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Differentiation and characterization of MafA-GFP reporter iPS cells into beta cell lineage

Master's thesis in Biotechnology

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To Ehsan

"Loss is nothing else but change, and change is nature's delight."

– Marcus Aurelius –

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Abstract

Diabetes is a chronic disease characterized by increased blood sugar because of dysfunctional glucose homeostasis by the beta cells in the pancreas. Functional beta cells can be generated by primary cells or stem cells. Primary cells are isolated directly from tissues (blood or bone marrow) and retain the morphological and functional characteristics of their tissue of origin. However, they have a finite period of cell culture, a limited potential for self-renewal and differentiation and are more sensitive than stem cells, they often require additional nutrients and growth factors. In contrast, stem cells allow to investigate basic biological processes, manipulate cellular functions, establish new methods, or perform preliminary screenings. Considering the limitations of the primary cells, stem cells can be an alternative source. Stem cells are at the forefront of research in cell therapy, drug discovery, and disease-modelling. Generation of pancreatic beta cells is possible by differentiating human induced pluripotent stem cells (hiPS cells), although in stem cell research, efficiency of mature beta cells generation is a key problem (optimal efficiency at 80%, current efficiency at 20%). This project first aims to improve efficiency of pancreatic beta cell generation from hiPS cells by performing six differentiation processes following the same protocol and later screening for beta cell production at different stages of the differentiation process. It uses a wild type and two MafA-GFP reporter iPS cell lines, where MafA as a critical beta-cell-specific transcription factor is tagged with GFP by using CRISPR/Cas9 technology. Second, flow cytometry and immunofluorescence analysis are used at different stages of the differentiation process to characterize the differentiated cells to confirm faithful expression of MafA. Expression of GFP corresponds to expression of MafA in adult beta cells, since MafA is only present in mature beta cells, which this confirms complete differentiation and maturation of iPS cells into beta cells. The results obtained from the differentiation processes showed low level of reproducibility, although this project was successful in showing the GFP and MafA expression of MafA reporter lines. The MafA reporter lines successfully expressed GFP signals (~11% efficiency) at stage 7 of beta cell differentiation.

Keywords: induced pluripotent stem cells, MafA, beta cells differentiation, pancreas, FACS.

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Contents

Abbreviations

°C	°celsius
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
AA	Activin A
Alk5i II	activin receptor-like kinase 5 inhibitor
ATP	Adenosine Triphosphate
BSA	bovine serum albumin
BTC	betacellulin
DE	definitive endoderm
DM	diabetes mellitus
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic acid
ESCs	embryonic stem cells
GFP	green fluorescent protein
GSIS	Glucose-Stimulated Insulin Secretion
ELISA	enzyme-linked immunosorbent assay
FACS	Fluorescent Activated Cell Sorting
Fbx15	F-box protein 15
g	gram
h	hour
HHEX	hematopoietically expressed homeobox
hiPSCs	human induced pluripotent stem cells
HNF1B	hepatocyte nuclear factor 1B
HNF4A	hepatocyte nuclear factor 4 alpha
HNF6	hepatocyte nuclear factor 6 known as ONECUT1
IBC	Immature beta cell
ICC	Immunocytochemistry
ICM	inner cell mass
iPSCs	induced pluripotent stem cells
ITS-X	Insulin-Transferrin-Selenium-Ethanolamine
KGF	Keratinocyte growth factor
KLF4	Krüppel-like factor 4
MafA	muscululo aponeurotic fibrosarcoma oncogene homolog A
MBC	Mature beta cell
min	minute(s)
NGN3	neurogenin 3
NGS	Next Generation Sequencing
NKX2.2	NK2 homeobox 2
NKX6.1	homeobox protein NK–6 homolog A
Oct3/4	octamer-binding transcription factor 3/4
ONECUT1	one cut homeobox 1
PBS (-/-)	phosphate-buffered saline without bivalent ions
PDX1	pancreatic-duodenal homeobox factor 1
PDBU	Phorbol 12,13-dibutyrate
P/S	Penicilin/Streptomicin
PAX6	paired box 6
PE	Pancreatic endoderm

PEP	Pancreatic endoderm precursors
PF	Posterior foregut
PFA	paraformaldehyde
PGT	Primitive gut tube
RA	retinoic acid
RCF	Relative Centrifugal Force
ROCK inhibitor	Rho-associated protein kinase inhibitor
RT	room temperature
Sant-1	Sonic hedgehog Agonist-1
Sox2	SRY (sex determining region Y)-box 2
T1DM or T2DM	type 1 or type 2 Diabetes mellitus
TGF-β	transforming growth factor-beta
XXI	γ-Secretase inhibitor

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1. Introduction

1.1. Overview

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia. The metabolic abnormalities of diabetes are based on the deficient insulin action resulting from either inadequate insulin secretion, decreased response from tissues to insulin, or both. Factors involved in the development of diabetes can have either genetic or non-genetic origin [1]. The epidemic level of DM is increasing to a degree to become prominent, hence it is necessary to screen for and diagnose it while it is still in its early stage.

Diabetes development is associated with many pathogenic processes ranging from pancreatic beta cells autoimmune destruction to abnormal insulin resistance based on deficient insulin action on target tissues. The two most common etiopathogenetic classifications of DM are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM) [2, 3].

In T1DM, deficient insulin production results from an autoimmune pathologic destruction of beta cells through T-cell mediated inflammatory response and humoral B cell response in pancreatic islets. It comprises 5-10% of DM patients, predominantly children and adolescents [2]. The rate of beta cell destruction is quick in infants and children and slow in adults. To maintain normoglycemia, patients require insulin therapy, and in individuals with extensive beta cell destruction, it is a vital requirement for survival [1, 2, 3].

In T2DM, which is the most prevalent one, the condition is characterized by insulin resistance, although it may result from a combination of insulin action resistance and inadequate insulin secretion response. The insulin concentration is increased in the plasma, although it is not enough to control the blood glucose level, and during time, beta cell dysfunction and insulin deficiency follows. [2, 3].

Treatment strategies for DM are implicated by its classification, though it is not always an easy task to classify patients into one class [1]. The duration of a disease process can affect/change the degree of hyperglycemia over time. In individuals, the disease process may be present but is not too advanced to lead to hyperglycemia [2, 3]. Depending on the type and the duration of diabetes, the severity of symptoms manifested in patients may differ, as in either being asymptomatic or showing hyperglycemia. However, the severity of symptoms is higher in children than in adults [1].

Regarding the severity of DM issue and the effects it can impose on numerous individuals, its prevention has become an urgent medical concern, and further biomedical research is necessary to find an effective and lasting treatment. Current approaches including islet cell transplantation and pancreas transplantation are clinically approved [4]. One therapeutic approach is stem cell

therapy, where hiPS cells can provide cells for DM research as well as treatment. This therapy promises a novel treatment modality for advanced DM, and avoids concerns linked to daily injections of insulin. It is expected to be producing, storing, and supplying insulin to keep glucose homeostasis, by aiming to create functional glucose-sensing, insulin-producing beta cells.

With only 21.72% of patients showing adverse effects, this approach seems to be a safer form of therapy, in comparison to whole organ or islet transplantation [4, 5, 6, 7]. Currently, there is a possibility to obtain skin cells from T1DM patient and reprogram them into iPS cells. Researchers has created many iPS cell lines from diabetic patients with various genetic background. However, the ending stage of transforming iPS cells into beta cells is not complete for humans yet [5].

Against this background, AstraZeneca (AZ) as a science-led biopharmaceutical company carries out research to investigate the role of stem cells in drug discovery. In 2015, AZ began a five-year collaboration with the Harvard Stem Cell Institute (HSCI) to gain insight into declining pancreatic beta cell function and resulting diabetes, through engineered stem cell research. Although the engineered stem cells will not be used as a therapeutic candidate under the AZ/HSCI agreement, a better understanding of beta cell function and decline will aid AZ in discovering and developing drugs that can better target some of the underlying causes of diabetes [8].

As discussed earlier, insulin deficiency in T1DM is caused by autoimmune destruction of beta cells. Beta cells are found in islets of Langerhans in pancreas and their role is to produce, store, and release insulin for blood glucose regulation [9]. The promising stem cell therapy utilizes human pluripotent stem cells (hPSCs). The advantage is when these cells can be isolated and cultured for many rounds of proliferation while sustaining their pluripotency. However, the current struggle is to direct reliable differentiation of stem cells into specific cell type. Many obstacles need to be overcome in order to be able to use stem cells to cure human ailments such as better understanding of the common feature of unlimited cell division in order to avoid cancer formation, mastering the ability to acquire large number of the right cells at the right stage of differentiation, as well as development of specific protocols for enhanced production [10]. This project focuses on investigating the induction efficiency of differentiated cells and characterizing the differentiated cells.

The morphology and gene expression in induced pluripotent stem (iPS) cells are similar to embryonic stem (ES) cells, and it is enough to express transcription factors especially in human fibroblasts to produce iPS cells [11]. The critical transcription factor in this project is MafA which is a beta-cell-specific member of the Maf family of transcription factors responsible for activation of insulin gene expression as well as regulation of the glucose-stimulated insulin secretion (GSIS) in vivo. It binds to the conserved C1/RIPE3b element of the insulin promoter in the insulin gene, with critical role in beta-cell-specific and glucose-regulated expression of insulin [12, 13, 14]. It is only expressed in adult beta cells and is one of the markers for maturity and age [15].

In this project, CRIPSR/Cas9 technology was used to tag MafA gene with GFP to create MafA-GFP reporter iPS cell lines. By using the CRISPR-based precise tagging, GFP as a marker protein

was tagged next to MafA gene in order to be able to follow expression and hence differentiation in live cells which can continue to be differentiated and used in laboratory testing. The two MafA-GFP reporter iPS cell lines used in this project are MafA2.6 and MafA7.1. In MafA2.6 reporter line, GFP is inserted on both alleles of MafA gene which can yield a higher GFP signal while inMafA7.1 reporter line, GFP is inserted on only one allele of MafA gene which leaves the other MafA allele intact and unmodified, but it can yield lower signal than MafA2.6. This makes each report line unique, powerful, and advantageous in its own way. The reason to study these 2 reporters and comparing them to wild type is to examine the lifetime and persistency of expression during cell growth and differentiation.

1.2. Aims and approach

The first aim of this project is to differentiate two human MafA-GFP reporter iPS cell lines (MafA2.6 and MafA7.1) and the wild type (AD3-01) in order to compare and investigate the differentiation efficiency in these three iPS cell lines as well as to confirm faithful expression of MafA which this proves that the iPS cells are differentiated fully to mature and functional beta cells. Both MafA2.6 and MafA7.1 reporter iPS cell lines were generated from wild type AD3-01. MafA2.6 was identified as a double copy clone (GFP was inserted on both gene copies) and MafA7.1 as a single copy clone (GFP was inserted only on one gene copy). There is an interest to compare single copy vs double copy clones to study whether there is a risk or expectation that one will work better than the other or not. All three iPS cell lines will be differentiated into beta cell lineage in six separate differentiation experiments, and all six experiments follow the same structure and differentiation protocol. Due to cell culture handling (inevitable human error), biological complexity, and sensitivity of stem cells, the results from one experiment may vary from another, therefore the results and induction efficiency gathered from the experiment will be compared and studied. I want to see which cell line is the most robust and the most successful to yield the highest number of mature and functional beta cells.

The second aim of this project is to characterize the differentiated beta cells using defined methods including *flow cytometry* for cell analysis and measuring expression of the target proteins within mixed population of cells, *FACS analysis* for cell sorting and enriching for beta cells, *ICC analysis and imaging techniques* to evaluate whether or not the cells express the protein in question, *functional assays such as glucose-stimulate insulin secretion (GSIS) analysis* to measure insulin secretion from isolated human islets and *molecular next generation sequencing (NGS)* for expression profiling by sequencing RNA of the differentiated beta cells and later compare with human primary beta cells for quality assurance purpose (unfortunately the results from GSIS and NGS analyses were not able to be analyzed and performed respectively, due to COVID-19 pandemic situation). Later, depending on the results obtained, the differentiation protocol would possibly be optimized after thorough studying of all obtained data and analyzing the characterization results.

1.3. Specification of issues under investigation

The following concepts have been studied in this project.

1. Differentiation of iPS cells into pancreatic mature beta cells by following an 8-week differentiation protocol.
2. Characterization of differentiated beta cells through flow cytometry, FACS analysis and ICC analysis at 2 different timepoints (stage 4 and 7) of differentiation process and later evaluating the expression level of target proteins.
3. Functional analysis of beta cells through GSIS analysis by measuring insulin secretory output of cells which this determines the functionality level of mature beta cells (unfortunately the results from GSIS analysis were not analyzed & completed and hence not included in this thesis report as a result of COVID-19 pandemic).
4. Optimization of differentiation protocol mainly based on the data and results gathered (this step also was not approachable, since more data and experimental results are needed for an optimization).

