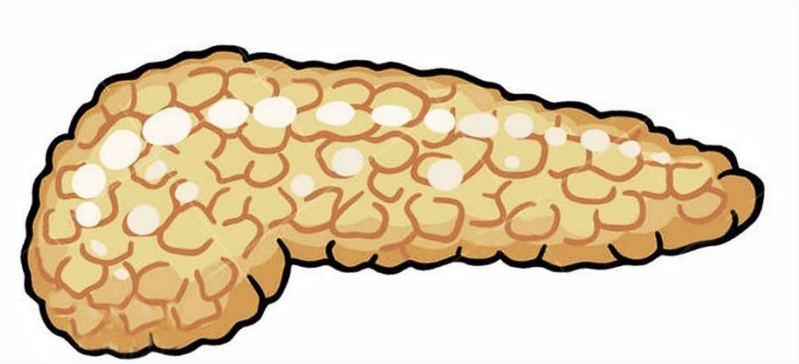
# Pluripotent Stem Cell derived Beta Cells for treating Diabetes Type I

*Bachelor thesis Biomedical Technology by*
Kevin Quang Doan



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10-07-17

UNIVERSITY OF TWENTE.

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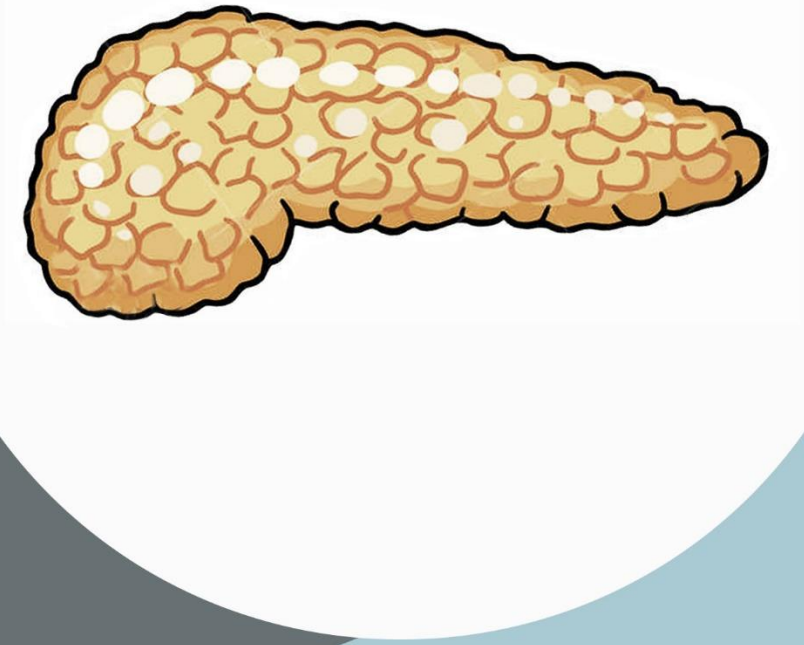
(s1602853) | 10-07-17



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## ABSTRACT

Type 1 diabetes patients suffer from an autoimmune destruction of pancreatic islets, resulting in dysfunctional insulin production as a response to fluctuations in sugar blood levels. Recently, multi-staged protocols have been developed to differentiate insulin-producing β-cells *in vitro* from pluripotent stem cells to use them for islet transplantations. The aim of this study is to differentiate definitive endodermal cells from embryonic stem cells under different conditions, thereby testing effective differentiation conditions for our embryonic stem cell line. In this study, definitive endoderm gene markers (CXCR4, SOX17, FOXA2 and NANOG) expressions were analyzed of undifferentiated and differentiated cells, mainly using qPCR and FACS. From all the testing conditions, best results were obtained from embryonic stem cells that were initially passaged with cell densities between 140K and 150K cells/cm$^2$ (with confluency between 85% and 95% before differentiation started using well-defined differentiation media). Furthermore, an increase of CXCR4 and decrease of NANOG expressions was observed compared to undifferentiated embryonic stem cells. Whether high percentage of the differentiated cell populations were expressing the definitive endoderm gene markers or not remains unanswered. In addition, since no cDNAs of definitive endodermal cells were available to our experiment to compare absolute values with our differentiated cells, we cannot conclude with absolute certainty to have differentiated definitive endoderm, or rather we succeeded in differentiating cells that expresses the correct gene markers with possibly correct relativity expressions of SOX17 and FOXA2.

## SAMENVATTING

Diabetes type 1 patiënten lijden aan een auto-immuunziekte, waarbij het immuunsysteem van de patiënt het lichaamseigen alvleesklier aanvalt. Hierdoor wordt er niet genoeg insuline geproduceerd als reactie op het fluctuerende bloedsuikerspiegel. Recent zijn er multi-fase protocollen ontwikkeld om insuline producerende β-cellen *in vivo* te differentiëren uit pluripotente stamcellen om te gebruiken voor transplantatie van eilandjes van Langerhans, zodanig om het genoemde probleem op te lossen. Het doel van dit onderzoek is het differentiëren van definitieve endoderm cellen met embryonale stamcellen onder verschillende condities, waardoor de effectiviteit van differentiëren wordt getoetst op onze embryonale stamcel lijn. In dit onderzoek worden definitieve endoderm gen markers (CXCR4, SOX17, FOXA2 en NANOG) expressies geanalyseerd bij ongedifferentieerde en gedifferentieerde cellen, voornamelijk met qPCR en FACS. Van al de getoetste condities lieten de embryonale stamcellen, die aanvankelijk gepasseerd waren met een cel dichtheid van 140.000 en 150.000 cellen/cm2 (met de confluentie tussen 85% en 95% voordat differentiatie startte met goed gedefinieerde differentiatie media) de beste resultaten zien. Ook was er een toename van CXCR4 en afname van NANOG geobserveerd vergeleken met de ongedifferentieerde embryonale stamcellen. Het is echter niet duidelijk of een groot deel van de gedifferentieerde cel populaties alle definitieve endoderm gen markers laat zien. Verder kan er niet met zekerheid worden geconcludeerd of met onze protocol daadwerkelijk definitieve endoderm cellen zijn gedifferentieerd, wegens het feit dat er geen cDNAs van definitieve endoderm cellen aanwezig waren om de absolute waardes van de gen expressie met elkaar te vergelijken. Wel kunnen we suggereren dat het is gelukt om cellen te differentiëren die definitieve endoderm gen markers laten zien met mogelijk de juiste SOX17 en FOXA2 expressie verhoudingen.

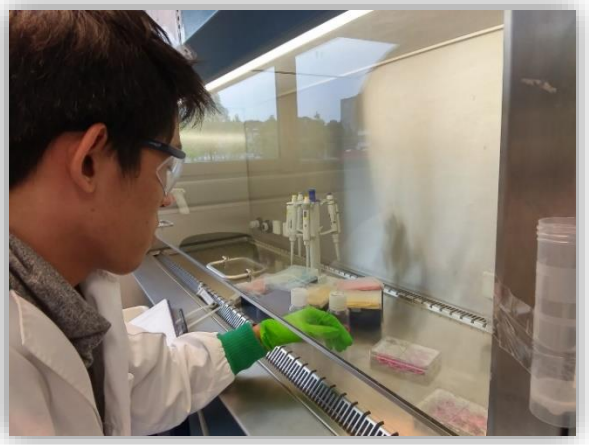
## ACKNOWLEDGEMENTS

In this bachelor thesis, I present to you my research regarding efficient differentiation of insulin-producing β-cells from pluripotent stem cells to treat type 1 diabetes patients. In the past ten weeks I learned to independently conduct my own research at the Developmental BioEngineering (DBE) and the Applied Stem Cell Technologies (AST) research groups, while I received the privilege to interact with many promising intellectuals. It has been a lovely experience where I actually felt as if my contribution will make a difference for society to benefit of. The last thing that I expected before applying for these two research groups, is to develop a small moral feeling toward my embryonic stem cells. Basically, I felt responsible for the cells as if they are my own child, but then million times more.

