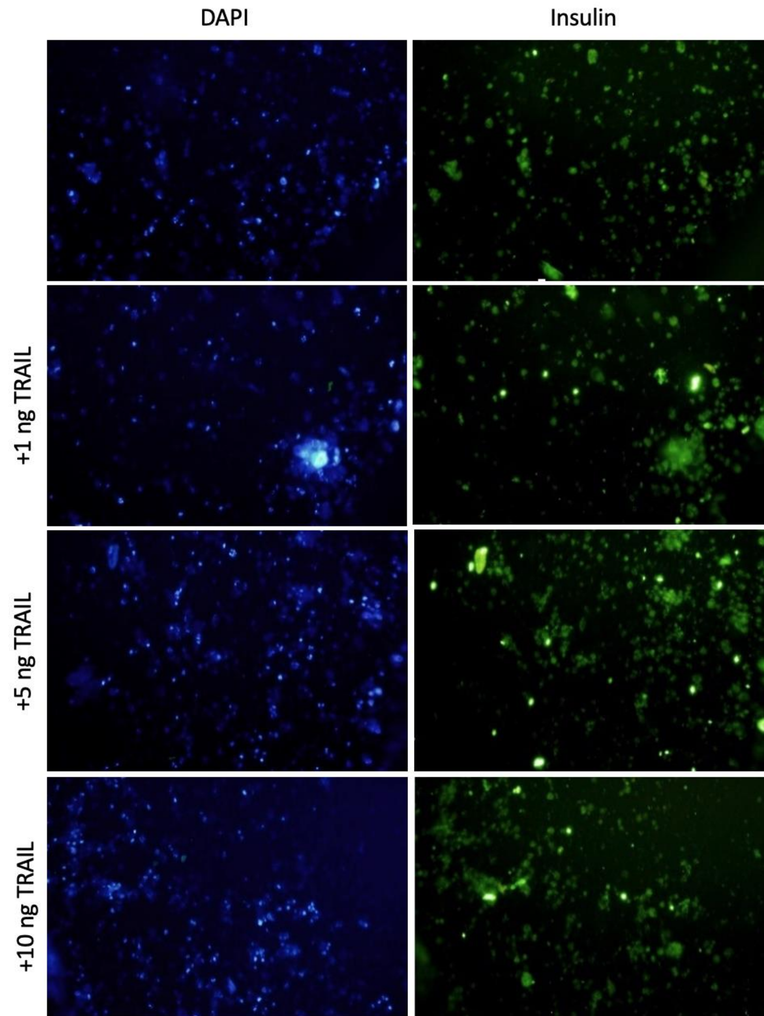

Figure 4.8. Insulin positive cells resulting from experimental settings which included or did not include sTRAIL application

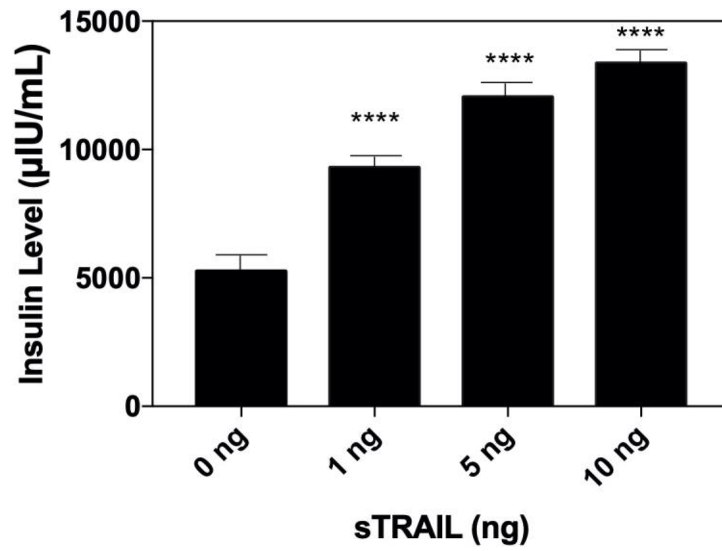

Figure 4.9. Insulin levels released to the culture medium following sTRAIL applications.