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2. Background

2.1. Pancreas & Langerhans islets

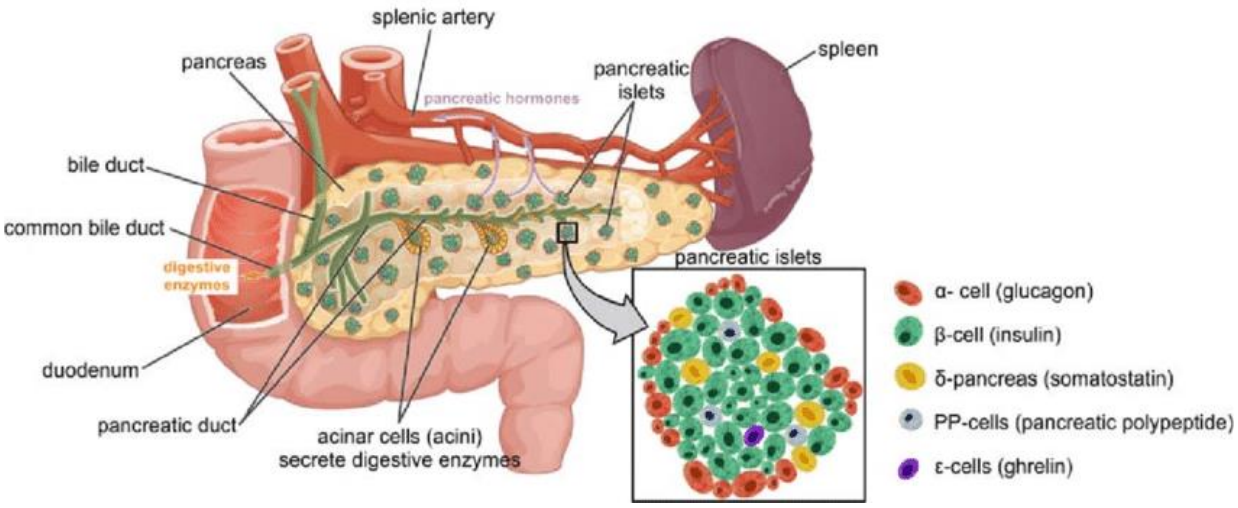


Figure 2.1. Representative overview of the pancreas, its components, localization and anatomy [16]**.**

The pancreas, as a retroperitoneal gland, has both endocrine and exocrine functions, for instance glucose homeostasis and nutrient digestion, respectively. Due to pathologic conditions or aging, the size of pancreas may change, but in adult humans its approximate length is 15cm and weight is 80g [17]. Glucose homeostasis is maintained by the islets of Langerhans, the functional units of endocrine pancreas, scattered uniformly in the parenchyma of the pancreas while adopting an overall spherical structure. In a healthy adult, there are on average 3.2 million islets evenly distributed and accountable for 1-2% of the pancreas's mass with a mean diameter of 108.92μm [18]. The size of the islets is between 50 and 300 μm, containing hundreds to thousands endocrine cells [17].

The five main kinds of cells in islets of Langerhans are alpha, beta, delta, gamma, and epsilon. In humans, pancreatic beta cells are the most common (~55% of islet cells), which are responsible for insulin and amylin secretion. The next most abundant types are alpha cells (~35%) and delta cells (~10%) secreting glucagon and somatostatin, respectively. The least common types are gamma cells or PP-cells (~5%) and epsilon cells (~1%) responsible for secretion of polypeptide and human islet cells respectively [18]. Between and within species, pancreatic islets may present different cellular compositions and distributions. The most studied cell type of the islets is the beta cell. It is a cell that creates and secretes insulin for the purpose of glucose regulation. In humans, most of the beta cells exist along the islet blood vessels [19]. Through a certain staining

process, light microscopy can identify different cell types in the islets of the human pancreas. Insulin can be demonstrated in the beta cells through fluorescent antibody technique, discussed later.

2.2. Diabetes

Diabetes mellitus, a disorder characterized by increased blood sugar because of dysfunctional glucose homeostasis, is known to be one of the most common chronic disorders in the western world. Its prevalence varies around the world, and with dramatic increase in its prevalence, estimations show that by the year 2030 it will increase more than 50% from 19 million to 55 million people. In the United States, the cost of diabetes will increase from $490.2 billion per year in 2020 to $622.3 billion per year in 2030 [20].

Diabetes mellitus as a genetically heterogenous group of disorders that share impaired glucose tolerance in common, is not a single disease. Certain genetic patterns, and etiologic as well as pathophysiologic mechanisms are involved. Several prolonged complications can result from diabetes chronic elevated glucose levels such as peripheral vascular disease, coronary heart disease, and neuropathy [21]. Hyperglycemic coma may happen because of built-up ketone bodies, which are poisonous and created from metabolism of fat when glucose metabolism is non-existent. Unmetabolized glucose accumulates in the blood when insulin level is insufficient. Thus, homeostasis starts acting, by diluting blood concentration, using osmosis to draw water from the body cells to excrete with excessive glucose. This will consequently result in dehydration as soon as it surpasses glucose renal threshold [22]. Excessive thirst, unexplained itch, fatigue, unexplained loss of weight, recurrent infections and excessive urination are some of diabetes characteristic–related symptoms, which they reoccur in case of inadequate control of the condition by treatment.

2.3. iPS cells

Embryonic stem cells (ESCs) are derived from inner cell mass (ICM) of blastocyst with extensive proliferation capacity while keeping pluripotency. Tissue rejection and using human embryos are the issues with ESC transplantation which can be avoided by reprogramming the nuclei of differentiated cells to pluripotent cells. Several strategies have been reported to generate induced pluripotent stem cells [23]. One method is to reprogram by defined factors; where induced pluripotent stem (iPS) cells are generated by mouse embryonic or adult fibroblasts when four transcription factors Oct3/4, Sox2, c-Myc and Klf4 (OSMK) are introduced through retrovirus mediation, and the expression for Fbx15, a target of Sox2 & Oct3/4, is selected [24]. The iPS cells are like ESCs in terms of teratoma formation, morphology, and proliferation. Thus, full reprogramming can be obtained by expression of four factors and using an appropriate selection procedure.

Disease-specific and patient-specific iPSCs can be generated for disease-modelling, drug screening & development, cytotoxicity studies, and regenerative medicine (cell replacement therapy). Modelling of human diseases aims to discover the molecular mechanism of diseases and develop drugs ultimately for their treatment. Cells obtained from patients afflicted with diseases can

generate genetically matched iPSC lines which can be differentiated *in vitro* into the affected cell types. iPSCs can be used as disease models owing to their extensive proliferation in culture and differentiation into all cell types in the human body. Drug screening and discovery can be facilitated by iPSCs. They may also test the toxicity levels of therapeutic drugs. Human disease phenotype cannot be reflected completely in animal models and its full recapitulation can be prevented due to various drug toxicity responses. iPSCs can be used in regenerative medicine to induce endogenous regenerative repair or for cellular transplantation replacing injured tissues. Recent gene editing technologies offer a chance to introduce genetic changes into iPSCs, allow to repair disease-causing gene mutations in patient-derived iPSCs, and finally generate healthy iPSCs for iPSC-based cell therapy purposes [25].

Prior to the commencement of the project, human iPSC lines were generated with a GFP reporter inserted in the endogenous MafA locus using CRISPR/Cas9 technology. MafA, a key marker for mature beta cells, is a transcription factor that binds to the promoter in an insulin gene to regulate insulin transcription in response to serum glucose levels. Genetically modified iPSCs designed to express a fluorescent reporter gene under the control of cell-type specific promoters facilitate identification and isolation of relevant cell types in otherwise heterogenous cultures. Once differentiated into beta cell lineage, these CRISPR modified iPSC lines will express GFP in mature insulin producing functional beta cells.

2.4. Proteins in question

Here I describe the roles and regulation of MafA, PDX1 and NKX6.1 in islet beta cells. **MafA** is a beta-cell-specific member of the Maf family of transcription factors. The role of Maf family proteins is regulation of cell differentiation & gene expression in different tissues. MafA binds to *C1/RIPE3b*, which is an element of the insulin promotor with critical role in beta-cell-specific and glucose-regulated expression of insulin [13].

PDX1, as a beta-cell-enriched transcription factor, has two critical roles: activation of the insulin gene promotor establishing beta-cell-specific insulin expression, and regulation of beta cell differentiation. MafA and PDX1 are weak transactivators of the insulin promoter when expressed alone. However, their co-expression leads to synergetic and strong activation of the insulin promoter. They are involved in both insulin gene transcription and proliferation & survival of beta cells in pancreas [13].

NKX6.1 as a potent transcription regulator, is generally expressed in pancreatic epithelium during development and it is later required restrictively in the beta cells to express genes critical for beta cell function and identity. It is also critical for specification of pancreatic endoderm as well as beta cells during hPSC differentiation to the pancreatic lineage. hESC-derived pancreatic endoderm expressing NKX6.1 and PDX1 are capable to differentiate into functional beta cells and other pancreas lineages [26].

In short, PDX1 as the earliest marker for pancreatic differentiation is necessary for beta cell maturation & duodenal differentiation. However, NKX6.1 is essential for maintaining the

functional state of mature pancreatic beta cells. MafA, as a key regulator of genes implicated in maintaining beta cell function, regulates both NKX6.1 and PDX1 [26, 27].

C-peptide is a marker for detecting endogenous insulin production. In pancreatic beta cells, proinsulin as a precursor is cleaved into equal amounts of c-peptide and insulin molecules. C-peptide is widely used to assess pancreatic beta cell function. It can reflect the expression of beta cells more accurately than insulin since its degradation rate is slower than that of insulin. C-peptide is shown to correlate with diabetes type, duration of disease, and age of diagnosis [28].

On the other hand, **glucagon** was also stained since it is a polypeptide processed from proglucagon in pancreatic alpha cells. It is studied that diabetic patients show higher glucagon levels, and the combination of hyperglucagonemia and hypoinsulinemia establishes a fundamental pathophysiological basis for diabetic hyperglycemia [29]. It often happens that cultures are heterogenous and contain beta-like cells, polyhormonal cells expressing several hormones and other undefined cell types [26]. Glucagon staining approves if the cells differentiated into more than one cell type, as in alpha cells here.

In the MafA-GFP reporter iPS cell lines, GFP acts as a marker protein. It is CRISPR-based precise tagging where I know the exact location, which is next to the MafA gene. The differentiation of these lines together with wild type line was compared and no difference was found, it can be said that it does not affect the differentiation propensity of the cells. The single allele GFP insertion in MafA7.1 reporter and double allele GFP insertion in MafA2.6 reporter makes each reporter unique. Single GFP insertion is good because the other MafA allele is intact and unmodified, but signal is low, while in double GFP insertion it is the other way around.

2.5. Differentiation protocol and stages

Here I explain the main stages in differentiation and their duration and discuss the importance of stages 4 and 7, and later give reasons to why I change the cell cultures from adherent (2D) to suspension (3D) and why I stain for certain protein markers. In **Stage 0**, the iPS cells are seeded in culture plates and are adjusting in the new environment for 48 h before differentiation starts. See Appendix F and K for the formulation and role of stage-specific media and and see Appendix K for the role and definition of the signaling proteins used during differentiation process. Table 2.1 gives an overview of each stage of the differentiation process.

Table 2.1. Differentiation of iPS cells to hormone-expressing pancreatic endocrine cells. Through these stages, iPS cells can obtain pancreatic endocrine phenotype and ability of glucose responsive insulin secretion.

Stage 1	Differentiation to definitive endoderm
Stage 2	Establishment of primitive gut endoderm
Stage 3	Patterning of posterior gut
Stage 4	Specification and maturation of pancreatic endoderm and pancreatic endocrine precursors
Stage 5	
Stage 6	Further maturation
Stage 7	Harvest of mature beta cells

Stage 1, known as Definitive Endoderm (DE) with 3-day duration, is a prerequisite for efficient differentiation to mature endoderm derivatives. DE gives rise to the epithelial lining of the respiratory and digestive tracts and to the thyroid, thymus, lungs, liver, and pancreas. It is formed during gastrulation, in which pluripotent cells are allocated to the three principal germ layers—ectoderm, mesoderm and definitive endoderm. Cultures with up to 80% DE cells are produced where stem cells are differentiated in the presence of AA (Activin A), see Appendix K and low serum [30].

Stage 2, known as Primitive Gut Tube (PGT) with 3-day duration, is the stage where primitive gut tube/foregut is eventually formed from anterior-posterior axis of DE. The DE-derived PGT induces the pharynx, esophagus, stomach, duodenum, small and large intestine along the anterior-posterior axis as well as associated organs, including pancreas, lung, thyroid, thymus, parathyroid, and liver [31].

Stage 3, known as Posterior Foregut (PF) with 2-day duration, is the stage where pancreas, liver and duodenum originate from posterior portion of foregut. The PF starts expressing PDX1 and other developmental markers such as HHEX, HNF6 and HNF4A. In an efficient differentiation process both stage 2 and stage 3 convert DE derived from stem cells into pancreatic lineage endocrine cells expressing pancreatic hormones [31].

In short, in this stepwise differentiation protocol, these three stages are former steps in generating pancreatic beta cells. First, iPS cells are differentiated into definitive endoderm with AA. Removal of AA induces the progression from definitive endoderm to primitive gut endoderm which expresses primitive gut tube markers HNF1B and HNF4A. Next, cells were treated with KGF (keratinocyte growth factor) which significantly promotes primitive gut tube differentiation and further beta cell differentiation. Then the PGT cells were treated with LDN, KGF, SANT, PDBU and RA (retinoic acid), see Appendix K.

The differentiated iPS cells from these stages express PDX1, NKX6.1 alongside with HNF6, NKX2.2, NGN3, and PAX6. This clearly shows why I am interested in staining for PDX1 and NKX6.1 at this stage. The cells also have functional ATP-sensitive potassium channel and voltage-dependent calcium channels, which are the key components of insulin secretory machineries. However, glucose- responsive insulin secretion is absent; thus, they need to be more matured [31].

The reason that I change from adherent to suspension culture is that, adherent cultures (2D) are more efficient for differentiation of iPS cells into pancreatic endocrine progenitor cells, while suspension cultures (3D) are more effective for maturation of beta cell progenitors into functional ones. Hence, a combination of 2D and 3D culture may lead to derive more functional pancreatic beta cells [31].

Stage 4, known as Pancreatic Endoderm (PE) with 5-day duration, and **Stage 5**, known as Pancreatic Endoderm Precursors (PEP) with 7-day duration, together are the stages during which cells become specified and mature, the pancreatic endoderm is formed and endocrine is induced with growth factor treatment, see Appendix K and Figure 3.1 [31].

Stage 6, known as Immature Beta Cells (IBC) with 21-day duration, is the stage where islet-like clusters and aggregates mature only by stage-specific medium replenished every 48 h, no growth factor is added here.