In this section of my thesis, I would like to take this opportunity to thank the people who have provided me support in many aspects of my study in chronological order. I would like to thank Dr. Janneke Alers for helping me to choose a bachelor assignment that suits my taste as well as giving me many useful tips for my colloquium. I would like to thank prof. dr. P.C.J.J. Passier and prof. dr. H.B.J. Karperien for providing me feedback on my work as well as giving me helpful advice during the entire internship period. I am thankful for Miguel Koch E Silva Do Carmo, also known as the forgetful Pablo, for being a good sport to introduce me to this assignment. I would like to show gratitude towards ing. Kim Vermeul and Rolf Slaats, MSc for providing me the basic skills and supplements to maintain my embryonic stem cells. Thank you Lydia Bolhuis-Versteeg for providing me access to the ML-II lab with your brief but effective introduction. I am thankful for dr. Jacqueline Plass and Karin Roelofs-Haarhuis for their efforts helping me with FACS. Usually Saturdays are a day of rest and when I lose access to the lab, however thanks to Yao Fu, MSc and Kannan Govindaraj, MSc I was still able to have access to the lab several times.

Finally, my family and friends have been supportive throughout my entire internship, and deserve lots of love for making who I am today.

Kevin Quang Doan
July, 2017.

CONTENTS

# ACRONYMS

| | |
|---|---|
| T1D | Type 1 diabetes |
| T2D | Type 2 diabetes |
| hESC | Human Embryonic Stem Cell |
| iPSC | Induced Pluripotent Stem Cell |
| hPSC | Human Pluripotent Stem Cell |
| S1 | Stage 1 |
| DE | Definitive Endoderm |
| AST | Applied Stem Cell Technologies |
| DBE | Developmental BioEngineering |

# 1 INTRODUCTION

## 1.1 CLINICAL RELEVANCE

Pancreatic β-cells respond to high blood sugar levels by secreting the peptide hormone insulin, which acts on other tissues to promote glucose uptake from the blood, therefore reducing the high blood sugar level. Diabetes mellitus, however, is a metabolic disease in which malfunctioning of blood sugar level regulations occurs. Tissues and organs in diabetic patients are then not able to take up glucose, causing severe hyperglycemia, i.e. abnormal high blood sugar levels [1].

This chronic disease comes in two distinctive forms with different causes. Type 1 Diabetes (T1D) is characterized by an autoimmune destruction of the insulin-producing β-cells in the islets of Langerhans of the pancreas. This disease is usually associated with the presence of islet-autoantibodies, which causes islet-autoreactive CD8+ T-cells to respond with an attack. Since T-reg cells help control effector T-cells and maintain immune tolerance, dysfunction could lead to autoimmunity [2]. However, the exact causes for the autoimmune response have not fully been understood yet. Studies suggest that native insulin and its precursors act as primary autoantigens, and fragments of the signal peptide of the preproinsulin (PPI) are main targets for these cytotoxic T-cells [3]. Other theories suggest several viral infectious events contribute to the autoimmune destructive response [4]. According to Wang et al. [5], diabetes is a polygenic disorder with over 50 loci known to affect disease susceptibility, where the strongest association to T1D is located within the HLA class I and II gene. It has also been suggested that epigenetic modifications (caused by e.g. environmental factors) have a contribution as well.

Type 2 Diabetes (T2D) results from lowering insulin resistance in peripheral tissue, usually due to an unhealthy lifestyle and/or obesity. The β-cells compensate by upregulation of insulin secretion. Whether these are able to do so is based on their genetic constitution, which determines whether an obese individual develops diabetes or not [6]. Therefore, a deficiency in insulin secretion has its contribution as well. New studies suggest that the subsequent occurring chronic systemic inflammation will eventually lead to an autoimmune response, resulting in loss of pancreatic β-cells [7].

The effects of having malfunctioning glucose homeostasis is characterized by polydipsia (excessive thirst), polyphagia (excessive eating) and polyuria (excessive urination) in diagnosis [8]. Persistent misregulation leads to a variety of secondary complications such as retinopathy causing blindness, nephropathy leading to renal failure and neuropathy that can result in amputation [1]. It has also been suggested that having type 1 diabetes is accompanied by sleep disruption, thereby increasing chances of developing cardiovascular and microvascular disease [9].

In 2015, the International Diabetes Federation reported that an estimated 415 million adults suffer from diabetes mellitus globally, suggesting in 2040 this number will be 1.5 times larger. An estimated 36 million people were living with T1D in 2015, half a million of which were children. In that same year nearly 5 million people died from this chronic disease [10]. The World Health Organization predicts that diabetes will be the 7$^{th}$ leading cause of death in 2030 [11]. It is therefore of great importance to enhance management of diabetes, eventually leading to effective treatment methods. In this thesis, however, the emphasis is put on type 1 diabetes.

## 1.2 TREATMENT OPTIONS FOR TYPE 1 DIABETES

As mentioned in paragraph 1.1, the exact mechanism behind the development of type 1 diabetes is not fully understood yet. However, it has been thought to be a predictive disease since islet autoantibodies

could be present months or years before symptomatic onset, and dysregulation of metabolism (detectable in serum) precedes islet autoantibodies positivity [12]. Usually when symptomatic onset is detected, 90-95% of β-cells have already been lost, making it difficult to treat the disease. Therefore, some studies propose to predict disease susceptibility using biomarkers combined with first-degree relative genetic history [13]. Additionally, several large trials are currently investigating methods to prevent or delay clinical manifestation, including primary prevention studies (dietary trials), secondary prevention studies (immune modulatory therapies) and tertiary intervention (immune suppression) [14].

Currently there is no cure for diabetes type 1. Glycemic control can be improved by administration of exogenous insulin through daily injection or computerized pumps. The latter could be an insulin pump, continuous glucose monitoring or those two combined. Although it has been shown that these methods increase life expectancy significantly [15], the lack of physiological response still remain, resulting in hyper- or hypoglycemia episodes [16] and possible secondary complications.

The ultimate cure for T1D lies in restoring a patient's ability to produce its own insulin. This motive result in alternative therapeutic options that can be found in the field of regenerative medicine. Efforts have been made to regenerate β-cells *in vivo*, either by converting related cell types (other pancreatic cell types, and cells of the liver and gastrointestinal tract) into β-cells through transdifferentiation or by promoting expansion of residual β-cells in diabetic patients [17]. Although some studies show promising results, these methods are not designed to replace faulty tissue completely. Intrahepatic allogeneic transplantation of islet-cells does, and has been proven to be a successful therapy. However, availability of donated human cadaveric pancreata (from which the islets are taken from) is limited, especially as single patients often require more than one donor. In addition, islet transplant requires lifelong immune suppression. Another complication would be declining long-term insulin independence, due to progressive islet loss in the posttransplantation period when transplanted intrahepatically. The decrease in islet mass loss is caused by mechanical stress and lack of oxygen due to impaired vascularization. Therefore, current studies are looking for appropriate scaffolds that provide a protective environment while maintaining islet morphology and islet functionality extrahepatically [18, 19].