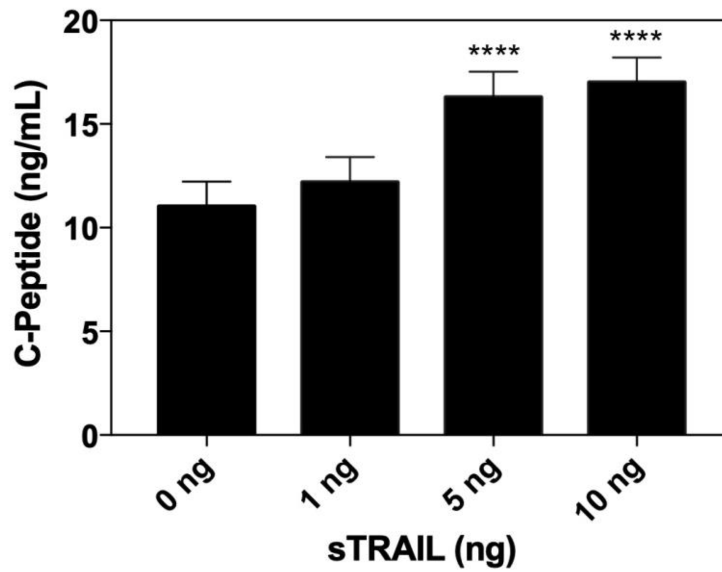

Figure 4.10. C-peptide levels released to the culture medium following sTRAIL applications.

5. DISCUSSION

Pancreatic islets are crucial endocrine microorgans for glucose homeostasis in the body, with the highly specialized cells they include, which secrete vital hormones in response to different stimuli. The essential balance in the islet functions is disrupted upon autoimmune attack or gluco- and lipotoxicity, leading to serious complications such as diabetes. In this regard, accumulating the necessary knowledge for generation of novel beta cell sources to compensate for the beta cell loss in different settings constitutes a highly significant target of scientific research, where studies with pluripotent stem cells have recently taken the center stage. Pluripotent stem cell-based studies accordingly hold great promise for regenerative medicine. Introduction of the induced pluripotent stem cells (iPSCs) was a breakthrough achieved by Yamanaka and colleagues in 2006, quickly surpassing embryonic stem cells (ESCs) with the several advantages they provide. iPSCs brought excitement to many different fields of study by combining the advantages of adult stem cells with the unique properties of ESCs, reflected in their high potential of differentiation and self-renewability along with low immunogenicity (Martin 2017).

iPSCs can be differentiated into beta cell-like insulin-producing cells (IPCs) by ectopic expression of a series of defined factors, for research or treatment purposes, as well as for identification of novel targets for antidiabetic drug development. Several studies have reported generation of IPCs from pluripotent stem cells via stepwise protocols mimicking pancreatic development (Kunisada, Tsubooka-Yamazoe et al. 2012, Maja B.K. Petersen 2018). One of the main concerns in such protocols is emphasized as establishment of efficient and stable protocols eliminating the need for complicated processes. In our study, we have referred mainly to a protocol by Kunisada et al. reporting a stepwise process for differentiation of human iPSCs into IPCs via use of specific small molecules (Figure 3.1) (Kunisada, Tsubooka-Yamazoe et al. 2012). We combined this protocol with use of a hit compound, sodium cromoglycate, reported by Kondo et al. to improve generation of pancreatic endocrine cells (Kondo, Toyoda et al. 2018). Each small molecule used in different approaches for IPC production has its own contribution to the IPC generation process, with new small molecules likely to be discovered that will improve the current

strategies further. We have tested the TNF-Related Apoptosis-Inducing Ligand (TRAIL) as a newly introduced molecule to such protocols, and observed that increasing doses of TRAIL applied in the third stage of our protocol provided enhanced insulin release by the IPCs generated, compared to the basal levels measured (Figure 4.9). Yet fully functional beta cell-like IPCs will only be confirmed after glucose-induced insulin secretion analysis, which we will be applying to the IPCs to be generated in our ongoing experimental procedures to obtain adequate number of cells for further analyses. Also, comparison of this strategy with results form a beta cell line such as NIT-1 and/or MIN6 will provide more information regarding versatility of this approach.