Stage 7, known as Mature Beta Cells (MBC) is the final stage where cells are ready to be harvested and processed for characterization analysis.

A fully differentiated and developed beta cell can secrete c-peptide in response to glucose stimulation. To test the successfulness of this protocol at generating cell populations that express the pancreatic hormone insulin, I want to measure the degree of cellular heterogeneity. This clearly shows why I am interested in staining for c-peptide, and glucagon (produced by alpha cells).

A previously developed differentiation protocol in 2005 suggested a simple three-step experimental approach based on the combination induction by AA, all-trans RA, and other mature factors that was able to induce murine Embryonic Stem Cells (ESCs) to differentiate into insulin-producing cells in 2 weeks, and that insulin release of these induced cells is regulated by the glucose concentration. Their findings offered a novel model to study the differentiation mechanism of pancreatic beta cells *in vitro* [32].

A recent study in 2019 suggested that 3D induction method can further increase the efficiency of cell differentiation, since cell-cell interactions in clusters can play critical roles on beta cell differentiation. In this study, 3D PDX1 positive colonies were generated at stage 4 and the efficiency increased significantly. During stage 6 the percentage of PDX1 positive was 50-60% [33]. Another study in 2020 showed that *in vitro* differentiation of hPSCs to PDX1 and NKX6.1 co-expressing pancreatic progenitors has the efficiency of ~80–90% [34]. However, a previous study in 2015 reported that applying the same pancreatic progenitor protocol on eight different hPSC lines results in a variation in NKX6.1 induction ranged from 37% to 84%, mainly due to the fact that the duration of the stage 3 induction step influences the ratio of polyhormonal cells and NKX6.1 positive progenitors that develop within the culture [35].

2.6. Analytical techniques

The high number of differentiation steps makes the current in vitro beta cell differentiation protocols very complex. The process requires almost 20 signaling proteins and small molecules to regulate the growth and differentiation of the cells and lasts for more than four weeks. Within this multi-step process not all cells differentiate into the targeted cells but take wrong differentiation paths. This can lead to a highly heterogeneous cell population with beta cells which are not completely functional [36]. Here I give a brief background on characterization analysis FACS, ICC, and ELISA, I performed on stage 7 differentiated cells.

The terms **flow cytometry** and fluorescence-activated cell sorting **(FACS)** are often used interchangeably. In practice, there are differences between the two methods. In a flow cytometry experiment, every cell that passes through the flow cytometer and is detected will be classified as a distinct event [37]. It is a rapid and quantitative method for analysis and purification of cells in

suspension, which allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second [38]. I will use flow cytometry for quantitative analysis of the cells obtained from stage 4 and 7 which are expressing the target proteins. On the other hand, FACS is a derivative of flow cytometry that adds an exceptional degree of functionality. Using FACS a researcher can physically sort a heterogeneous mixture of cells into different populations. By using highly specific antibodies tagged with fluorescent dyes, a researcher can perform FACS analysis and simultaneously gather data on and sort a sample by a nearly limitless number of different parameters [37]. I will perform FACS cell sorting to sort the mixed population of cells from stage 7 to two populations of GFP positive cells and GFP negative cells. These will be discussed in more details in Methodology section.

Immunocytochemistry (ICC) is a common laboratory assay that can confirm the expression and location of target peptides or protein antigens in the cell via specific combination of antibodies and target molecules. These bound antibodies can then be detected using several different methods. Immunocytochemistry (ICC) allows to evaluate whether or not cells in a particular sample express the antigen in question [39]. In this case, I want to detect and visualize the target proteins, PDX1, NKX6.1, c-peptide, Glucagon, GFP and MAFA in differentiated cells obtained from stage 4 and stage 7. More details are given in the Methodology section.

3

3. Methodology

3.1. Overview

Figure 3.1 gives information on each stage of the differentiation process. It approximately takes 6 to 8 weeks to complete a differentiation process. It also shows that during stage 4 cells are processed to go from adherent culture to suspension culture. See Appendices A – J for further details on the materials used in this project.

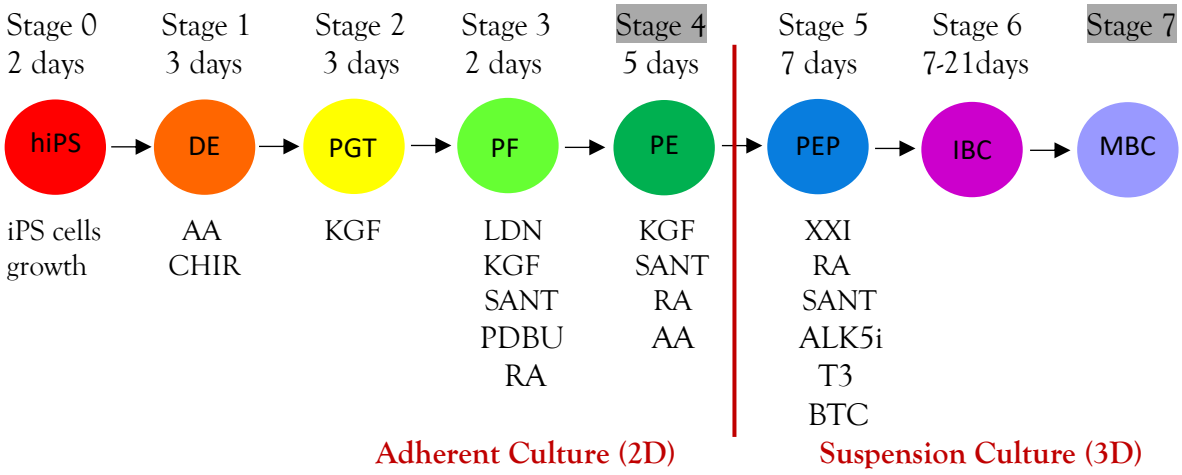


Figure 3.1. Representative overview of the differentiation process for generation of human beta cells. The significance and duration of each stage of the differentiation process and a summary of growth factors used in each stage is shown.

Table 3.1 represents a timeline for one differentiation process and events that take place during one experiment. Although each experiment follows the same differentiation protocol and the cells are cultured under the same condition all through the project, the induction efficiency is compared between six experiments, and it is due to the sensitivity of stem cells and their biological complexity that can affect the amount of mature beta cells yielded.

Table 3.1. Representative fate of one Differentiation process and the analyses performed.

Weeks →	1	2	3	4	5	6	7&8
Activity ↓							
1 single differentiation process							
Adherent culture							
Suspension culture							
Fix cells for ICC & FACS analysis at stage 4							
Fix cells for ICC & FACS analysis at stage 7							
Stain fixed cells and perform ICC analysis							
Perform FACS analysis on fixed cells			Stage 4				Stage 7
GSIS analysis and NGS analysis							

3.2. Gene editing of iPS lines

MafA-GFP reporter lines were generated using wild type AD3-01 iPSC line. These cell lines were previously created in the lab, prior to this thesis project. In brief, AD3-01 cells were harvested at passage P11, cells were transfected with CRISPR/Cas9 plasmid vector DNA and targeting plasmid vector. Transfected cells were selected and after 24 hours they were passaged. When the cells expanded, pure clones were isolated via single cell cloning. Junction PCR and ddPCR analysis was used to validate the correctly targeted clones. For clone validation, single cell clones were analyzed, and primer pairs were used to identify clones with insert at correct location. After validation, clone MafA2.6 was identified as a double copy clone (GFP was inserted on both gene copies) and MafA7.1 as a single copy clone (GFP was inserted only on one gene copy). Next, the clones were transfected with GFP and FACS sorted based on GFP expression. Single cell cloning and ddPCR analysis was performed for identification of pure clones and clone validation.

3.3. iPSCs differentiation into beta cells

A total of six differentiation experiments were performed, with approximately 2-week interval between each experiment, to differentiate AD3-01, MafA2.6 & MafA7.1 cell lines into pancreatic lineage.

Briefly, iPSCs were thawed, and 500K cells per well were seeded as single cell solution on 6-well cell culture plate coated with growth factor reduced Matrigel in mTeSR™1 medium with 10µM ROCK inhibitor. Depending on the cell density, the cells were passaged every 3-4 days using Accutase and ROCK inhibitor (here, ROCK inhibitor enhances cell survival when they are dissociated to single cells by preventing dissociation-induced apoptosis, thus increasing their cloning efficiency. It also improves survival of cell monolayers at the initiation of differentiation protocols [40]).

Next, iPSCs were expanded and 6 million cells per well were seeded on a new 6-well plate and differentiation started 48 h post seeding of cells. Specific cell culture media was prepared for each stage of the differentiation, see Appendix F for medium formulation. A total of eleven different growth factors were used during the differentiation, see Appendix K for the used growth factors & reagents and their role. Cells were rinsed once in PBS (-/-) before starting the differentiation. Cells were differentiated in the culture plates for all stages of the differentiation and medium was replenished every 24 h. Cells were in adherent culture from stage 0 and they were changed to suspension culture at stage 4. During stage 6, medium was replenished for 3 weeks every 48 h (MCDB131-S3, without factors).

At stage 7, which marks the endpoint and the day the cells are harvested, the mature beta cells were collected, dissociated to single cells using Accutase and counted using Cedex-HiRes Analyzer device. This cell count was used later to compare the number of cells obtained after cell sorting and purification. Later the cells were processed for further analysis. For detailed information on the 7-stage differentiation process see Appendix L, and for further details on cell processing protocols see Appendices M – U.

3.4. Immunofluorescence imaging and flow cytometry

For flow cytometry analysis, pancreatic differentiated cells are collected from stages 4 and 7 of the differentiation processes. According to the protocol seen in Appendix Q, collected cell clusters were washed with PBS (-/-), fixed by using PFA 4%, permeabilized with permeabilization buffer and stained by using specific antibodies. The cells at stage 4 were stained for NKX6.1 and PDX1 protein expression, and they were stained for c-peptide, glucagon and GFP expression at stage 7. In brief, cells were stained with primary antibody during an overnight incubation at 4°C. The following day, the unbound antibodies were removed by washing steps using permeabilization buffer and cells were stained with secondary antibody during a 1 h incubation at RT. Further washing steps were followed by resuspension in FACS buffer, and cells were ready for analysis. See Appendix G for details on the antibodies used.

3.5. Fluorescence-activated cell sorting (FACS)

For cell sorting, stage 7 differentiated cell clusters were dissociated to single cell solution using Accutase and washed once. Cells were resuspended in differentiation medium (MCDB131.D3, without factors). Cells were sorted on a Sony SH800 cell sorter using a 100µm nozzle. The Sort Mode was Normal. Target Ratio [%] for GFP positive population and GFP negative population was ~2-3% and ~90% respectively. As I can see the gaiting for GFP positive cells is very conservative, meaning that I aim to collect cells with true GFP positive signal. The Sorted Count was set to stop at 10,000,000 events. Details on processing stage 7 clusters is given in Appendix R.

I analyzed sorted cell populations with unsorted population to see whether it makes a difference to the cells when I perform live cell analysis on them such as GSIS analysis. Running the cells through cell sorter would be the best control, although the stresses imposed by sorting could affect the cells.

3.6. Image acquisition

For immunocytochemistry (ICC) analysis, differentiated cells were collected from stages 4 and 7 of the differentiation processes. Collected cells were seeded on a 96-well plate and fixed by using PFA 4% 24 h post seeding of cells. Then the cells were treated with blocking solution for 1 h at RT, for reduction of background interference, improvement of the signal-to-noise ratio & assay sensitivity, and later they were stained by using specific antibodies. The cells at stage 4, were stained for NKX6.1 and PDX1 protein expression, but at stage 7, they were stained for MafA, GFP, c-peptide and glucagon protein expression. In brief, cells were stained with primary antibody during an overnight incubation at 4°C. The following day, the unbound antibodies were removed by washing steps using PBS (-/-) and cells were stained with secondary antibody during a 1 h incubation at RT. Further washing steps were performed and cells were ready for analysis using ImageXpress Micro system (See Appendices H, S, and T).

3.7. Definition and calculation of differentiation efficiency

One wonders how the differentiation efficiency of each cell line is defined and calculated. As explained previously, at the end of each differentiation experiment, the cells are harvested and dissociated into single cells. First, the cells are counted using cell-count machinery to see how many live cells were obtained at the end of each protocol of differentiation and maturation. Next, they are taken for being processed for characterization analysis, after which based on the results obtained from the number cells expressing the target proteins (MafA, GFP, c-peptide, glucagon, PDX1 and NKX6.1), the differentiation efficiency is measured as a percentage of the cells I counted after harvesting. The guideline to see how well the cells are differentiated, weather they have become mature and functional beta cells or nor, is to measure the percentage of cells expressing the target proteins, PDX1 and NKX6.1 for stage 4, and MafA, GFP, c-peptide and glucagon for stage 7. These measurements can be obtained by using specific analytical techniques such as flow cytometry to quantify cells expressing target proteins, FACS cell sorting to sort the cells into cells expressing GFP marker which is only expressed in adult beta cells with MafA gene expression, and ICC to confirm the expression and location of target proteins. Each of these techniques are discussed in detail in the Background section.

4

4. Results

4.1. Pancreatic differentiation of MafA-GFP reporter iPSC lines

An established seven-stage differentiation protocol [41] was applied to differentiate MafA-GFP iPSC lines to the pancreatic lineage, to confirm faithful expression of MafA which this proves that the iPS cells are differentiated fully to mature and functional beta cells. Wild type AD3-01 and two MafA-GFP iPSC clones (MafA2.6 and MafA7.1) were differentiated to pancreatic endoderm where MafA is known to be expressed. A GFP signal was detected in live MafA-GFP pancreatic endoderm both by flow cytometry and fluorescence microscopy, while no signal was observed in wild type iPSC line differentiated to the same stage which was to be expected (Figure 4.1). All six experiments were successful in differentiating iPSCs into beta cells, yet with various induction efficiencies.