### 1.3 APPLICATION OF hPSCs FOR T1D TREATMENT

Islet transplantation is a promising method to cure type 1 diabetes. With the introduction of human embryonic stem cells (hESCs) any cell type from the germ line can theoretically be generated. This would solve the shortage of cadaveric pancreata donors. However, ethical issues remain with the use of hESCs and lifelong immune suppression is then still required. In 2006 Takahashi and Yamanaka [20] succeeded to reprogram human somatic cells (in their research skin fibroblasts) using viral transduction of pluripotency-related genes. These so called induced pluripotent stem cells (iPSCs) could solve the ethical and graft rejection issue, since these cells can be harvested from the patient's own somatic cells (besides fibroblast e.g. from keratinocytes, also peripheral blood cells and urine samples [21]).

Based on studies of the past decades, it seems that human pluripotent stem cells (hPSCs) like hESCs and human iPSCs are potential sources for β-cells regeneration. By inducing sequential stimulation or inhibition of key signaling pathways through growth factors and small molecules, early protocols have successfully differentiated pancreatic progenitor cells *in vitro*, which can mature *in vivo* into insulin-producing cells that resemble mature human β-cells [17]. Recently, protocols have successfully been developed for differentiating hESCs as well as T1D human iPSCs into β-like cells *in vitro* [22, 23]. These functional β-like cells can produce insulin while responding to fluctuations in blood sugar levels. In

addition to their clinical value, these cells can provide *in vitro* studies of metabolism of β-cell function as well as novel drug-screening platforms for diabetes.

Current human clinical trials are ongoing, using hPSCs derived pancreatic progenitor cells to mature *in vivo* in non-endogenous sites into cells that resemble the pancreas, which is able to produce its major hormones such as insulin, glucagon and somatostatin (ViaCyte Inc. clinical trials identifier: NCT02239354). However, clinical application of hESCs and iPSC have their own risks and limitations. Pluripotent stem cells are characterized as being able to form teratomas. Therefore, it is essential that graft tissue does not contain undifferentiated cells [24]. In addition, protocols using viral vectors for iPSC development could lead to viral integration into the endogenous genes, therefore risking unwanted activation of genes that could result in cancer. Studies are still on going to develop and enhance cell reprogramming techniques that involve a non-genomic integration. According to Warren et al. [25], it is possible to produce iPSCs by applying a non-integrating strategy based on administration of synthetic mRNA modified to overcome innate antiviral responses. To avoid possible severe complications for T1D patients, current clinical trials utilize encapsulated immune-protecting devices to carry the hPSCs derived pancreatic progenitor cells.

## 1.4 PANCREATIC β-CELLS DEVELOPMENT

In the past decade, studies have been trying to develop a protocol which allows generation of insulin-producing β-cells that resemble mature residual β-cells as accurate as possible with glucose homeostasis. In order for pancreatic β-cells to produce insulin, glucose is sensed and taken up primarily by its glucose transporters GLUT2 [26]. The subsequent glucose metabolism generates glutamate and ATP, with the latter one causing $K_{ATP}$ channels to close. This results in $Ca^{2+}$ influx through $Ca^{2+}$ channels, leading to depolarization and exocytosis of insulin.

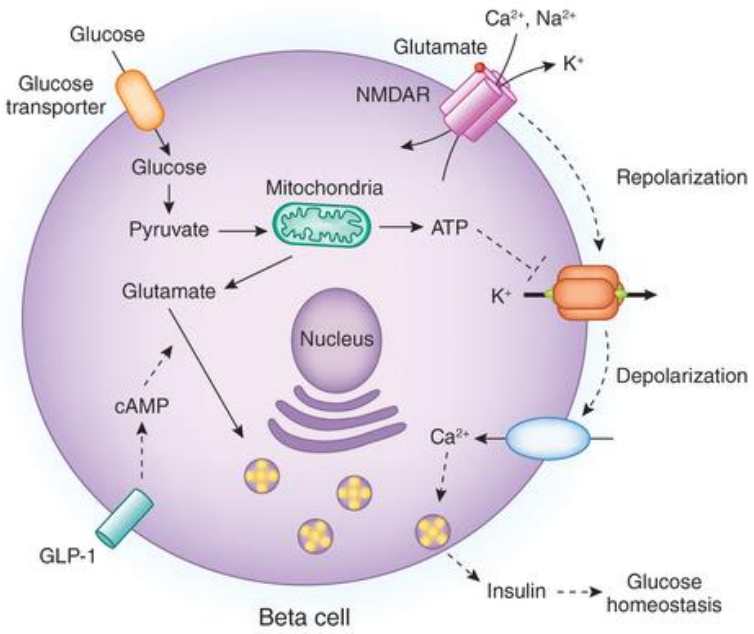



*Figure 1 - β-cell insulin secretion pathways [27].*

The GLP-1 receptor on the surface of β-cells modulate nutrient-stimulated insulin secretion through the incretin signaling pathway. More specifically, stimulations are brought by hormones i.e. the GLP-1 hormones from gut L cells in the presence of glucose and other nutrients in the gut lumen [6]. Together with glutamate concentrations, the insulin secretion can be amplified. Moreover, glutamate (which is also produced by the glucagon-producing counterpart of β-cells) may activate receptor NMDAR to open $K_{ATP}$ channels, resulting in repolarization and inhibition of insulin secretion [27]. This whole process can be visualized in Figure 1.

In the past decade, Rezania et al. [22, 28-30] have been developing and subsequently improving protocols in which hPSCs go through seven stages (S1-S7) in order to develop glucose responsive insulin-producing β-like cells *in vitro*. Subpopulations of the S7 cells are able to mimic most of the characteristics of residual mature pancreatic, including GLP-1 receptor expression, intact incretin signaling pathways and functional voltage-gated $Ca^{2+}$ channels. One major difference of these β-like cells, however, is a deficiency in glucose metabolism and/or the $K_{ATP}$ channels triggering of electrical activity. This would cause a slower $Ca^{2+}$ response to glucose, therefore a delay (and smaller in magnitude) of glucose-stimulated secretion of insulin. Despite these differences from adult human beta cells, the S7 cells are able to reverse diabetes in mice rapidly within 40 days.

The seven-staged protocol mimics the blueprint of the embryonic development of pancreatic cells [1]. Cells of the fertilized embryo first select which germ layer fate to acquire: mesoderm, ectoderm or endoderm. For generation of pancreatic cells, the endodermal pathway is activated, whereas the other two pathways will be inhibited. This phenomenon is a response to the activation of the TGF beta signaling and canonical Wnt signaling pathway by signaling of adjacent developing tissue during gastrulation [16, 31]. As embryo folding continues, the so called produced DE cells or definitive endoderm cells (S1) move towards the formation of the primitive gut tube (S2), following the posterior foregut (S3) and subsequently pancreatic endoderm (pancreatic progenitor cells), as can be seen in Figure 2 [22].

The pancreas is composed of two major compartments: the exocrine compartment (ductal and acinar cells) and the endocrine compartment (the islets of Langerhans, including insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, ghrelin-releasing ε-cells and pancreatic polypeptide-releasing PP cells). As pancreatic progenitor cells select the endocrine fate (S5), they become one of the five mentioned endocrine cells. For insulin production, the formation of β-cells (S6-S7) is crucial.