Although studies predominantly point out to a protective role for TRAIL in diabetes, many issues, including the mechanism of this benefit is still to be clarified. TRAIL has been referred to in various studies related to obesity and diabetes (Harith, Morris et al. 2013). It is a molecule that stood out from all other members in the TNF superfamily, shortly after its discovery with its potential to induce selective apoptosis in many transformed cells, the fact that its mRNA is widely expressed in human tissues, and that it does not exert a toxic effect upon systemic administration (Guimaraes, Gaglione et al. 2018). Studies revealing significant results such as earlier and more severe development of type 1 diabetes (T1D) in mice in the absence of TRAIL and various diabetes-related complications reported in whole body TRAIL$^{-/-}$ mice brings out the question regarding the mechanisms behind the anti-diabetic actions of TRAIL (Lamhamedi-Cherradi, Zheng et al. 2003, Di Bartolo, Chan et al. 2011). We have observed that increased doses of TRAIL enhanced insulin and c-peptide secretion by IPC cells, the mechanism of which remains to be investigated. This appears as a significant finding that may contribute to understanding of the protective actions of TRAIL in the diabetic setting.

The process of IPC production from iPSCs is divided in recent studies in a simple pattern of three main parts: differentiation into definitive endoderm, pancreatic differentiation, and further differentiation into IPCs. We also followed the same pattern in our study, and successfully generated IPCs, with completion of the critical steps of differentiation into definitive endoderm followed by generation of PDX1-positive pancreatic progenitor cells and formation of IPCs. For the initial stage of differentiation into the definitive endoderm,

we cleaned off the possibly differentiated cells around the iPSC colonies, for synchronization of the cells at the pluripotent stage, also confirmed by the Alkaline Phospatase and Tra-1-60 live stainings (Figures 4.1 and 4.2). Live staining gave us the opportunity to continue our iPSC cultures without terminating the cultures, as the stains used in live staining are cleared off from the cultures within a short time. Furthermore, as an important indication of healthy cells, our starting cells did not contain any mycoplasma (Figure 4.3). Mycoplasma analysis is considered as a means of characterization for iPSCs for testing of their suitability for use in subsequent processes. Our colonies were also dissociated into single cells for even exposure to the small molecules used in the further processes, yet single cells as well as cell groups were also evident in our cultures following this step, which did not affect confirmation of any three stages of differentiation (Figure 4.4-4.7).

We believe it is also significant that the IPCs generated in our study have the potential to be used in disease model sytems. In a significant portion of human diseases, including diabetes, it is difficult to get access directly to patient tissues and many problems occur with the culturing processes. Establishment of well-designed *in vitro* disease models is also important in its potential to decrease the need for animal models. In fact, more improved disease models may be formed with IPCs differentiated from iPSCs generated from primary cells belonging to the patients bearing known or unknown mutations related to diabetes. iPSCs have been established from individuals with diabetes-related diseases such as the Mature Diabetes of the Young (MODYs), insulin receptor mutations, Wolfram syndrome, diabetic foot ulcers, etc., which reflect the features of the various underlying complications (Kondo, Toyoda et al. 2017). Overall, IPCs generated from iPSCs are considered to have the potential to consitute very improved disease models of diabetes particularly if they are used as a part of a model system where they are organized together with other cells that have a role in the diabetic setting, such as the immune cells.