In short, experiment 1 was the most productive one, yielded the greatest number of cells of all three lines. The differentiation took 41 days, where cells changed from adherent culture to suspension culture on S4D4 (stage 4 day 4). ICC & FACS (for stages 4 & 7) and GSIS analysis (for stage 7) were performed and protein secretion was determined by ELISA assay (data for GSIS analysis and ELISA assay is not included as a result of COVID-19 pandemic).

Experiment 2 started only with wild type and MafA7.1 iPSC lines since MafA2.6 suffered bacterial contamination prior to differentiation start. The differentiation took 43 days with cells suspended on S4D5. FACS analysis (for stages 4 & 7) was performed, although after cell sorting the number of cell clusters formed did not suffice to perform GSIS analysis.

Experiment 3 began with uneven number of cells for each line. The differentiation took 58 days and cells were suspended on S4D5. Due to handling error, many cells were lost during differentiation, although at stages 4 & 7 FACS analysis was performed on all three lines. Obviously, GSIS analysis and ELISA assay were not performed.

The differentiation process in experiment 4 and 5 each took 52 and 55 days, and the cells formed suspension culture on S4D5 and S4D3, respectively. In both experiments, an unusual black color appeared in some of the clusters only in wild type & MafA7.1 iPSC lines. However, the cells appeared to be alive and differentiating (Figure 4.2). The clusters from stage 7 of experiment 4 were harvested and prepared for FACS cell sorting, although due to human error the cells were fixed using PFA 4% and killed accidentally before FACS analysis cell sorting. Hence, they lost their means for further live cell analysis. The same fate for stage 7 clusters of experiment 5 was avoided, and the harvested cells were sorted successfully. Later, samples underwent RNA-prep for PCR analysis. This step was out of scope of the project and would be performed independently.

Experiment 6 yielded better results than former experiments. The differentiation took 50 days with cell suspension on S4D5. The clusters from stage 7 were harvested & sorted and prepared for next generation sequencing (NGS) analysis. As a result of COVID-19 pandemic, NGS analysis was excluded from the project plan and would be performed independently.

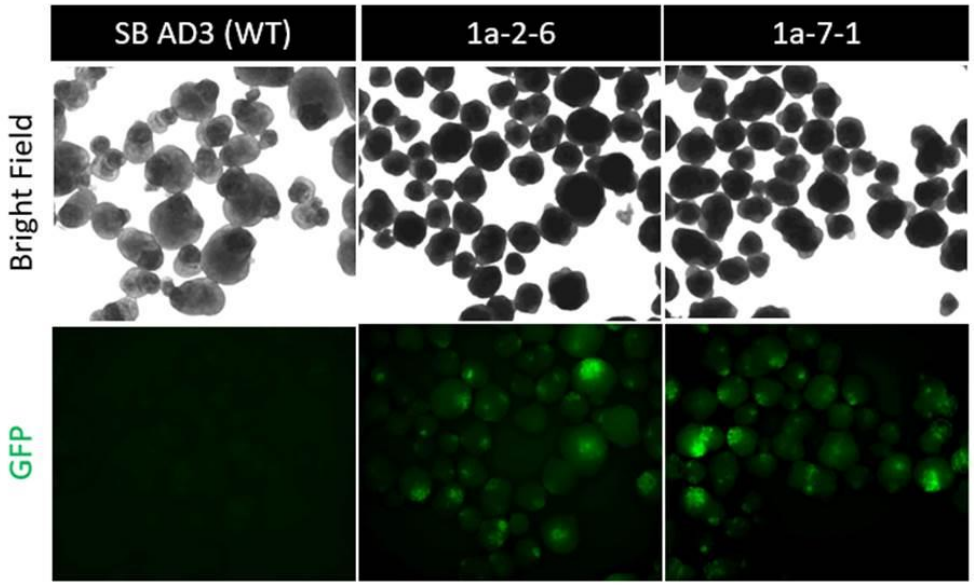


Figure 4.1. Representative bright field and fluorescence microscopy images of live cultures of wild type AD3-01, MafA2.6 and MafA7.1 pancreatic endoderm from stage 7 of experiment 1 (20x magnification).

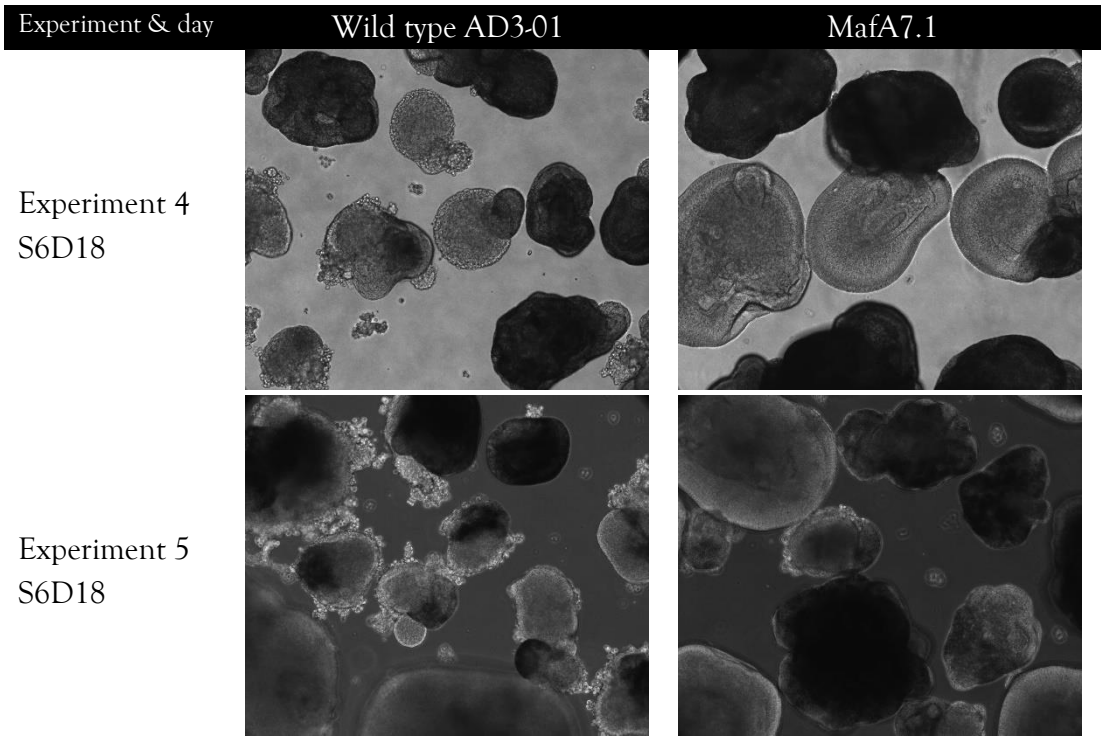
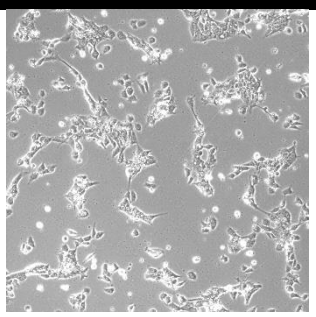
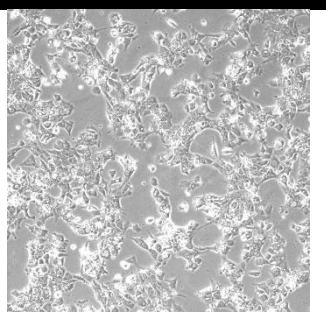
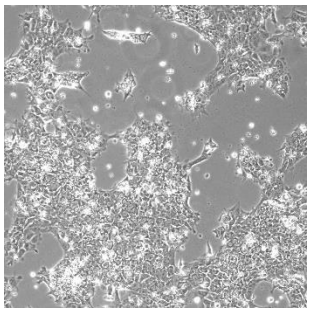
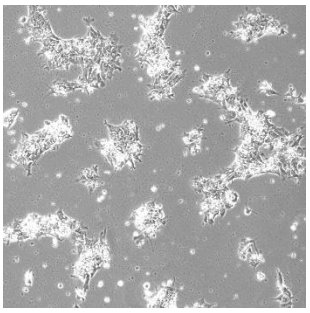
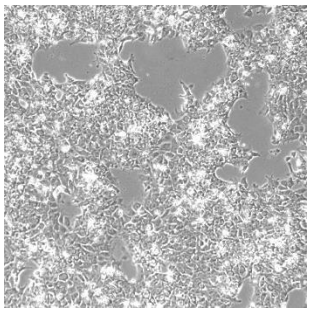
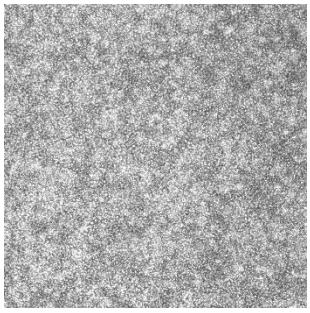
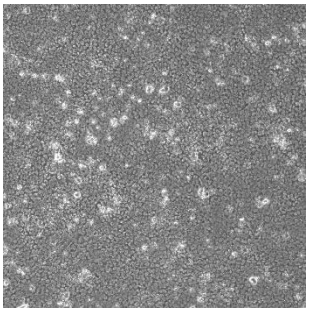
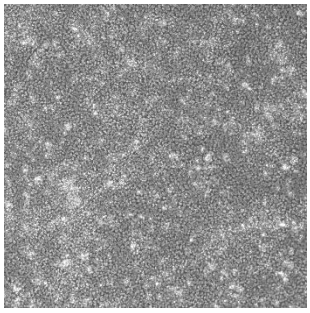
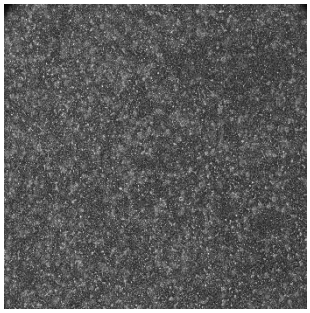
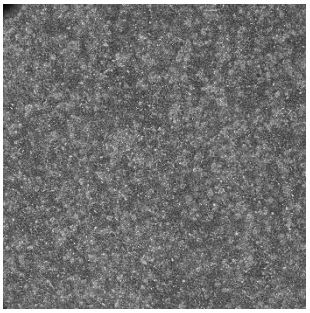
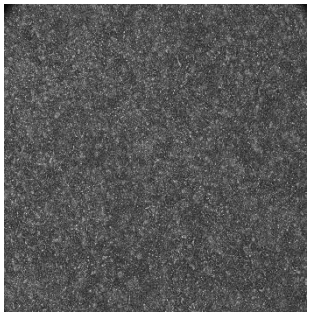
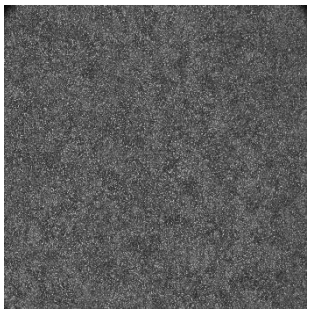
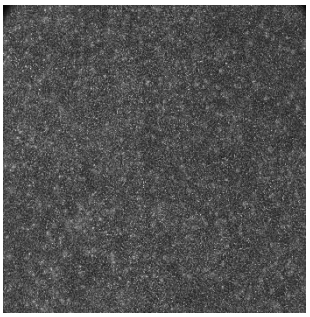
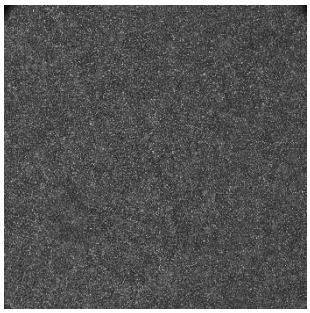
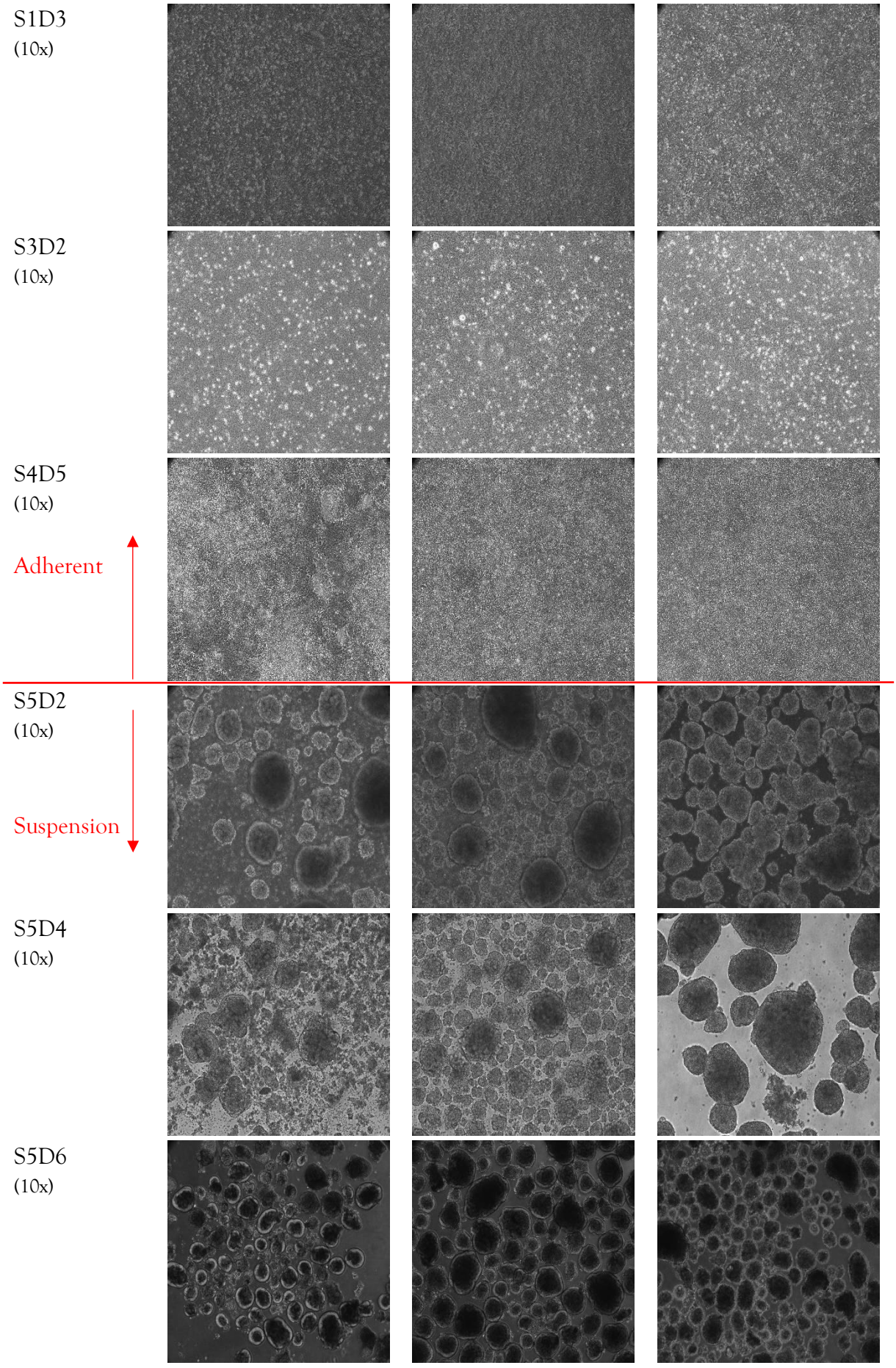
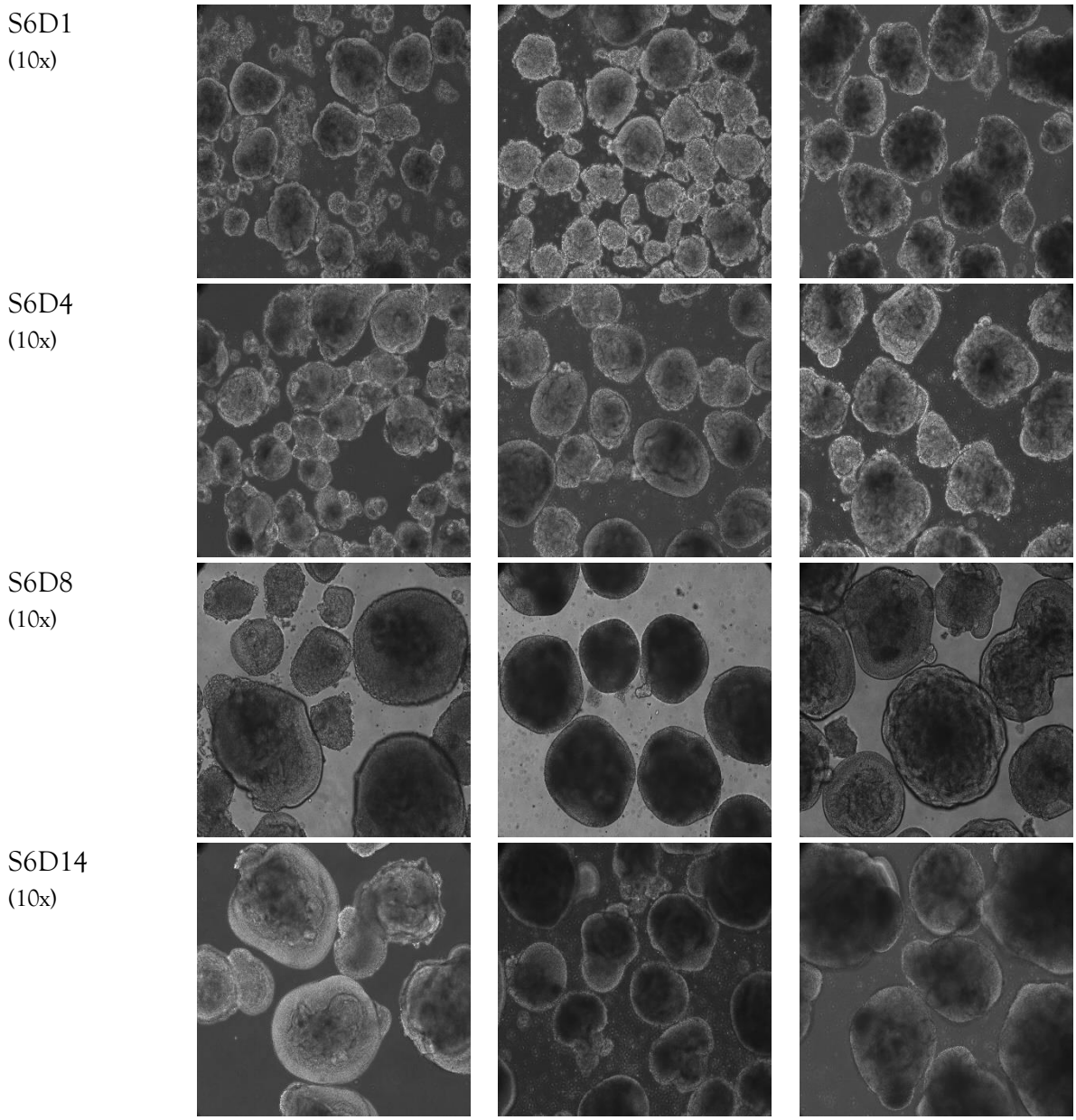