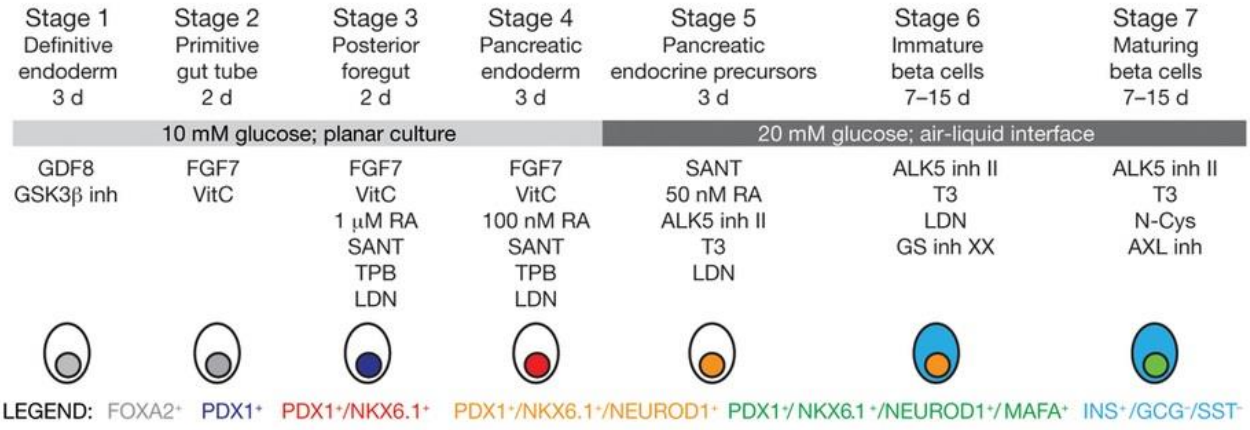



*Figure 2 – Overview of the seven-staged (S1-S7) protocol for developing S7 cells, representing insulin-producing β-like cells. In each stage expressed key markers define its cell type [22].*

## 1.5 DEFINITIVE ENDODERM FORMATION

As mentioned before, the formation of definitive endoderm is regulated by two important pathways during gastrulation: the TGF signaling pathway and the canonical WNT signaling pathway. In the TGF beta signaling pathway, in particular growth factors Nodal, Activin A and GDF-8 [29, 32, 33] are proven to play an important role in definitive endoderm differentiation. However, this same signaling pathway is also responsible for maintaining pluripotency by blocking expression of mesodermal and neuroectodermal markers with the pluripotency factor NANOG interacting with Smad2/3 [34]. It has not been fully clear yet why these two cell fates are being controlled by the same pathway. According to Brown et al. [35], both are dependent on the location of Smad2/3 to bind on the genome with its protein partners (ex. NANOG). Upon endodermal differentiation however, NANOG expression decreases [35, 36], suggesting subsequently Smad2/3 to bind with different protein partners on different locations on the genome. In case of interacting with endodermal protein partners, transcriptional activity of a broad number of endodermal genes can therefore be controlled.

In conjunction of the Nodal/Activin A signaling pathway for endodermal formation is the WNT signaling pathway. This controls the expression of endodermal and mesodermal markers, dependent on the amount of GSK3β that is inhibited. According to Naujok et al. [37], at high concentration of GSK3β inhibitor CHIR-99021, ESCs were directed to mesodermal cells, whereas at low concentrations mesodermal and endodermal cell fate were permitted. Essentially, when inhibiting the GSK3β, the (canonical) WNT pathway will be activated by preventing complex formation and increasing of β-catenin levels [38]. This allows β-catenin to bind with transcriptional factor SOX17, which potentiates its transcriptional activation target genes (such as FOXA2) [39]. When β-catenin is bonded to Smad2/3 however, mesodermal cell fate will be stimulated. Therefore, it has been suggested that both the Nodal/Activin A signaling pathway as well as the WNT signaling pathway share the same downstream effectors: Smad2 and Smad3 (Smad2/3).

According to Miller et al. [40], CXCR4 has a role in the development of stem cells (such as embryonic stem cells) as well as its progenitors such as definitive endoderm [30], ranging from an initial role in regulating the migration and functions of stem cells to tissue specific effects on differentiated cells.

When both pathways are activated, pluripotent stem cells such as iPSCs and ESCs are able to differentiate into definitive endoderm, expressing the right gene markers: NANOG, SOX17, FOXA2 and CXCR4 [29, 41]. According to Bruin et al. [29], using GDF-8 instead of Nodal/Activin A induces higher percentages of CXCR4 expression. Therefore, it is recommended to use CHIR-99021 and GDF-8 for DE differentiation from pluripotent stem cells. In the past protocols described using WNT3A to affect the WNT pathway. However, CHIR-99021 has been shown to be more effective in feeder- and serum-free conditions [42]. In addition, keeping cell density low during endoderm induction is important for the efficiency.

According to Hoveizi et al. [36], it has been shown that there is an increase of proliferation, differentiation and survival of stem cells when using Matrigel as a basement membrane for the ESCs. Matrigel is a natural extracellular matrix (usually containing laminin, type IV collagen, entactin/nitrogen).

## 1.6 THESIS OUTLINE

Recently, the Developmental BioEngineering (DBE) research group of the University of Twente succeeded in developing a micro-fabricated scaffold as a niche for β-cells to guarantee islet retention and long-term insulin independence with extrahepatic transplantation in diabetic induced mice [19]. Subsequently, the group wants to do more research with human β-cells. However, sufficient quantities of human β-cells are quite difficult and expensive to obtain. Therefore, in my thesis I test a proven protocol of Rezania et al. from 2014 [22] with their past protocols as extra support [28-30] (all combined, from now on referred to as the proven protocol) by producing insulin-positive β-cells using human pluripotent stem cells, specifically human ESCs. The protocol is mainly a guideline, since the DBE research group has access to other human embryonic stem cell lines as well as tools from different sources. Note that embryonic stem cell populations harvested from different sources responses differently from each other. Therefore, it is important to adjust the protocol for effective differentiation results.

Since following the proven protocol takes at least 30 days divided over 7 stages (see Figure 2), it is unrealistic for me to evaluate the entire protocol for my bachelor's thesis. Therefore, the main goal of this study is to differentiate a sufficient amount of definitive endoderm cells (Stage 1/S1 differentiation cells) from human ESCs *in vitro* using the proven protocol as a guideline. Subsequently, three different conditions are used to be able to find the best DE differentiation conditions for our ESCs. To achieve this goal, the following goals must be met;

- The S1 differentiation cells need to express gene markers for definitive endoderm (CXCR4, SOX17, FOXA2 and NANOG).
- CXCR4 expression should increase in DE cells compared to ESCs.
- NANOG expression should decrease in DE cells compared to ESCs.
- The correct concentration of CHIR-99021 should be used in culturing media.
- The expression percentage of CXCR4/FOXA2 is approximately 98-99%.

Since it is a proven protocol acknowledged by many studies, I hypothesize that our human ESCs can be differentiated into definitive endoderm in high concentrations with high gene marker expression rates.

# 2 MATERIALS AND METHODS

## 2.1 EXPERIMENTAL DESIGN

*General DE differentiation timeline*

The whole differentiation process of ESCs to DE cells (S1 differential cells) took 5 days. On the first day cells were passaged on the desired density. After 48 hours (on day 3), the actual differentiation was initiated for three days straight using S1 Differentiation Medium. On day 6 RNA isolation was performed to synthesize cDNA for qPCR analysis. In addition, some differentiated cells were passaged to be maintained for immunostaining and FACS. More details on the exact procedures will be explained in the following paragraphs.