Thus we have generated IPCs from iPSCs via a combined improved approach with the TRAIL molecule tested for the first time within such a protocol pattern. Although with limitations, we believe that results of our study may be useful as a starter setting to be improved towards generation of fully functional IPCs, which may also be used in optimized disease model systems.

6. CONCLUSION and SUGGESTIONS

Induced pluripotent stem cells (iPSCs) which highly resemble embryonic stem cells (ESCs) in many aspects, can be established form adult somatic cells via direct reprogramming. Studies with iPSC technology has the potential to contribute greatly to development of novel gene and cell therapies. Generation of beta cell-like insulin-producing cells (IPCs) from iPSCs constitute a significant area of research in this regard, with the potential to provide the beta cell source necessary to compensate for the beta cell loss evident in various diabetes settings. In our study we successfully generated IPCs by introduction of spesific small molecules triggering the required differentiation steps starting from formation of the definitive endoderm, followed by pancreatic differentiation and finally generation of beta cell-like IPCs which expressed insulin, PDX1, PAX6, NeuroD1 and Islet-1.

This strategy, if also proves successful in iPSCs originating from primary human cells, will have the potential to be tested in generation of autologous IPCs as well. Autologous IPCs are correlated with many advantages over allogeneic beta cells obtained via pancreatic islet isolation from donated pancreases, via surpassing enzymatic and mechanical stress and possibly also the requirement for immunosuppression (Sanlioglu 2016)(Schuetz, Anazawa et al. 2018). The fact that the number of beta cell-like IPCs to be transplanted may be adjusted would also be advantageous. Overall, optimally efficient and safer approaches are still needed.

Our combined approach may be useful in further improvement of IPC generation protocols from iPSCs.

REFERENCES

Akinci, E., A. Banga, L. V. Greder, J. R. Dutton and J. M. Slack (2012). "Reprogramming of pancreatic exocrine cells towards a beta (beta) cell character using Pdx1, Ngn3 and MafA." Biochem J **442**(3): 539-550.

Akinci, E., A. Banga, K. Tungatt, J. Segal, D. Eberhard, J. R. Dutton and J. M. Slack (2013). "Reprogramming of various cell types to a beta-like state by Pdx1, Ngn3 and MafA." PLoS One **8**(11): e82424.

Atkinson, M. A., M. Campbell-Thompson, I. Kusmartseva and K. H. Kaestner (2020). "Organisation of the human pancreas in health and in diabetes." Diabetologia **63**(10): 1966-1973.

Avolio, F., A. Pfeifer, M. Courtney, E. Gjernes, N. Ben-Othman, A. Vieira, N. Druelle, B. Faurite and P. Collombat (2013). "From pancreas morphogenesis to beta-cell regeneration." Curr Top Dev Biol **106**: 217-238.

Chen, S., M. Borowiak, J. L. Fox, R. Maehr, K. Osafune, L. Davidow, K. Lam, L. F. Peng, S. L. Schreiber, L. L. Rubin and D. Melton (2009). "A small molecule that directs differentiation of human ESCs into the pancreatic lineage." Nat Chem Biol **5**(4): 258-265.

D'Amour, K. A., A. G. Bang, S. Eliazer, O. G. Kelly, A. D. Agulnick, N. G. Smart, M. A. Moorman, E. Kroon, M. K. Carpenter and E. E. Baetge (2006). "Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells." Nat Biotechnol **24**(11): 1392-1401.

Di Bartolo, B. A., J. Chan, M. R. Bennett, S. Cartland, S. Bao, B. E. Tuch and M. M. Kavurma (2011). "TNF-related apoptosis-inducing ligand (TRAIL) protects against diabetes and atherosclerosis in Apoe (-)/(-) mice." Diabetologia **54**(12): 3157-3167.