Figure 4.2. Representative irregular black color manifestation in wild type AD3-01 and MafA7.1 iPSC lines (10x magnification).

Table 4.1. Representative fate of wild type and two reporter iPS cell lines from undifferentiated to differentiated state over the course of differentiation process.

Day	Wild type AD3-01	MafA2.6	MafA7.1
1 Undifferentiated (10x)			
2 Undifferentiated (10x)			
3 Undifferentiated (10x)			
S0D2 (10x)			
S1D1 (10x)			


S1D3
(10x)
S3D2
(10x)
S4D5
(10x)
Adherent
S5D2
(10x)
Suspension
S5D4
(10x)
S5D6
(10x)



4.2. Flow cytometry

Harvested cells from stage 4 and 7 were analyzed by flow cytometry. The expression level of NKX6.1 and PDX1 was measured at stage 4 of the protocol, Figure 4.3 A from experiment 1. Here, two distinct populations of cells are seen which follow a similar pattern in wild type and two reporter lines. The cell populations observed in Q2 square represent cells expressing both PDX1 and NKX6.1 (AD3-01 3.7%, MafA2.6 21.5% and MafA7.1 8.6%) meaning that these cells are successfully differentiated at stage 4 and are on the path to become functional beta cells.

At stage 7, the expression level of c-peptide was measured to be robust in both wild type (from Experiment 2) and MafA-GFP iPSC lines (from experiment 1), Figure 4.3 B. To detect endogenous insulin production, c-peptide was used as a marker. The GFP expression was measured in the wild type to be used both as a guidance for comparing the results from reporter

lines and to detect any possible cross-contamination between reporter lines and wild type due to human error. Here, no GFP signal was detected in wild type, which is what is expected since there is no GFP tagged in wild type. However, the cell populations in Q2 square represent cells expressing both GFP and c-peptide in two reporter lines (MafA2.6 23.8% and MafA7.1 29.2%). Moreover, the combination of Q2 and Q3 squares represent the total expression of c-peptide in wild type (11.9%) and in reporter line (MafA2.6 31.7% and MafA7.1 35.7%).

The position of gates was decided, before running the stained cell samples through flow cytometer and analyzing them. Alongside with each stained cell sample, a control or unstained cell samples were also prepared, and these controls were used to set the gate, starting with wild type AD03-01 control sample and then reporter control samples. It was after this gate setting that the stained samples were run and the images in Figure 4.3 were obtained. In Figure 4.3 B, the gate setting in AD03-01 differs from that of the reporters, due to two facts; first, the analysis was obtained from experiments 1 (reporters) and 2 (wild type) in which the results obtained were different already, and second, the gate setting in AD03-01 was chosen to be very conservative to include cells that truly expressed c-peptide but no GFP.

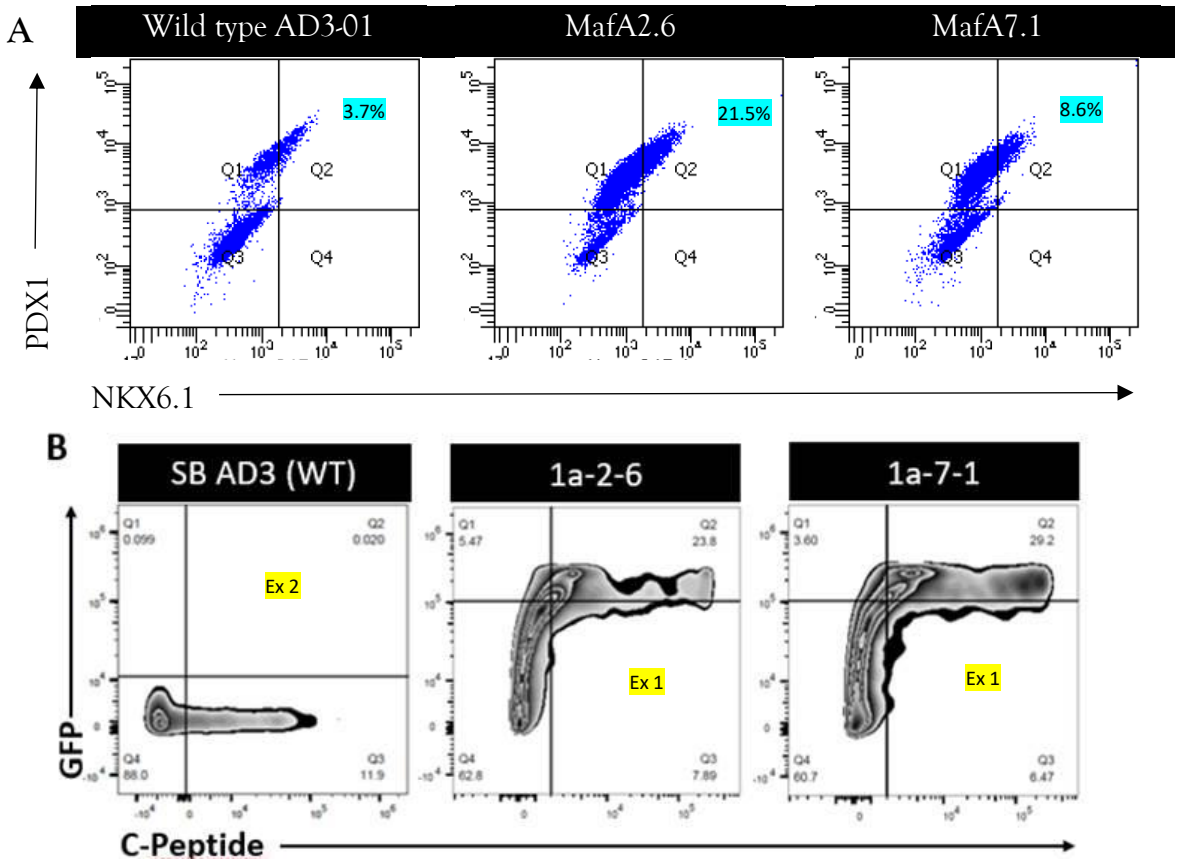


Figure 4.3. Differentiation of MafA-GFP lines to insulin-producing beta cells. A) Representative flow cytometry showing expression of PDX1 and NKX6.1 in wild type AD3-01 and MafA-GFP reporter lines differentiated to stage 4 of the differentiation protocol in experiment 1. B) Representative flow cytometry showing expression of c-peptide and GFP in wild type AD3-01 in experiment 2 and MafA-GFP reporter lines differentiated to stage 7 of the differentiated protocol in experiment 1.

Tables 4.2 and 4.3 give information on the quantitative analysis of flow cytometry performed in experiments 1 to 4 at stages 4 and 7, respectively. 'Data not available' in these tables means the analysis was not performed.

Table 4.2 Representative quantitative analysis of the flow cytometry results shows percentage of cells expressing the differentiation marker for each experiment at stages 4.

Stage 4	AD03-01			MafA2.6			MafA7.1		
Ex.	PDX1	NKX6.1	Joint Expression	PDX1	NKX6.1	Joint Expression	PDX1	NKX6.1	Joint Expression
1	8.5%	0%	12.2%	51.5%	0%	73%	28.8%	0%	37.4%
2	0%	43.2%	27%	Data not available			0%	50.5%	20.1%
3	0.2%	3.7%	19.6%	0.3%	5.4%	30.8%	0.3%	17.3%	1.3%
4	0%	0%	0%	0%	0%	0%	0%	0%	0%

Table 4.3 Representative quantitative analysis of the flow cytometry results shows percentage of cells expressing the differentiation marker for each experiment at stages 7. The squares with yellow shade in each cell line correspond to flow cytometry results in Figure 4.3 B. The columns 'C-peptide & GFP' and 'Glucagon & GFP' in this table are showing the sum of the cells from Q2 and Q3 in Figure 4.3 B.