*Differentiation conditions*

In order to evaluate the proven protocol, three different conditions were tested in duplicate following the "*General DE differentiation timeline*" on three 12-wells plates, referred to as: The Cell Density plate, the NANOG plate and the CHIR-99021 plate. The Cell Density plate tested four different ESC concentrations (110K, 140K, 150K and 170K cells/$cm^2$) that were passaged on day 1. The NANOG plate was used to confirm the decrease of NANOG expression in DE cells compared to undifferentiated ESCs. Despite the name of the plate suggests, increase of CXCR4 expression levels were tested as well. Therefore, some undifferentiated ESCs underwent RNA isolation first before following the "*General DE differentiation timeline*" to start DE differentiation. After confirming pluripotency through NANOG expression in ESCs (and the presence of CXCR4 expression), these same passage line was plated on 140K cells/$cm^2$. On day 4 (in other words, 1 day of actual differentiation has passed) some cells underwent RNA isolation for gene expression analysis after cDNA synthesis and performing qPCR. These included undifferentiated ESCs and undergoing differentiated cells. The same happened on day 6 (basically, three days of actual differentiation has passed), but only for differentiated cells.

As mentioned before, ESCs are directed to mesodermal cells when exposed to high concentration of GSK3β inhibitor CHIR-99021, whereas at low concentrations mesodermal and endodermal cell fate is permitted [37]. However, no primers for mesodermal cells were available to use. Therefore, the CHIR-99021 plate was designed to test if different concentrations of CHIR-99021 (0.6 μM, 1.0 μM, 1.4 μM and 2.0 μM) had any influence on DE differentiation only, since this GSK3β inhibitor affects expression of DE gene markers as well. ESCs on this condition plate were also plated at 140K cells/$cm^2$. However, during execution of the experiment cell populations seem more confluent than cells passaged on 170K cells/$cm^2$ on day 3, suggesting the cell density is at least higher than 170K cells/$cm^2$ (>170K).

In this design, ESCs were defined as both positive as negative control. Since ESCs don't express SOX17 and FOXA2, for these gene markers particularly it is a negative control. However, no positive control for these target genes (e.g. cDNA of DE) were available for this experiment. For the expression evaluation of NANOG and CXCR4, these particular gene markers were defined as positive control since it is being expressed in large and small rates respectively in ESCs.

## 2.2 MATERIAL COLLECTION & PREPARATION

*Matrigel and Vitronectin coated plates*

For testing three different DE differentiation conditions plates, three 12-wells plates (Greiner Bio-One, Cat#M8687) were coated with 1:121 diluted Matrigel (Corning, Cat#354230) in 1x DMEM/F-12 (Gibco, Cat#31331028). In addition, multiple 6-wells plates (Greiner Bio-One, Cat#657160) were coated for 1 hour at room temperature with 1:101 diluted Vitronectin (Thermo Fisher Scientific, Cat#A14700) in DPBS without $Ca^{2+}$ and $Mg^{2+}$ (Gibco, Cat#14190144) for maintaining the ESC line.

*ESCs (general maintenance)*

Embryonic stem cells were obtained from Leids Universitair Medisch Centrum (Leiden, The Netherlands) called Marcelo ΔN3, from which I started with passage P13+46. The ESCs were cultured on 1:101 diluted Vitronectin treated 6-wells plates in Essential 8 (E8) Medium (Life Technologies, Cat#A1517001) supplemented with 1% Penicillin/Streptomycin (Thermo Fisher Scientific, Cat#15140) solution. This medium is refreshed daily with 2 ml each well. At ~70-80% confluency, cultures were rinsed once with 2 ml 1x DPBS without $Ca^{2+}$ and $Mg^{2+}$ following incubation for 3 minutes at 37 °C 3-5% $CO_2$ with 1 ml 0.5 mM EDTA (0.5 M UltraPure™ EDTA, pH 8.0, Thermo Fisher Scientific, Cat#15575020) in DPBS solution to detach cells from the plate. After rinsing the released single cells with E8 Medium (forming a single cell suspension), the harvested cells were counted and subsequently passaged on a new 1:101 diluted Vitronectin treated 6-wells plate. ESCs were incubated at 37 °C 3-5% $CO_2$.

*S1 Differentiation Medium*

Differentiation from ESCs to DE requires special medium called S1 Differentiation Medium. This contains 1.5 g/l sodium bicarbonate (Sigma-Aldrich, Cat# S6297), 0.5% BSA without fatty-acid (Sigma-Aldrich, Cat#A8806), 10 mM final Glucose (Gibco, Cat#15023021) concentration, 1x Glutamax (Thermo Fisher Scientific, Cat#35050), 1% Penicillin/Streptomycin and 1x MCDB 131 medium (Gibco, Cat#10372019). After assembling the medium, a syringe was used with a filter tip to sterilize the medium, following storage at 4 °C.

## 2.3 CELL CULTURING FOR DE DIFFERENTIATION

*ESCs (experiment day 1-2)*

On day 1 single cell suspension of ESCs were cultured on 1:121 diluted Matrigel treated 12-wells plates in E8 Medium with the desired cell density (dependent on the differentiation condition according to my experimental design; ranging from 110K-170K cells/$cm^2$).

*S1 differentiation cells (experiment day 3-5)*

After 48 hours, the E8 Medium of the ESCs were replaced with S1 Differentiation Medium for three days straight. On day 3 specifically, 100 ng/ml GDF-8 (PeproTech) in distilled water (Gibco, Cat#15230) solution was added for all three the differentiation condition plates. In addition, 1.0 μM CHIR-99021 (Axon Medchem, Cat#Axon1386) was added in the cell density and NANOG plate as well; the CHIR-99021 plate used different concentrations (ranging from 0.6-2.0 μM). On day 4, again 100 ng/ml GDF-8 was added for all three the differentiation condition plates. Furthermore, 0.1 μM CHIR-99021 was added in the cell

density and NANOG plate; the cells of the CHIR-99021 plate received 10% of the used CHIR-99021 concentrations of day 3 for the same wells (ranging from 0.06-0.2 μM). On day 5, again 100 ng/ml GDF-8 was added for all three the differentiation condition plates.

### 2.4 RNA ISOLATION AND QPCR

Immediately after the required differentiation period has passed, the cells of the three differentiation plates were killed for DE gene markers expression analysis through qPCR on the same day (except for the day 1 and day 4 conditions of the NANOG plate, which happened in the past already, but followed the same steps as described here). Therefore, first the cells underwent RNA isolation using the RNeasy® Mini Kit as directed by manufacturer (Qiagen, Hilden, Germany). Quantification of total extracted RNA was measured with NanoDrop™ (Thermo Fisher Scientific). A total of 1 μg RNA was used per sample for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad) as directed by manufacturer in a MJ Mini Thermal Cycler (Bio-Rad) under the following conditions: 25 °C for 5 min; 42 °C for 30 min, 85 °C for 5 min and 4 °C for forever. After incubation, the cDNA samples were 1:15 diluted in $ddH_2O$.