Di Bartolo, B. A., J. Chan, M. R. Bennett, S. Cartland, S. Bao, B. E. Tuch and M. M. Kavurma (2011). "TNF-related apoptosis-inducing ligand (TRAIL) protects against diabetes and atherosclerosis in Apoe / mice." Diabetologia **54**(12): 3157-3167.

Dirice, E., S. Kahraman, G. O. Elpek, C. Aydin, M. K. Balci, A. Omer, S. Sanlioglu and A. D. Sanlioglu (2011). "TRAIL and DcR1 expressions are differentially regulated in the pancreatic islets of STZ- versus CY-applied NOD mice." Exp Diabetes Res **2011**: 625813.

Dirice, E., A. D. Sanlioglu, S. Kahraman, S. Ozturk, M. K. Balci, A. Omer, T. S. Griffith and S. Sanlioglu (2009). "Adenovirus-mediated TRAIL gene (Ad5hTRAIL) delivery into

pancreatic islets prolongs normoglycemia in streptozotocin-induced diabetic rats." Hum Gene Ther **20**(10): 1177-1189.

Doble, B. W. and J. R. Woodgett (2003). "GSK-3: tricks of the trade for a multi-tasking kinase." J Cell Sci **116**(Pt 7): 1175-1186.

Griffith, T. S. and D. H. Lynch (1998). "TRAIL: a molecule with multiple receptors and control mechanisms." Curr Opin Immunol **10**(5): 559-563.

Guimaraes, P. P. G., S. Gaglione, T. Sewastianik, R. D. Carrasco, R. Langer and M. J. Mitchell (2018). "Nanoparticles for Immune Cytokine TRAIL-Based Cancer Therapy." ACS Nano **12**(2): 912-931.

Harith, H. H., M. J. Morris and M. M. Kavurma (2013). "On the TRAIL of obesity and diabetes." Trends Endocrinol Metab **24**(11): 578-587.

J.B Gurdon, S. D., Daniel St Johnston, (1998). "Cells' Perception of Position in a Concentration Gradient." Cell **95**(2): 159-162.

Jing Shena, Y. C., Qingwang Hanb, Yiming Mua, Weidong Hanb (2013). "Generating insulin-producing cells for diabetic therapy: Existing strategies and new development." Ageing Research Reviews **12**: 469– 478.

Kahraman, S., O. Yilmaz, H. A. Altunbas, E. Dirice and A. D. Sanlioglu (2021). "TRAIL induces proliferation in rodent pancreatic beta cells via AKT activation." J Mol Endocrinol.

Kavurma, M. M., M. Schoppet, Y. V. Bobryshev, L. M. Khachigian and M. R. Bennett (2008). "TRAIL stimulates proliferation of vascular smooth muscle cells via activation of NF-kappaB and induction of insulin-like growth factor-1 receptor." J Biol Chem **283**(12): 7754-7762.

Kondo, Y., T. Toyoda, N. Inagaki and K. Osafune (2018). "iPSC technology-based regenerative therapy for diabetes." J Diabetes Investig **9**(2): 234-243.

Kondo, Y., T. Toyoda, R. Ito, M. Funato, Y. Hosokawa, S. Matsui, T. Sudo, M. Nakamura, C. Okada, X. Zhuang, A. Watanabe, A. Ohta, N. Inagaki and K. Osafune (2017). "Identification of a small molecule that facilitates the differentiation of human iPSCs/ESCs and mouse embryonic pancreatic explants into pancreatic endocrine cells." Diabetologia **60**(8): 1454-1466.

Kroon, E., L. A. Martinson, K. Kadoya, A. G. Bang, O. G. Kelly, S. Eliazer, H. Young, M. Richardson, N. G. Smart, J. Cunningham, A. D. Agulnick, K. A. D'Amour, M. K. Carpenter and E. E. Baetge (2008). "Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo." Nat Biotechnol **26**(4): 443-452.