Stage 7	AD03-01			MafA2.6		MafA7.1	
Ex.	C-peptide & GFP	Glucagon & GFP		C-peptide & GFP	Glucagon & GFP	C-peptide & GFP	Glucagon & GFP
1	0.2%	0.3%		31.7%	13.5%	35.7%	16.1%
2	11.9%	Data not available		Data not available		Data not available	
3	45.3%	Data not available		51.2%	Data not available	68.1%	Data not available
		GFP	C-peptide	Data not available		Data not available	
4	13.5%	82.6%	9.9%				

Table 4.4 gives information about cell populations of GFP positive and GFP negative in two reporter lines. Once again, presence of GFP expression confirms expression of MafA gene which is only expressed in adult beta cells. After FACS cell sorting of cells from stage 7, experiment 1 yielded the highest number of GFP positive cells of all experiments in both reporter lines. The percentage of GFP positive cells (shaded in green) in MafA2.6 (10.89%) and MafA7.1 (10.95%) was very close which makes it challenging to conclude anything about the influence of GFP inserting in one versus two gene copies on gene expression level at this moment. When comparing the results obtained from differentiated beta cells in experiment 1, GFP positive expression of ~11%, to published data from Pagliuca et al. (2014), which is mentioned to be ~33% [41], it seems quite low. They studied different biological batches of human embryonic stem cell line (HUES8), 2 human induced pluripotent stem cell lines (hiPSC-1 and hiPSC-2), polyhormonal cells, and primary beta cells. Although the studied cells were not GFP modified, they stained HUES8 for c-peptide and NKX6.1 expression to measure differentiation efficiency and their flow cytometry results revealed that the protocol they used (the same protocol I followed in this project) produces an average of 33±2% NKX6.1 and c-peptide cells [41].

GFP expression corresponds to expression of MafA gene, since GFP is tagged next to MafA gene in the reporter lines. MafA gene is only expressed in adult beta cells, and accordingly I am looking for percentage of GFP positive expression to define the differentiation efficiency. Overall, experiment 1 was the best of all experiments, generating ~11% adult beta cells.

The results from experiment 2 were measured but not recorded unfortunately in FACS cell sorting instrument, hence the data is not available to be presented. It can only be mentioned that the results were like the experiments 4 to 6.

In experiments 4 to 6, it is shown that in both reporters, GFP negative population is significantly higher than GFP positive. Directed differentiation was inefficient as not more than 2.5% of the cells were GFP positive (range from 0.006% to 2.54%). Since these lines are brand-new and generated by scientists at AstraZeneca, there is no published data on their efficiency yet to compare the results with.

Table 4.4. Representative summary of results obtained from FACS cell sorting analysis.

	MafA 2.6		MafA 7.1	
Ex.	GFP +	GFP –	GFP +	GFP –
1	10.89%	88.76%	10.95%	88.62%
2	Data not recorded		Data not recorded	
4	0.32%	~30.52%	0.64%	50.15%
5	0.006%	5.88%	0.079%	9.92%
6	2.54%	67,55%	0.16%	51,32%

Table 4.5 Summary of analyses performed on each experiment.

Experiment	Differentiation	Flow cytometry	FACS cell sorting	ICC analysis
1	✔	✔	✔	✔
2	✔	✔	✔	
3	✔	✔		
4	✔	✔	✔	
5	✔		✔	
6	✔		✔	

5

5. Discussion

5.1. Significance of results

Differentiation results clearly demonstrated that in this multistage protocol, the cell population has ~11% beta cells, and majority of cells are relatively uncharacterized cells that can be undifferentiated progenitors or other types of untargeted cells. Differentiated and adult beta cells were identified by analyzing the results obtained from flow cytometry and FACS cell sorting analysis which they measured the percentage of cells expressing GFP signal (Figure 4.3 B, Table 4.3 and Table 4.4 – wild type from experiment 2 and reporter lines from experiment 1). Since GFP is tagged next to MafA gene, using CRISPR/Cas9 technology, GFP positive expression corresponds to MafA gene expression, and this MafA gene is only expressed in adult beta cells. Thus, percentage of GFP positive cells is directly linked to the percentage of adult beta cells. The results in this project was compared to a study from 2018 that stated the induction efficiency is to be 30-60% [42]. They studied $INS^{GFP/w}$ hESCs reporter lines where GFP was inserted into the insulin (INS) locus [43] and the protocol they used was a combination of two protocols from Schulz et al, 2012 (Stage 1 to Stage 4) [44] and Rezania et al, 2012 (Stage 5 to Stage 6) [45]. In another study from 2014, they optimized their previous differentiation protocol by adding factors such as vitamin C, protein kinase C activators, transforming growth factor-beta receptor inhibitors, and thyroid hormones to generate insulin-producing cells at an induction rate of approximately 50% [46]. The current protocol used in this project also optimized a differentiation method to generate beta cells from hESC/iPSC in vitro at an induction efficiency of > 30% [41]. Thus, improving efficiency, in terms of the percentage of differentiated cells that become beta cells, remains an important challenge. These three lines were chosen to be studied to see if there is any difference in the differentiation efficiency obtained between two reporters and the wild type will appear or not, since only GFP tagging was performed on the wild type to create the reporters, not any type of enhancement gene editing. Hence, I am not expecting to see any adverse difference between reporters and wild type. As mentioned previously, these lines are brand-new and generated by scientists at AstraZeneca, thus other studies are non-existent in this case.

Different results obtained for each six differentiation experiments, while all followed the same protocol and had the same cell culturing conditions, can be as a result of biological complexity and sensitivity of stem cells, some of which are not fully understood yet. Certain human errors can inadvertently happen as a result of poor cell culturing techniques; such as any type of contamination (bacterial, viral, etc.), or cell loss (especially during changing adherent culture to suspension culture, during media change of cells in suspension, and during washing steps of cells when preparing them for characterization analysis). Another contributing factor to this can be that the length of the differentiation protocol (almost 2 months) can make this multistage protocol even more complicated which also provides a lot of opportunities for mistakes; such as

inaccuracy in adding growth and differentiation factors, and wrong and stress-induced decisions on days with a heavy workload that can change the fate of an experiment and the analysis performed on it, like what was explained to happen for experiment 4 (stage 7 cells were accidentally fixed and killed, and were no more useful for further live cell analysis).

Recent flow cytometry and FACS analyses identified that the protocol used in this project could generate beta cells from iPS cells in vitro [41]. The differentiation efficiency obtained was not as high as was expected from previous results published in other studies, and it also varied from one experiment to another which is concluded to be a result of the poorly performed cell culturing and analytical techniques used in this project. The signals from different target proteins of interest is not consistent to draw a conclusion. In Table 4.2 (cells at stage 4), it is expected to see some signals from PDX1 and some signals from NKX6.1 in the cell samples. It is impossible to have 0% signal from PDX1 and some signal from NKX6.1, since PDX1 is the earliest marker of beta cell successful and healthy differentiation path. In Table 4.3 (cells at stage 7), it is expected to see some c-peptide/GFP signal and some glucagon/GFP signal in the reporter cell samples, more signal in the former. It is impossible to see GFP signal from AD03-01 wild type, since GFP in the wild type is absent. Whereas, considerable amount of GFP expression is encountered which can be a result of poor antibody staining, or cross-contamination of cell samples during sample staining preparation, or due to using expired antibody stains. In Table 4.4, the population of cells with GFP negative signal is obviously more than the cells with GFP positive signal, which this approves the low induction efficiency obtained following this differentiation protocol. The GFP negative cell population can be polyhormonal cells, undifferentiated cells or other type of cells that are not of interest for this project. In that connection, it cannot be concluded at this moment that the efficiency of which reporter line is better than the other.

The differentiation protocol can be considered as a two-phase protocol, where phase one is the differentiation phase and phase two is the maturation phase. Phase one is from the beginning of stage 1 till the end of stage 4, and phase two is from the beginning of stage 5 till the end of stage 6. As discussed, stage 7 is only one day and is the day where cells are harvested. During phase one, the cells are growing and differentiating for 13 days in adherent culture, whereas during phase two, the cells are maturing for 28 days in suspension culture. It is somewhere between these two phases that the type of cell culture changes from adherent (2D) to suspension (3D). As discussed, adherent culture is better for cell differentiation and suspension culture is better for cell maturation. Importantly, the cell proliferation is arrested in the phase two and it is worthy of note that, as seen in Figure 3.1 the schematic of differentiation stages, at stage 5, ALK5i is added. ALK5i addition suppresses the differentiation and proliferation of beta-cell-derived cells [47]. ALK5i is an inhibitor of transforming growth factor β (TGF-β) signaling during the last stages of differentiation [48, 49]. TGF-β has a critical role in cellular responses, such as development, proliferation, and differentiation. TGF-β signaling pathway is known to play a multifunctional role in the regulation of embryonic development, and in normal conditions it maintains tissue homeostasis via the regulation of cell proliferation [50]. Hence, its inhibition leads to growth arrest in the cells in phase two of the differentiation process.

5.2. MafA2.6 versus MafA7.1

The major difference between the iPS reporter cell lines MafaA2.6 and MafA7.1 used in this project, is the GFP tagging. MafA2.6 has two GFP tagged on both copies of MafA gene, whereas MafA7.1 has one GFP tagged on one copy of MafA gene. With respect to these differences, this project planned to gain a deeper understanding of the two iPS reporter cell lines. Having double the amount of GFP tagged in MafA2.6 than that of in MafA7.1, one expects to observe brighter GFP signal during fluorescence microscopy in MafA2.6 than in MafA7.1 at later stages of the protocol. Figure 3.1 shows almost no difference in the intensity of GFP signal received at stage 7, and indeed no difference was observed when the cells were examined under the microscope at any time of the later stages.

Single GFP tagging only modifies one MafA gene and leave the other gene intact and unmodified, which is the case for MafA7.1, whereas double GFP tagging alters both MafA genes, which is the case for MafA2.6. This notion is speculated that single or double GFP tagging can have effects on the efficiency of cell proliferation and differentiation. From the experience and knowledge gathered in culturing these both reporters, it is worthy to mention that MafA2.6 (double GFP tagged), when compared to MafA7.1 (single GFP tagged), had lower cell proliferation rate. During cell detachment from the bottom of the well, MafA2.6 would detach and dissociate into single cells faster than MafA7.1 and needed less time for treatment by Accutase (agent for cell detachment). This can be due to less of number of cells in MafA2.6 than in MafA7.1, which only means higher number of cells causes more cell-cell interactions and consequently makes it harder to break the interactions and make the cells single again. Furthermore, from stage 5 onwards, where it is expected of suspended cells to form clusters, MafA2.6 formed smaller aggregates and a lot of single cells were floating in the media, they seemed almost passive and reluctant to cell-cell interactions and this led to losing cells during media change (it is easier to aspirate old media from a well where cells are suspended in cluster, visible without microscope, and not in single form), whereas with MafA7.1, big cell clusters easily were shaped, due to higher number of cells or cells being more active and social to form clusters, later leading to the issue of clusters getting too big now that had to be broken down by gently pipetting up and down so that the factors and nutrition would reach the cells in the center of cluster and they would stay healthy and alive.

One potential negative side-effect of having GFP tagged next to the MafA gene, as observed in this project, could possibly be lower cell proliferation efficiency since the genes are not intact anymore. A review in 2016 [51] presented current evidence for cellular toxicity of GFP in *in vivo* studies. In brief, initiation of the apoptosis cascade has been postulated as a possible mechanism for the toxicity of GFP and cellular death. In addition to initiating the apoptosis cascade, reactive oxygen production induced by GFP has been linked to cellular toxicity and eventual death in GFP expressing cells. Cells transfected with GFP plasmid have increased cellular permeability following the initiation of cellular death. Furthermore, to detect GFP in living cells, researchers must utilize light/laser or photoactivation, which uses precise light wavelengths, to excite the GFP. Photoactivation have been found to induce phototoxic effects. As a consequence, GFP's

cytotoxic effects can be complemented with the phototoxic effects of wavelength and light intensity during GFP excitation [51].

From the results gathered, it cannot be concluded that reporter lines were indeed doing better than the wild type due to lack of getting consistent results. It should not be expected to see any significant difference between the reporter lines and the wild type, since no genetical enhancement was done on the wild type to create the reports lines in the first place, only GFP tagging. As I compared the reporter lines in the above paragraphs, it can be only noted that the wild type stood somewhere between the reporter lines. Sometimes it would be as efficient as MafA2.6 and sometimes as efficient as MafA7.1. This variability is not understood completely yet, and further research is required in future, since these lines are all brand-new and generated by scientists at AstraZeneca.

5.3. Source of experimental errors

Some of the experimental errors that happened in this project and their possible source will be discussed here. First, it could be having poor cell culturing techniques which causes losing cells. Cells, as obvious it is, are extremely valuable in this project and losing them at any stage of the proliferation and differentiation is equal to losing an opportunity of obtaining desirable and significant results. Cells are most prone to be lost during cell splitting (passaging), during changing culture type from adherent to suspension, and during media change of cell clusters, especially if the cells are in the early period of suspension culture and have not formed aggregates fully yet, a lot of single cells can be taken out and discarded. Cells are also prone to be damaged and killed, if not handled and treated vigilantly. During cell detachment, if they are left for longer than normal time with Accutase treatment, they start dying. During passaging the cells, they are very stressed and most sensitive, thus after seeding them in a new cell culture plate it is advised to leave them in the incubator and do not disturb them for at least 4 hours (24 hour is more preferable).

Second error and fortunate low frequent error encountered during this project was bacterial contamination. It sourced directly from mishandling, carelessness, and lack of using antibiotics in media. This error can only be overcome by gaining more experience in handling cell cultures.

Thirdly, in flow cytometry analysis several issues were observed, such as no signal or weak fluorescence intensity, false GFP positive in wild type, or low event rate. If the intensity of fluorescence signal is weak or absent, it can be a result of several factors including; insufficient antibody present for detection (increase amount/concentration of antibody), intracellular target not accessible (for internal staining, ensure adequate permeabilization), target protein not present/expressed at low level (ensure tissue/cell type expresses target protein and that it is present in a high enough amount to detect), fluorochrome fluorescence has faded (antibody may have been kept for too long or left out in the light, and fresh antibody will be required), the primary antibody and the secondary antibody are not compatible (use secondary antibody that was raised against the species in which the primary was raised, for instance primary is raised in rabbit, use anti-rabbit secondary). Receiving GFP positive signal in wild type AD03-01 can be due

to cross-contamination of cell samples during sample staining preparation. Receiving low event rate can be a result of either there is low number of cells/ml (run 1x106 cells/ml, and ensure cells are mixed well, but gently), or cells are clumped and blocking tubing (ensure a homologous single cell suspension by pipetting gently several times before staining and ensure mixing again before running. In extreme cases, cells can be sieved or filtered to remove clumps, using 30 µl Nylon Mesh) [52].