QPCR was performed using SYBR green Supermix (Bio-Rad) and the synthesized cDNA, as well as the following used primers (ordered from IDT):

- CXCR4 Forward: 5'- ACG CCA CCA ACA GTC AGA G -3'
  CXCR4 Reversed: 5'- AGT CGG GAA TAG TCA GCA GGA -3'
- SOX17 Forward: 5'- GGC GCA GCA GAA TCC AGA -3'
  SOX17 Reversed: 5'- CCA CGA CTT GCC CAG CAT -3'
- FOXA2 Forward: 5'- GGA GCA GCT ACT ATG CAG AGC -3'
  FOXA2 Reversed: 5'- CGT GTT CAT GCC GTT CAT CC -3'

The following primers were ordered from Sigma Aldrich:

- NANOG Forward: 5'- GAT TTG TGG GCC TGA AGA AA -3'
  NANOG Reversed: 5'- CAG GGC TGT CCT GAA TAA GC -3'
- HARP Forward: 5'- CAC CAT TGA AAT CCT GAG TGA TGT -3'
  HARP Reversed: 5'- TGA CCA GCC CAA AGG AGA AG -3'

Per qPCR sample, 12 μl Mastermix was used (10 μl SYBR green + 1 μl primer forward + 1 μl primer reversed) and 8 μl cDNA in $ddH_2O$ solution. Primer HARP was used as a reference gene (qPCR positive control). Negative controls for qPCR were NTC (qPCR sample with cDNA substituted with $ddH_2O$) and NAC (qPCR sample with primers forward and reverse substituted with $ddH_2O$). Analysis were performed with the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) under the following thermal conditions: 1 cycle of 95 °C for 3 min, 40 cycles of 95 °C, 60 °C and 72 °C for 10, 15 and 15 seconds respectively, 1 cycle of 95 °C for 10 seconds, melt curve 65 °C - 95 °C with an 0.5 increment 5s/°C. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method, normalized on the undifferentiated ESCs (with HARP as the reference gene). Since there is no SOX17 and FOXA2 (DE markers) expressed in the undifferentiated ESCs (for these target genes ESCs acts as negative control), different S1 differential cells conditions are normalized to S1 differential cells from the Cell Density plate 110K cells/$cm^2$ relative to HARP (while looking to the DE target gene of interest). The reason for this approach was due to the fact that there was no positive control, specifically cDNA of DE tissue, available to compare DE gene marker expressions in S1 differential cells with. The CHIR-99021 plate had the same approach: The S1

differentiation cells were normalized to 0.6 condition, as well relative to HARP (while looking at the DE target genes of interest). With the NANOG plate however, gene expression was normalized to the undifferentiated ESCs, relative to HARP, looking at NANOG only. For the qPCR results, the mean and standard deviation (SD) were calculated using MS Excel 2016.

### 2.5 FLUORESCENCE-ACTIVATED CELL SORTING

To determine the efficiency of S1 differentiation in terms of percentage of cell populations expressing the correct DE gene markers, Fluorescence-Activated Cell Sorting (FACS) was performed. Before RNA isolation happened on day 6 of the experiment, some S1 differentiation cells from the 140K cells/$cm^2$ were maintained on a 6-wells plate for FACS analysis. Originally, FACS analysis on CXCR4 and SOX17 was planned on S1 differentiation cells. However, negative isotype control was not available, following using ESCs as the negative control. Before harvesting these cells, microscopic observation showed many cell deaths. Combined with not having a negative isotype control, there was no use to test on antibody SOX17 since no valid results would come out. Therefore, CXCR4 antibody was used to obtain a suggestion if ESCs and S1 differentiation cells both express CXCR4. Each sample was washed once with 2 ml DPBS, following incubation with 0.5 ml 0.25% trypsin-EDTA (Gibco, Cat#252000) for 5 minutes at 37 °C 3-5% $CO_2$. Subsequently, 1.5 ml S1 Differentiation Medium (E8 Medium for ESCs) was added to inactivate the trypsin-EDTA. At least $1x10^5$ cells were then transferred to a tube, centrifuged for 7 minutes at 300g, the supernatant removed and cells fixated with 10% formalin for 20-30 minutes. After centrifuging, cells were washed twice with 2 ml DPBS. Next, cells were incubated with 2 ml of (cold) blocking buffer (DPBS, 5% BSA) for 40 minutes at room temperature, following centrifuging for 3 minutes at 3000 rpm 4 °C. Since primary human CXCR4 PE-conjugated antibody (R&D Systems, Cat#FAB170P-025) were used by the proven protocol [29], 1:30 diluted 50 µL of this antibody was added to each sample. The dilution buffer consisted of DPBS, 1% BSA, 1% normal donkey serum. However, since normal donkey serum was not available, it was substituted with BSA. Cells were incubated for 1 hour at room temperature in the dark before washed twice with 2 ml washing buffer (0.1% BSA in DPBS). Cells were then re-suspended with 300 µl DPBS and transferred to a FACS tube for FACS analysis using a flow cytometer (FACS Calibur, BD Biosciences).

### 2.6 IMMUNOCYTOCHEMISTRY

In order to evaluate if the DE gene markers of S1 differentiation cells were expressing at the correct location, immunocytochemistry was performed in ESCs and S1 differentiation cells. Each sample was washed twice with 2 ml DPBS, and fixated in 10% formalin for 20-30 minutes. Subsequently, cells were washed twice with 2 ml DPBS and incubated with 2 ml of (cold) blocking buffer (DPBS, 10% normal donkey serum) for 45 minutes at room temperature. Since normal donkey serum was not available, it was substituted with BSA. Next, the blocking buffer was removed and stored in wash buffer (0.1% BSA in DPBS). After three days, wash buffer was then removed from each sample and 2 ml DPBS was added with 50 µL of 1:30 diluted (same dilution buffer was used as FACS) primary human CXCR4 PE-conjugated antibody, following incubation for 1 hour at room temperature in the dark before washed twice with 2 ml washing buffer (0.1% BSA in DPBS). Microscopic observations were performed with Invitrogen™ EVOS™ using filter RFP and Texas Red® since these were the only available filters near by the excitation and emission wavelengths of PE fluorophore.

# 3 RESULTS

## 3. 1 S1 DIFFERENTIATED CELLS EXPRESS DE GENE MARKERS

To assess if ESCs are able to develop into DE cells, differentiation of ESCs passaged with different cell densities (100K, 140K, 150K, 170K and >170K cells/cm$^2$) on day 1 of the experiment were evaluated. On day 3 (48 hours after the passaging occurred) cell populations of 110K, 140K, 150K, 170K and >170K cells/cm$^2$ showed a confluency of 40-50%, 85-95%, 85-90%, ~98% and 100% respectively (see Figure 3). On day 4 cell populations of the 110K and 140K condition, confluency increased to 85-90% and 95-99% respectively. However, cells passaged on a higher cell density reached a 100% confluency. All cells of the cell density conditions had a confluency of 100% after three days of differentiation (day 5).