Kunisada, Y., N. Tsubooka-Yamazoe, M. Shoji and M. Hosoya (2012). "Small molecules induce efficient differentiation into insulin-producing cells from human induced pluripotent stem cells." Stem Cell Res **8**(2): 274-284.

Kunisada, Y., N. Tsubooka-Yamazoe, M. Shoji and M. Hosoya (2012). "Small molecules induce efficient differentiation into insulin-producing cells from human induced pluripotent stem cells." Stem Cell Research **8**(2): 274-284.

Lamhamedi-Cherradi, S. E., S. Zheng, R. M. Tisch and Y. H. Chen (2003). "Critical roles of tumor necrosis factor-related apoptosis-inducing ligand in type 1 diabetes." Diabetes **52**(9): 2274-2278.

LeBlanc, H. N. and A. Ashkenazi (2003). "Apo2L/TRAIL and its death and decoy receptors." Cell Death Differ **10**(1): 66-75.

M, B. (2010). "The new generation of beta-cells: replication, stem cell differentiation, and the role of small molecules." The review of diabetic studies **7(2)**: 93–104.

Maja B.K. Petersen, C. A. C. G., Yung Hae Kim, Anne Grapin-Botton (2018). Recapitulating and Deciphering Human Pancreas Development From Human Pluripotent Stem Cells in a Dish. Current Topics in Developmental Biology. A. H. Brivanlou, Academic Press. **129:** 143-190.

Martin, U. (2017). "Therapeutic Application of Pluripotent Stem Cells: Challenges and Risks." Frontiers in Medicine **4**(229).

McLean, A. B., K. A. D'Amour, K. L. Jones, M. Krishnamoorthy, M. J. Kulik, D. M. Reynolds, A. M. Sheppard, H. Liu, Y. Xu, E. E. Baetge and S. Dalton (2007). "Activin a efficiently specifies definitive endoderm from human embryonic stem cells only when phosphatidylinositol 3-kinase signaling is suppressed." Stem Cells **25**(1): 29-38.

Mi, Q. S., D. Ly, S. E. Lamhamedi-Cherradi, K. V. Salojin, L. Zhou, M. Grattan, C. Meagher, P. Zucker, Y. H. Chen, J. Nagle, D. Taub and T. L. Delovitch (2003). "Blockade

of tumor necrosis factor-related apoptosis-inducing ligand exacerbates type 1 diabetes in NOD mice." Diabetes **52**(8): 1967-1975.

Murtaugh, L. C. (2008). "The what, where, when and how of Wnt/beta-catenin signaling in pancreas development." Organogenesis **4**(2): 81-86.

O'Dowd, J. F. and C. J. Stocker (2013). "Endocrine pancreatic development: impact of obesity and diet." Front Physiol **4**: 170.

Pagliuca, F. W., J. R. Millman, M. Gürtler, M. Segel, A. Van Dervort, J. H. Ryu, Q. P. Peterson, D. Greiner and D. A. Melton (2014). "Generation of functional human pancreatic β cells in vitro." Cell **159**(2): 428-439.

Pitti, R. M., S. A. Marsters, S. Ruppert, C. J. Donahue, A. Moore and A. Ashkenazi (1996). "Induction of apoptosis by Apo-2 ligand, a new member of the tumor necrosis factor cytokine family." J Biol Chem **271**(22): 12687-12690.

Rezania, A., J. E. Bruin, P. Arora, A. Rubin, I. Batushansky, A. Asadi, S. O'Dwyer, N. Quiskamp, M. Mojibian, T. Albrecht, Y. H. Yang, J. D. Johnson and T. J. Kieffer (2014). "Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells." Nat Biotechnol **32**(11): 1121-1133.

Sakano, D., N. Shiraki, K. Kikawa, T. Yamazoe, M. Kataoka, K. Umeda, K. Araki, D. Mao, S. Matsumoto, N. Nakagata, O. Andersson, D. Stainier, F. Endo, K. Kume, M. Uesugi and S. Kume (2014). "VMAT2 identified as a regulator of late-stage beta-cell differentiation." Nat Chem Biol **10**(2): 141-148.