Lastly, unusual manifestation of certain greyish to black colors in stage 6 of some cell clusters formed the question about their origin. It was finally concluded that addition of inaccurate (10 times higher) of retinoic acid (RA) causes the change in cell pigmentation. RA is considered to control melanocytes. It is a vitamin A derivative and is involved in the differentiation, proliferation and maintenance of homeostasis of various cells including effects on the pigmentary system [53]. This miscalculation of factors used comes from sheer inattentiveness to the dilution of factors added to the media before media change. The RA factor that caused color change in clusters was borrowed from a colleague in the cell lab and the detail of factor diluting preparation differed from the norm used in this project, obviously the details were overlooked, and clusters changed pigmentation.

5.4. Suggestions for future research

The accumulated knowledge of pancreatic beta cell development has given anticipation to minimize cell loss through improving cell culturing skills, specifically being vigilant in handling cells during passaging, media change of suspended cells and when changing culture format from adherent to suspension. Bacterial contamination which causes disruptions in project plan can be avoided by working more vigilantly and using sterile techniques. Future studies will include a better antibody staining protocol for flow cytometry to avoid the overlap/mixed signal from the fluorophores used, enhance fluorescence signal intensity, and obtaining true positive and true negative signals. The efficiency of the differentiation protocol is indeed needed to be increased, although with the results obtained cannot conclude how to optimize the protocol at this step. It can only be said how to improve the results, but further analysis is required to make them more robust as well as make it possible to optimize the differentiation protocol. A longer time plan would be necessary to perform a more detailed NGS analysis and analysis of the gene expression differences between stem cell beta cells and primary beta cells. GSIS analysis and ELISA assay is a necessity in future work.

6

6. Conclusion

In conclusion, the results obtained from the differentiation processes showed low level of reproducibility, although it confirmed successful differentiation of iPS cells into beta cells at stage 4 and maturation of immature beta cells into mature beta cells at stage 7 with efficiency of 11% cells expressing GFP signal, where GFP was tagged next to MafA gene. The maturation factor MafA is critical for the homeostasis of mature beta cells and regulates cell plasticity. The MafA-GFP reporter lines were successful with the task it was created to perform, which is to express MafA tagged with GFP signals at stage 7 of beta cell differentiation. Further study including GSIS analysis and NGS analysis are required for better understanding as well as implementing and optimizing differentiation protocols towards pancreatic lineages.

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Appendix A: Chemicals and Reagents

Ascorbic Acid (Vitamin C) (0.25mM)	Sigma
FAF-BSA (2%)	Sigma
Glucose (2mM)	Sigma
Glucose (8mM)	Sigma
Glucose (20mM)	Sigma
Heparin	Sigma
Hoechst Solution (20 mM)	Invitrogen
KCl	Sigma
Sodium Bicarbonate (2,46 g/L)	Sigma

Appendix B: Commercial kits

Glucagon ELISA	Mercodia
Ultrasensitive C-peptide ELISA	Mercodia
Ultrasensitive Insulin ELISA	Mercodia

Appendix C: General solutions and buffers

Blocking solution *(already prepared)*	1x PBS 5% FBS 0.05% TritonX
FACS buffer *(already prepared)*	1x PBS 2-5% (v/v) FBS (or BSA) 2 mM EDTA 2 mM NaN_3
Fixation buffer *(already prepared)*	1x PBS PFA 4%
KRBH buffer *(already prepared)*	137 mM NaCl 4.7 mM KCl 1.2 mM KH2PO4 1.2 mM MgSO4-7H20 2.5 mM CaCl2-2H2O 25 mM NaHCO3 ddH2O
Permeabilization buffer *(already prepared)*	1x PBS 0.1% (w/v) Saponin 2-5 % (v/v) FBS (or BSA) 2 mM EDTA 2 mM NaN3
MSD Tris Lysis Buffer	Meso Scale Diagnostics LLC.
PBS (-/-)	Gibco
(Paraformaldehyde) PFA 4%	Sigma-Aldrich

Appendix D: Cell culture reagents

AA	Peprotech
Accutase	Sigma-Aldrich
Alk5i II	Enzo
BTC	STEMCELL Technologies
CHIR99021	Axon
DMEM	Gibco
GlutaMax (2mM)	Invitrogen
ITS-X (1:50000)	Invitrogen
KGF	Peprotech
LDN183189	Stemgent
Matrigel	Corning
MCDB131	Cellgro
mTeSR™1	STEMCELL Technologies
P/S 1%	Invitrogen
PKC (PDBU)	EMD
RA	Sigma
ROCK inhibitor Y-27632	STEMCELL Technologies
Sant-1	Sigma
T3	Sigma
XXI	Sigma

Appendix E: Cell culture materials

15mL Conical Sterile Centrifuge Tubes	Invitrogen
40µm Falcon Cell Strainer	Corning
5mL Round-Bottom Tubes with Cell Strainer Cap	STEMCELL Technologies
50mL Conical Sterile Centrifuge Tubes	Invitrogen
6-Well Cell Culture Plate	Invitrogen
6-Well Cell Suspension Plate	CELLSTAR
96-Well Assay Plate	Corning
Disposable serological pipettes	CELLSTAR
Dualfilter pipettes (10L, 100L, 1mL)	Eppendorf
Nalgene Rapid-Flow 75mm Filter Unit	Thermo Scientific

Appendix F: Cell culture media

hiPSCs culture medium	mTeSR™1 1:1000 ROCK inhibitor (for day 1 only)
Stage 1 differentiation medium	500 mL MCDB131 0.22 g Glucose 1.23 g Sodium Bicarbonate 10 g FAF-BSA 10 µL ITS-X *(added after filtration)* 5 mL GlutaMaX 0.022 g Ascorbic Acid 5 mL P/S
Stage 2 differentiation medium	500 mL MCDB131 0.22 g Glucose 0.615 g Sodium Bicarbonate 10 g FAF-BSA 10 µL ITS-X *(added after filtration)* 5 mL GlutaMaX 0.022 g Ascorbic Acid 5 mL P/S
Stage 3 differentiation medium	500 mL MCDB131 0.22 g Glucose 0.615 g Sodium Bicarbonate 10 g FAF-BSA 2.5 mL ITS-X *(added after filtration)* 5 mL GlutaMaX 0.011 g Ascorbic Acid 5 mL P/S
Stage BE5 differentiation medium	500 mL MCDB131 1.3 g Glucose 0.877 g Sodium Bicarbonate 10 g FAF-BSA 2.5 mL ITS-X *(added after filtration)* 5 mL GlutaMaX 0.022 g Ascorbic Acid 0.005 g Heparin 5 mL P/S

Appendix G: FACS

G.1. Primary antibodies

	Antibody	Species	Vendor	Catalogue no.	Dilution
Stage 4	NKX6.1	Mouse	DSHB	F55-A12-s	1:200
	PDX1	Mouse	BD Biosciences	562161	1:200
Stage 7	C-peptide	Rat	BD Biosciences	565831	1:100
	Glucagon	Rat	BD Biosciences	565860	1:500
	GFP	Rabbit	Abcam	Ab290	1:100

G.2. Secondary antibodies

	Antibody	Species	Vendor	Catalogue no.	Dilution
Stage 4	Mouse IgG-Alexa 647	Goat	ThermoFisher Scientific	A28181	1:500
	Goat IgG-Alexa 488	Donkey	ThermoFisher Scientific	A32814	1:500
Stage 7	Mouse IgG-Alexa 647	Chicken	Life technologies	A21201	1:500
	Rat IgG-Alexa 647	Chicken	Life technologies	A21472	1:500
	Rabbit IgG-Alexa 488	Goat	Life technologies	A11008	1:500

Appendix H: ICC

H.1. Primary antibodies

Antibody	Species	Vendor	Catalogue no.	Dilution
PDX1	Goat	Abcam	Ab47383	1:1000
NKX6.1	Mouse	DSHB	F55-A12	1:100
GFP	Goat	Abcam	Ab6673	1:200
MAFA	Rabbit	Abcam	Ab26405	1:100
Glucagon	Mouse	MilliporeSigma	G2654	1:500
C-peptide	Rat	DSHB	GN-ID4	1:100
MAFA (N)	Rabbit	NovusBio	NB400-137	1:300

H.2. Secondary antibodies

Antibody	Species	Vendor	Catalogue no.	Dilution
Mouse IgG-Alexa 647	Goat	ThermoFisher Scientific	A28181	1:500
Goat IgG-Alexa 488	Donkey	Invitrogen	A11055	1:500
Rat IgG-Alexa 647	Chicken	Life technologies	A21472	1:500
Mouse IgG-Alexa 594	Chicken	ThermoFisher Scientific	A21201	1:500
Rabbit IgG-Alexa594	Chicken	ThermoFisher Scientific	A21442	1:500

Appendix I: Software

FACSDiva	BD
SH800 Cell Sorter	Sony
IX Micro	Molecular Devices
FlowJo	FlowJo LLC
NIS-Elements	Nikon

Appendix J: Microscopes and devices

Axiovert 40CFL	Carl Zeiss
Eclipse TE2000-U	Nikon
ImageXpress Micro XL	Molecular Devices
Yokogawa CV700	CellVoyager CV700S
Grant Water bath	SUB
Cedex-HiRes Analyzer	Innovatis
Cell incubator	Thermo Scientific
New Brunswick S41i (Shaker incubator)	Eppendorf
Rotana 46 R	Hettich ZENTRIFUGEN
BD LSRFortessa™ Cell Analyzer	BD
Sony SH800 Cell Sorter	Sony
SpectraMax® Plus 384 Microplate Spectrophotometer	Molecular Devices LLC
Refrigerator	NINOLUX

Appendix K: Role of used reagents, chemicals and growth factors used during cell culture and differentiation process.

Purpose	Substance	Role and definition
Cell culture	DMEM	A medium for plate coating for adherent cells
	Matrigel	An optimal matrix for keeping self-renewal and pluripotency in stem cells
	PBS	Keeps pH constant
	mTeSR1	A complete, defined & serum-free medium
	Accutase	Used for cell detachment and creating single-cell suspension
	ROCK Inhibitor	Blocks apoptosis of dissociated cells & increase survival and efficiency without affecting pluripotency
Medium formulation	MCDB131	Reduced serum-supplemented medium which contains no proteins & growth factors and is often supplemented
	BSA (bovine serum albumin)	Used due to its stability and lack of interference within biological reactions for protection from oxidative damage and stabilization of other media components
	Glucose	An energy source & a metabolic intermediate
	Sodium bicarbonate (NaHCO3)	Causes a high buffer capacity & to keep the pH value in the physiological area during cultivation
	Ascorbic acid (vitamin C)	A primary antioxidant to support & promote cell proliferation & differentiation, Heparin to promote cell growth
	GlutaMax	Keeps cells healthier longer & remain stable across a wide range of temperature
	ITS-X (Insulin-Transferin-Selenium-Ethanolamine)	A basal medium supplement to reduce the amount of fetal bovine serum (FBS) needed to culture cells
	P/S (Penicillin-Streptomycin)	An antibiotic solution to maintain sterile conditions & control bacterial contamination
Cell growth and differentiation	CHIR99021	Promotes cell proliferation and self-renewal
	AA (Activin A)	A transforming growth factor (TGF) to regulate cellular homeostasis, promote cell proliferation & induce pancreatic differentiation
	KGF (Keratinocyte growth factor)	A fibroblast growth factor (FGF) to promote and stabilize cell proliferation & differentiation
	RA (Retinoic acid)	A potent metabolite of vitamin A to act as a growth & differentiation factor
	Sant-1 (Sonic hedgehog Agonist-1)	A potent sonic hedgehog pathway (Shh) antagonist to inhibit Hedgehog signaling pathway[1]
	ALK5i (Activin receptor-like kinase 5 inhibitor)	Blocks invasive phenotypes of cancer cells & affect tumor progression
	PDBU (Phorbol 12,13-dibutyrate)	A tumor promoting phorbol ester to activate protein kinase C (PKC)[2]
	LDN183189	A bone morphogenic protein (BMP)[3] receptor inhibitor
	T3 (Triiodo-L-thyronine)	A thyroid hormone to regulate cell differentiation & protein expression
	XXI (γ-Secretase inhibitor)	A cell-permeable, potent, selective, peptidomimetic, non-transition-state analog inhibitor of γ-secretase[4] and Notch processing[5]
	BTC (beta-cellulin)	An epidermal growth factor (EGF)-related polypeptide to regulate growth and differentiation in islet cells in human pancreas

[1] Hedgehog (Hh) signaling pathway is used during development for intercellular communication and is important for the organogenesis of almost all organs in mammals, as well as in regeneration and homeostasis. It regulates the survival and proliferation of tissue progenitor and stem populations. This function is linked to its role in tumor formation, proving Hh signaling is disrupted in diverse types of cancer [54, 55].

[2] PKCs regulate gene expression, protein secretion, cell proliferation, and inflammatory response in cell.

[3] BMPs are embryonic proteins and members of transforming growth factor (TGFβ) superfamily. They are expressed in many carcinomas. Dysregulation of the BMP signaling pathway can have drastic consequences during mammalian development [56, 57].

[4] Gamma-secretase is an enzyme complex that cleaves numerous substrates, best known for cleaving amyloid precursor protein (APP) to form amyloid-beta (Aβ) peptides leading to Alzheimer's disease [58].

[5] The notch receptor is a transmembrane protein & its deregulation is linked to many developmental disorders. In Notch signaling pathway, the Notch is a transcriptional regulator initially expressed as a membrane-bound cell surface receptor. Notch activity is regulated at the level of proteolytic processing of the membrane-bound form to allow release of the active intracellular fragment [59].