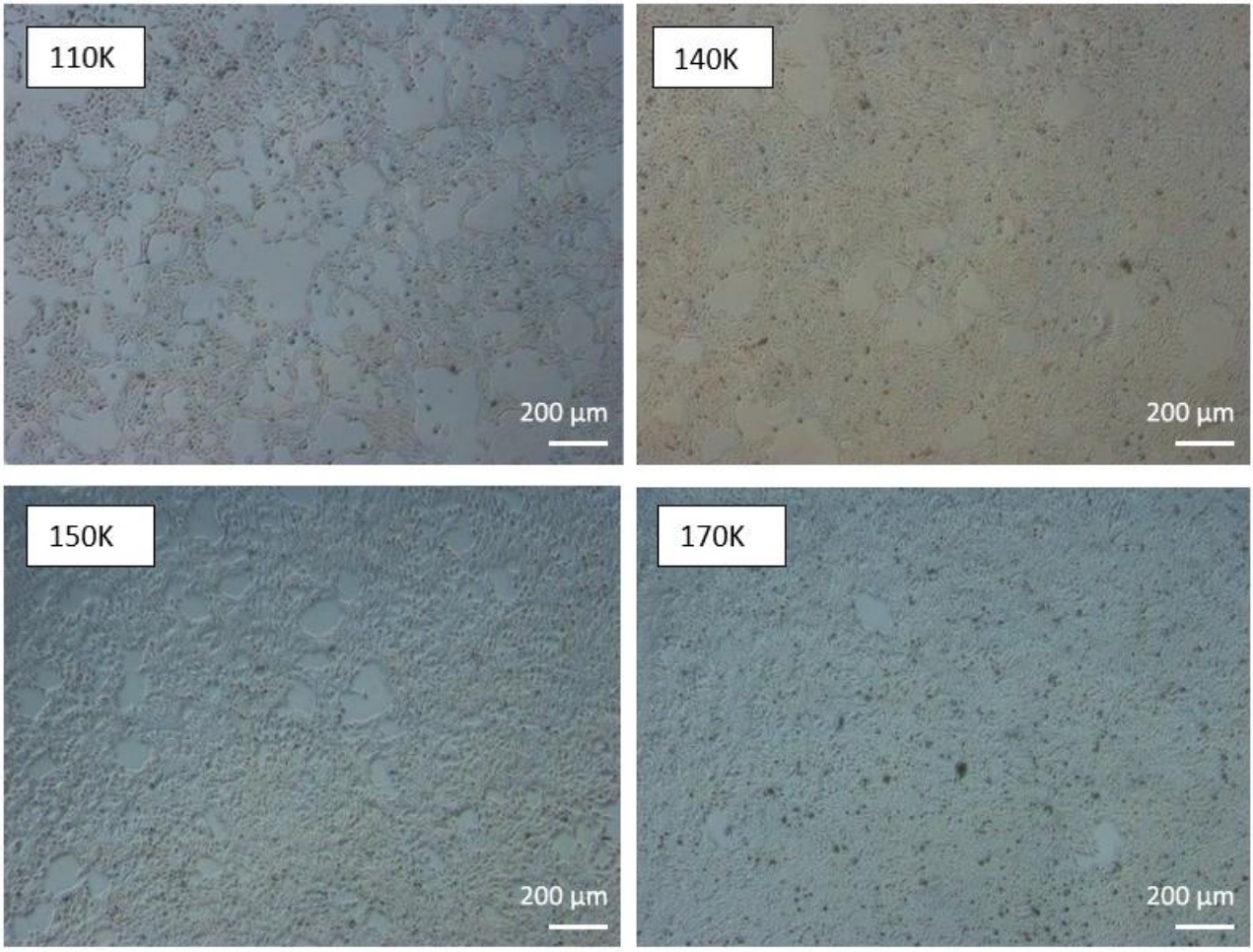



*Figure 3- ESCs (P13+54) on day 3 of the experiment before receiving S1 Differentiation Medium for the first time. The cell populations are showing a confluency of 40-50%, 85-95%, 85-90% and ~98% for increasing cell densities respectively. 40x magnification is shown.*

Based on qPCR analysis it was shown that the differentiated cells were expressing the DE gene markers CXCR4, SOX17, FOXA2 and NANOG when initially the ESCs were passaged according to the above-mentioned cell densities. However, the rate at which the expression occurs is different for every condition. In Figure 4 it can be shown that DE gene marker expression was higher for 140K and 150K, whereas expression rates of CXCR4, SOX17, FOXA2 and NANOG were relatively lower for S1 differentiation cells when initially plated on cell densities lower than 140K cells/cm$^2$ or higher than 150K cells/cm$^2$.

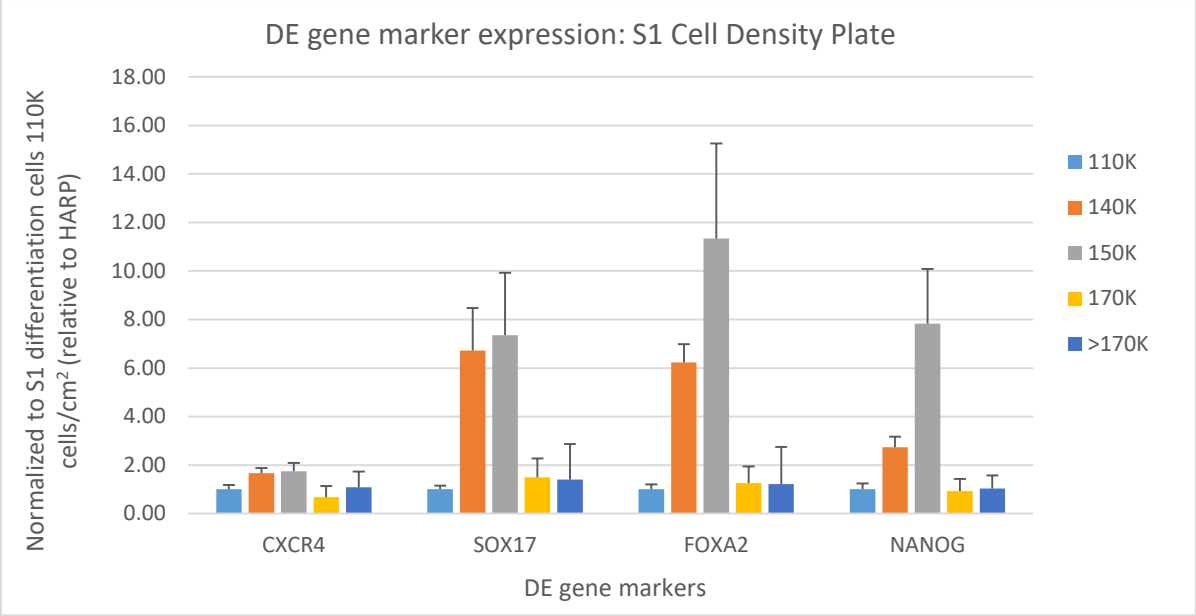



*Figure 4 - DE gene marker expression of the Cell Density condition plate, normalized to S1 differentiation cells (relative to housekeeper HARP), where its progenitor cell was initially passaged with a cell density of 110K cells/cm2. Data is shown as mean ±SD, and expression rate is higher in 140K and 150K than the rest of the cell density conditions.*

Furthermore, the expression levels of DE gene markers SOX17 and FOXA2 for ESCs, that did not undergo any differentiation, was insignificant since CT values of qPCR analysis are >35 cycles (see Figure 5). However, expression of CXCR4 and NANOG did occur. More details on these two target genes will be discussed in the next two paragraphs.