Sanlioglu, A. D. (2016). Pankreatik adacık reddini önleyecek yöntemler. 52. Ulusal Diyabet Kongresi ve Diyabet Yıllığı. M. T. Yılmaz. Antalya, Türkiye Diyabet Vakfı ve Türk Diyabet Cemiyeti.

Schuetz, C., T. Anazawa, S. E. Cross, L. Labriola, R. P. H. Meier, R. R. Redfield, 3rd, H. Scholz, P. G. Stock, N. W. Zammit and I. Y. Y. I. Committee (2018). "beta Cell Replacement Therapy: The Next 10 Years." Transplantation **102**(2): 215-229.

Schulz, T. C., H. Y. Young, A. D. Agulnick, M. J. Babin, E. E. Baetge, A. G. Bang, A. Bhoumik, I. Cepa, R. M. Cesario, C. Haakmeester, K. Kadoya, J. R. Kelly, J. Kerr, L. A. Martinson, A. B. McLean, M. A. Moorman, J. K. Payne, M. Richardson, K. G. Ross, E. S. Sherrer, X. Song, A. Z. Wilson, E. P. Brandon, C. E. Green, E. J. Kroon, O. G. Kelly,

K. A. D'Amour and A. J. Robins (2012). "A scalable system for production of functional pancreatic progenitors from human embryonic stem cells." PLoS One **7**(5): e37004.

Secchiero, P., A. Gonelli, E. Carnevale, F. Corallini, C. Rizzardi, S. Zacchigna, M. Melato and G. Zauli (2004). "Evidence for a proangiogenic activity of TNF-related apoptosis-inducing ligand." Neoplasia **6**(4): 364-373.

Secchiero, P., A. Gonelli, E. Carnevale, D. Milani, A. Pandolfi, D. Zella and G. Zauli (2003). "TRAIL promotes the survival and proliferation of primary human vascular endothelial cells by activating the Akt and ERK pathways." Circulation **107**(17): 2250-2256.

Secchiero, P., C. Zerbinati, E. Rimondi, F. Corallini, D. Milani, V. Grill, G. Forti, S. Capitani and G. Zauli (2004). "TRAIL promotes the survival, migration and proliferation of vascular smooth muscle cells." Cell Mol Life Sci **61**(15): 1965-1974.

Shapiro, A. M., J. R. Lakey, E. A. Ryan, G. S. Korbutt, E. Toth, G. L. Warnock, N. M. Kneteman and R. V. Rajotte (2000). "Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen." N Engl J Med **343**(4): 230-238.

Sheridan, J. P., S. A. Marsters, R. M. Pitti, A. Gurney, M. Skubatch, D. Baldwin, L. Ramakrishnan, C. L. Gray, K. Baker, W. I. Wood, A. D. Goddard, P. Godowski and A. Ashkenazi (1997). "Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors." Science **277**(5327): 818-821.

Song, K., Y. Chen, R. Goke, A. Wilmen, C. Seidel, A. Goke and B. Hilliard (2000). "Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is an inhibitor of autoimmune inflammation and cell cycle progression." J Exp Med **191**(7): 1095-1104.

Thomsen, S. K. and A. L. Gloyn (2014). "The pancreatic beta cell: recent insights from human genetics." Trends Endocrinol Metab **25**(8): 425-434.

Wiley, S. R., K. Schooley, P. J. Smolak, W. S. Din, C. P. Huang, J. K. Nicholl, G. R. Sutherland, T. D. Smith, C. Rauch, C. A. Smith and et al. (1995). "Identification and characterization of a new member of the TNF family that induces apoptosis." Immunity **3**(6): 673-682.