Appendix L: Schematic representation of the protocol for pancreatic beta cell differentiation from iPS cells.

Stage no.	Day no.	Medium	Factors	Conc.	Vendor	Catalogue no.
Stage 0	1(24 h)	mTeSR	ROCK inh	10uM	Stemcell	Y-27632
Stage 0	2(48 h)	mTeSR	-	-	-	-
Stage 1 Definitive Endoderm (DE)	1 2 3	MCDB131 **S1**	CHIR99021 AA AA AA	1.4ug/ml 100ng/ml 100ng/ml 100ng/ml	Axon Peprotech Peprotech Peprotech	1386 120-14 120-14 120-14
Stage 2 Primitive Gut Tube (PGT)	1-3	MCDB131 **S2**	KGF	50ng/ml	Peprotech	100-19
Stage 3 Posterior Foregut (PF)	1 2	MCDB131 **S3**	RA Sant-1 KGF LDN183189 PKC (PDBU) RA Sant-1 KGF PKC (PDBU)	2uM 0.25uM 50ng/ml 200nM 500nM 2uM 0.25uM 50ng/ml 500nM	Sigma Sigma Peprotech Stemgent EMD Sigma Sigma Peprotech EMD	R2625 S4572 100-19 04-0074 565740 R2625 S4572 100-19 565740
Stage 4 Pancreatic Endoderm (PE)	1-5	MCDB131 **S3**	RA Sant-1 KGF AA	0.1uM 0.25uM 50ng/ml 5ng/ml	Sigma Sigma Peprotech Peprotech	R2625 S4572 100-19 120-14
Stage 5 Pancreatic Endocrine Precursors (PEP)	1-4 5-7	MCDB131 **BE5**	XXI RA Sant-1 Alk5i II T3 BTC XXI RA Alk5i II T3 BTC	1uM 0.1uM 0.25uM 10uM 1uM 20ng/ml 1uM 0.1uM 10uM 1uM 20ng/ml	Sigma Sigma Sigma Enzo Sigma Stemcell Sigma Sigma Enzo Sigma Stemcell	SCP0004 R2625 S4572 ALX-270-445 T6397 78105 SCP0004 R2625 ALX-270-445 T6397 78105
Stage 6 Immature Beta Cells (IBC)	Change media every 48 h	MCDB131 **S3** -	- -	- -	- -	- -
Stage 7 Maturing Beta Cells (MBC)	1	MCDB131 **S3**	-	-	-	-

Appendix M: Coating for 1 well of 6-well plate

1. Aliquot 1ml cold (4°C) DMEM media into a conical tube to prepare for dilution of Matrigel.
2. Add 10µl Matrigel at 1:100 to 1ml cold DMEM.
3. After mixing, add 1ml of diluted Matrigel to 1 well of a 6-well plate.
4. Incubate coated plate at 37°C for at least 30 minutes.

Appendix N: Thawing process

1. Remove iPSC from liquid nitrogen vapor or dry ice and immerse the cryovial in a 37°C water bath. Thaw quickly by gently swirling until only a small piece of frozen material remains.
2. In a conical tube add 10ml warm mTeSR1 medium, containing 10µl ROCK inhibitor, and then add the thawed cell suspension dropwise gently and mix cells by swirling.
3. Centrifuge conical tube containing cells at room temperature at 250 rcf for 5 minutes.
4. Aspirate the supernatant and gently resuspend cells in 1ml of warm culture media supplemented with 10µM ROCK inhibitor.
5. Aspirate DMEM from the previously coated wells, add 2ml mTeSR1 media and place 1ml suspended cells and gently ROCK the plate to evenly distribute cells.
6. Next day remove ROCK inhibitor with media change.

Appendix O: Passaging for 1 well of 6-well plate

1. Aspirate old mTeSR1 media from the plate.
2. Wash with 1ml PBS (-/-).
3. Add 1ml Accutase per well for cell detachment.
4. Incubate at 37°C for 5 minutes.
5. Pipette the Accutase – cell mixture up and down 3x, visually inspect cell colonies are detached. Collect cell suspension in 15 ml Falcon tube.
6. Add 2ml mTeSR1 containing ROCK inhibitor 1:3 to the cell suspension to dilute the detachment agent and mix gently.
7. Centrifuge at RT at 250 rcf for 5 minutes.
8. Aspirate the supernatant and resuspend cell pellet in 1ml mTeSR1 containing ROCK inhibitor and pipette 10 times to break the pellet. Count the cells.
9. After cell count, aspirate DMEM media from the coated well and add 3ml mTeSR1 medium and seed 500K cells.
10. For the first day after passaging, inspect the cells under the microscope and exchange the media with only mTeSR1.
11. Passage again when cells reach 75-90% confluency.

Appendix P: Adherent to suspension culture for 5 wells

1. Aspirate old media from the well and wash with 1ml PBS (-/-).
2. Add 1ml Accutase per well and incubate at 37°C for 5 minutes or more.
3. Add detached cells to a conical tube containing 10ml S3 media with added factors plus ROCK inhibitor.
4. Centrifuge at RT at 250 rcf for 5 minutes.
5. Aspirate the supernatant and resuspend cell pellet in 12ml S3 media.
6. Pass through 40µm Falcon cell strainer. Count the cells.
7. Place the cells in a 6-well suspension plate and top up to 3ml media in each well.
8. Incubate them in a shaker incubator at 37°C to form clusters.

Appendix Q: Processing 1 well of differentiated cells for FACS analysis

1. Aspirate old media from the well and wash with 1ml PBS (-/-).
2. Add 1ml Accutase per well and incubate at 37°C for 5 minutes or more.
3. Add detached cells to a conical tube containing 2ml S3 media with added factors plus ROCK inhibitor.
4. Centrifuge at RT at 250 rcf for 5 minutes.
5. Aspirate the supernatant and resuspend in 3ml media.
6. Pass through 40µm Falcon cell strainer. Count the cells.
7. Take 1 million cells and centrifuge at RT at 250 rcf for 5 minutes.
8. Resuspend in 2ml PBS and centrifuge again at RT at 250 rcf for 5 minutes.
9. Aspirate the supernatant and resuspend in 1ml PFA 4% and wait for 30 minutes.
10. Centrifuge the cells at RT at 500 rcf for 5 minutes (or 800 rcf for 3 minutes).
11. Aspirate the supernatant and resuspend in 2ml PBS.
12. Repeat step 10.
13. Aspirate the supernatant and resuspend in 500µl permeabilization buffer and wait for 20 minutes.
14. Split each sample into 2 tubes marked positive control (stained) and negative control (unstained).
15. Repeat step 10.
16. Aspirate the supernatant, resuspend positive control tube in 100µl primary antibody* and resuspend negative control in 100µl permeabilization buffer.
17. Store the samples overnight at 4°C.
18. Next day, centrifuge the samples at RT at 500 rcf for 5 minutes (or 800 rcf for 3 minutes).
19. Aspirate the supernatant and resuspend in 1ml permeabilization buffer.
20. Repeat step 18.
21. Repeat step 19.
22. Repeat step 18.
23. Aspirate the supernatant and resuspend in 200µl secondary antibody**.
24. Store the samples for 1 hour at RT.
25. Repeat step 18.
26. Repeat step 19.
27. Repeat step 18.
28. Repeat step 19.
29. Repeat step 18.
30. Aspirate the supernatant and resuspend in 400µl FACS buffer.
31. Prior to performing FACS analysis by FACS machine (BD LSRFortessa™ Cell Analyzer), pass the samples through FACS filter tube with blue lid.

Table Q.1 Combination of antibodies used for FACS cell staining.

Tube	Primary antibody	Secondary antibody
Positive control	NKX6.1 and PDX1	Mouse IgG-Alexa 647 and Goat IgG-Alexa 488
Negative control	-	Mouse IgG-Alexa 647 and Goat IgG-Alexa 488

Appendix R: Processing Stage 7 clusters for cell sorting

1. Collect clusters in a conical tube.
2. Centrifuge the samples at RT at 250 rcf for 5 minutes.
3. Aspirate the supernatant and resuspend in 2ml PBS (-/-).
4. Repeat steps 2 and 3.
5. Centrifuge the samples at RT at 250 rcf for 5 minutes.
6. Aspirate the supernatant, resuspend in 3ml Accutase and incubate at 37°C for 7 minutes or more. At around minute 3, pipette the sample gently to break them more into single cells.
7. Add 6ml S3 media containing ROCK inhibitor.
8. Centrifuge the samples at RT at 250 rcf for 5 minutes.
9. Aspirate the supernatant and resuspend in 2ml S3 media.
10. Pass through 40µm Falcon cell strainer. Count the cells.
11. On a 6 or 12-well suspension plate, suspend 200K unsorted cells per well in fresh S3 media and incubate in a shaker incubator at 37°C to form clusters.
12. Sort the rest of the sample in to GFP positive and GFP negative by cell sorting machine (Sony SH800 Cell Sorter).
13. After sorting, suspend 200K of positive and negative sorted cells in fresh S3 media in the same plate with unsorted and incubate for 48 hours in a shaker incubator at 37°C to form clusters.

Appendix S: Processing 1 well of differentiated cells for ICC analysis

1. Aspirate old media from the well and wash with 1ml PBS (-/-).
2. Add 1ml Accutase per well and incubate at 37°C for 5 minutes or more.
3. Add detached cells to a conical tube containing 2ml S3 media with added factors plus ROCK inhibitor.
4. Centrifuge at RT at 250 rcf for 5 minutes.
5. Aspirate the supernatant and resuspend in 3ml media.
6. Pass through 40µm Falcon cell strainer. Count the cells.
7. Seed 100K cells per well in a 96-well plate (Greiner Bio-One), previously coated with DMEM and Matrigel.
8. Top up the media in each well to 100µl and incubate overnight before fixing the cells.

Fixing the cells (next day)

1. Aspirate old media gently and wash cells with 100µl PBS (-/-).
2. Aspirate PBS and apply 100µl PFA 4% and incubate at RT for 30 minutes.
3. Aspirate PFA and add 100µl PBS and wait 5 minutes.
4. Aspirate old PBS and add 100µl fresh PBS and wait 5 minutes.
5. Either aspirate old PBS, add fresh PBS, wrap the plate in parafilm and store at 4°C until analysis OR start staining the cells straight away.

Appendix T: Staining the cells for ICC analysis

1. Aspirate old PBS and treat the cells with 100µl blocking solution at RT for 1 hour.
2. Aspirate blocking solution and add 100µl primary antibody.
3. Store the plate overnight at 4°C.
4. Next day, aspirate primary antibody and wash the cells 3 times with 100µl PBS with 5-minute interval between each washing.
5. Aspirate PBS and add 100µl secondary antibody.
6. Store the plate for 1 hour at RT.
7. Aspirate secondary antibody and wash the cells 3 times with 100µl PBS with 5-minute interval between each washing.
8. The cells are ready for imaging analysis using ImageXpress Micro image system.

NB. Hoechst staining dye solution (1:1000) was used to label DNA.

Table T.1 Combination of antibodies used for ICC cell staining.

Row	Primary antibody	Secondary antibody
1	NKX6.1 and PDX1	Mouse IgG-Alexa 647 and Goat IgG-Alexa 488
2	C-peptide and Glucagon	Rat IgG-Alexa 647 and Mouse IgG-Alexa 594
3	GFP and C-peptide	Goat IgG-Alexa 488 and Rat IgG-Alexa 647
4	GFP and MAFA	Goat IgG-Alexa 488 and Rabbit IgG-Alexa594
5	GFP and MAFA (N)	Goat IgG-Alexa 488 and Rabbit IgG-Alexa594
6	Blocking solution – negative control	All secondary antibodies and Hoechst staining dye solution

Appendix U: Processing clusters for GSIS assay

1. Prepare KRBH buffer (Krebs-Ringer Bicarbonate Hepes) from stock KRBH buffer, supplement with 10% BSA, and store at 37°C.
2. For further treatment, prepare KRBH-BSA plus 2mM glucose (as low glucose), KRBH-BSA plus 20mM glucose (as high glucose), and KRBH-BSA plus 2mM KCl.
3. Wash the collected clusters with PBS (-/-).
4. Load them into columns of a 96-well plate and acclimatize in KRBH buffer without glucose for 30 minutes at 37°C. Find the loading layout in Table 6.
5. Expose the clusters to desired buffer (2mM & 20mM glucose and 2mM KCl).
6. Collect the secretory outputs of glucose stimulation at 0, 10 and 60 minutes.
7. Collect the secretory outputs of KCl stimulation at 60 minutes.
8. During each interval incubate the plate at 37°C.
9. Collect the supernatant from the wells and transfer into a new plate for further measurements.
10. Use MSD Tris Lysis Buffer (Meso Scale Diagnostics LLC.) to lyse the clusters and islets.

Table U.1 Plate layout for GSIS analysis.

		Unsorted				**GFP positive**				**GFP negative**			
MafA2.6	A	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)
	B	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)
	C	0 (20mM)	10 (20mM)	60 (20mM)		0 (20mM)	10 (20mM)	60 (20mM)		0 (20mM)	10 (20mM)	60 (20mM)	
	D	0 (20mM)	10 (20mM)	60 (20mM)		0 (20mM)	10 (20mM)	60 (20mM)		0 (20mM)	10 (20mM)	60 (20mM)	
MafA7.1	E	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)
	F	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)	0 (2mM)	10 (2mM)	60 (2mM)	60 (KCl)
	G	0 (20mM)	10 (20mM)	60 (20mM)		0 (20mM)	10 (20mM)	60 (20mM)		0 (20mM)	10 (20mM)	60 (20mM)	
	H	0 (20mM)	10 (20mM)	60 (20mM)		0 (20mM)	10 (20mM)	60 (20mM)		0 (20mM)	10 (20mM)	60 (20mM)	

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