Yang, Y., E. Akinci, J. R. Dutton, A. Banga and J. M. Slack (2013). "Stage specific reprogramming of mouse embryo liver cells to a beta cell-like phenotype." Mech Dev **130**(11-12): 602-612.

RESUME

Personal Information

Name	Büşra	Race	Turkish
Surname	ÇETİN	Tel no	
Date of Birth		e-mail	

Educational Information

Institution		Graduation Date
High School	Alanya Hüseyin Girenes Fen Lisesi	2013
University	Üsküdar University, Department of Molecular Biology and Genetics	2018

Work Experience

Mission	Organisation	Duration
Intern	Marmara University, GEMHAM	2016
Intern	University of Liverpool	2017

Foreign Languages	Name of the Exam	Score
English	TOEFL	100
English	YÖKDİL	91

Project Experience

Project name	Supporting Institution	Duration
Potential therapeutic efficacy of lentivirus-mediated Vasoactive Intestinal Peptide gene delivery in a retinal degenerative disease model.	TÜBİTAK	**2021-2022 (Scholarship Recipient)**
Generation of induced pluripotent stem cells from huma fibroblasts via mini plasmids and nonintegrated lentiviral vectors, differentiation into pancreatic beta cell-like insulin-producing cells, and strengthening against autoimmunity	TÜBİTAK	**2018-2021 (Actively involved as a researcher)**

Poster/Oral Presentations

- Seker G., Cetin B., Sanlioglu AD. Successful generation of induced pluripotent stem cells from HEK-293T cells via sodium butyrate and codon-optimized mini intronic plasmids encoding reprogramming factors. ESGCT Collaborative Virtual Congress, 2021, October 19-22, Online Congress (Poster presentation).
- Seker G., Cetin B., Sanlioglu AD. Yeniden programlama faktörlerini kodlayan kodon-optimize mini intronik plazmidler aracılığıyla insan embriyonik böbrek hücrelerinden uyarılmış pluripotent kök hücre üretimi. Medical Biology and Genetics Congress, 2021, October 28-31, Online Congress (Oral presentation).
- Yilmaz O., Seker G., Cetin B., Akinci E., Altunbas HA., Sanlioglu AD. Codon-optimized mini intronic plasmids encoding reprogramming factors combined with ascorbic acid and valproic acid successfully generates induced pluripotent stem cells from HEK293T cells. ISSCR (International Society for Stem Cell Research), 2021, July 21-26, Online Congress (Poster presentation).
- Eken Başak Funda, Yüksel İpek, Çetin Büşra, Can Rümeysa, Sercan Canan, Ulucan Korkut. Türk Erkek Futbolcularda Anjiyotensin Dönüştürücü Enzim (ACE) Polimorfizmi. 23rd National Biology Congress, September 5-9, 2016, Gaziantep (Poster presentation).
- Sercan Canan, Arslan Kadir Sinan, Eken Başak Funda, Çetin Büşra, Ulucan Korkut. Genç Basketbolcularda Anjiotensin Dönüştürücü Enzim (ACE I/D) Gen Polimorfizmlerinin Belirlenmesi 23rd National Biology Congress, September 5-9, 2016, Gaziantep (Poster presentation).
- Sercan Canan, Çetin Büşra, Yüksel İpek, Can Rümeysa, Ulucan Korkut. Türk Futbolcularda Alfa- Aktinin-3 (ACTN3 R577X) Gen Polimorfizmlerinin Belirlenmesi. 23rd National Biology Congress, September 5-9, 2016, Gaziantep (Poster presentation).
- Sercan Canan, Yüksel İpek, Eken Başak Funda, Çetin Büşra, Kulaksız Hamza, Kapıcı Sezgin, Ulucan Korkut. Türk Futbolcu ve Basketbolcularda Alfa- Aktinin-3 (ACTN3 R577X) Gen Polimorfizmlerinin Belirlenmesi. 23rd National Biology Congress, September 5-9, 2016, Gaziantep (Poster presentation).