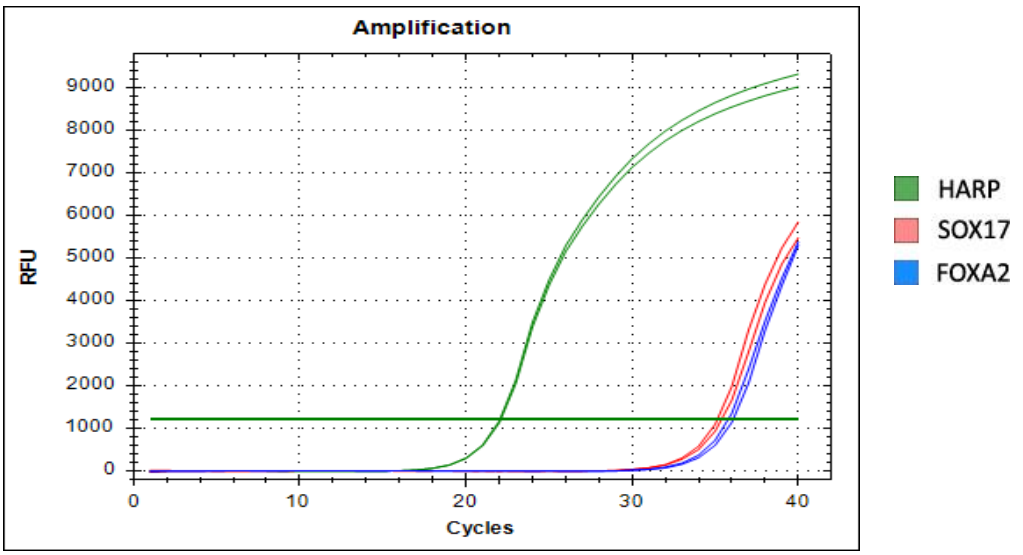



*Figure 5 – QPCR amplification curves of ESCs for target genes SOX17 and FOXA2, with HARP as the positive control. CT values of SOX17 and FOXA2 are >35 cycles, which suggests no expression at all.*

## 3.2 CXCR4 EXPRESSION INCREASES IN S1 DIFFERENTIATED CELLS

To assess if the S1 differentiation cells express more CXCR4 compared to its undifferentiated progenitors (ESCs), qPCR was used to determine mRNA expression levels of CXCR4. When comparing the S1 differentiation cells from the Cell Density plate with the ESCs, it showed a significant increase of CXCR4 gene expression (up to 13.63±2.7 times more with condition 150K) with cells exposed to S1 Differentiation Medium. However, CXCR4 expression is lower for conditions 110K and 170K compared to 140K and 150K. S1 differentiation cells from the CHIR-99201 plate (>170K cells/cm$^2$) as well expressed significant more of the target gene than the undifferentiated ESCs, however overall high error bars are shown (Figure 6).

Expression levels of CXCR4 were kept track of as well during the development of the S1 cells exposed to differentiation medium of cell density condition 140K cell/cm$^2$. On the second day of differentiation of the S1 cells (d4 S1), only 6±3% of the CXCR4 was expressed. On the third day, however, expression levels increased significantly up to 13 times more than what ESCs normally expresses.

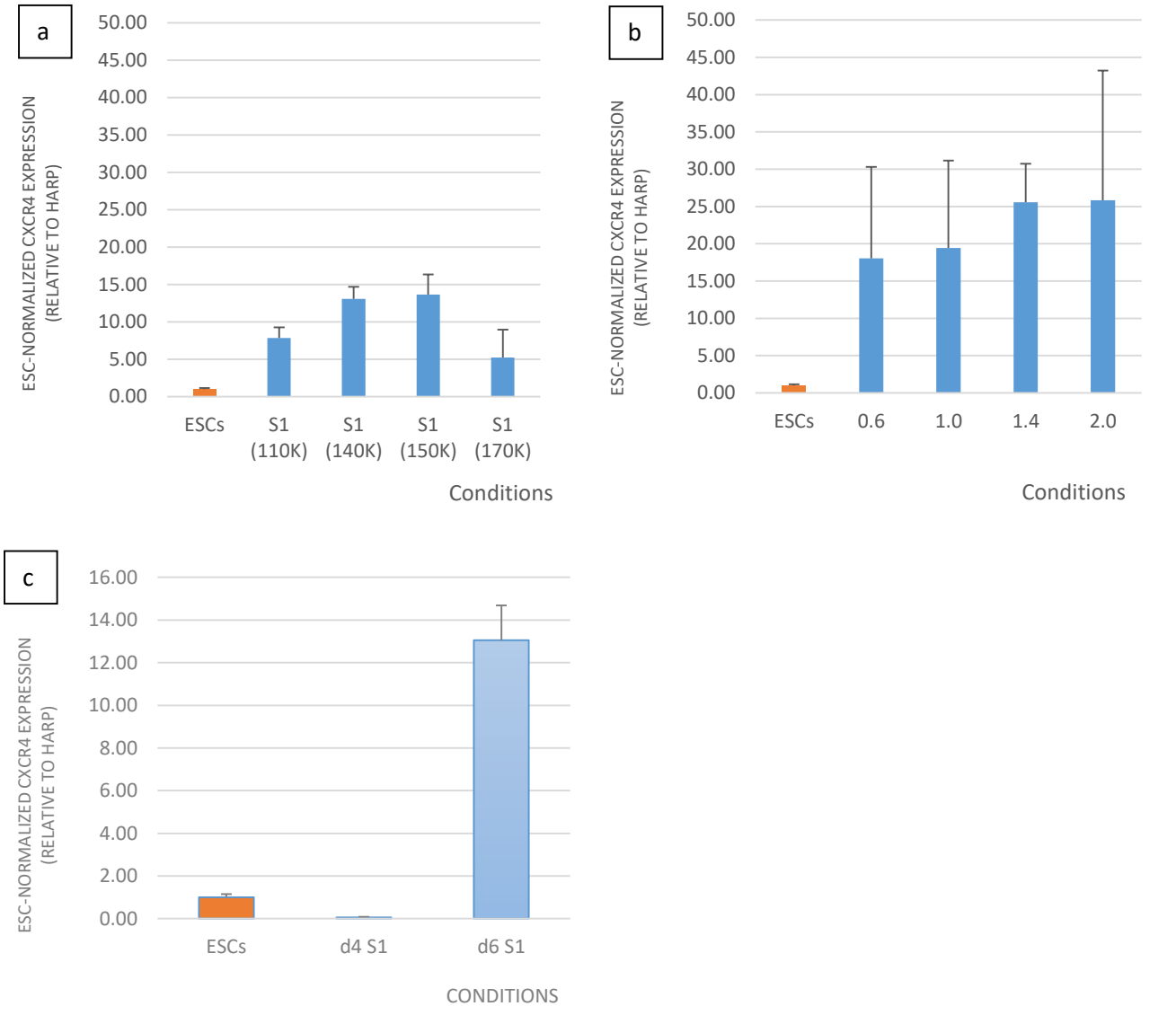



*Figure 6 – CXCR4 expression of S1 differentiation cells (normalized to ESCs, relative to housekeeper HARP) from (a) the Cell Density and (b) CHIR-99021 plate. (c) CXCR4 expression was kept track as well during S1 cell development for day 1 (d4 S1) and day 3 (d6 S1) of the differentiation. D4 S1 and d6 S1 were from the 140K condition. Data is shown as mean ±SD, with the error quite high for the CHIR-99021 plate.*

## 3.3 NANOG EXPRESSION DECREASES IN S1 DIFFERENTIATED CELLS

To determine whether NANOG expression decreases in S1 differentiation cells relative to ESCs, qPCR was used to determine mRNA expression levels of NANOG. When comparing the S1 differentiation cells from the Cell Density plate with the ESCs, it showed a decrease of NANOG gene expression for conditions 110K (0.28±0.07), 140K (0.76±0.12) and 170K (0.31±0.57) with cells exposed to S1 Differentiation Medium. However, condition 150K showed an increase of 2.17±0.63 times. A decrease of NANOG expression was observed at the CHIR-99021 plate (>170K cells/cm$^2$) as well for all its different conditions (see Figure 7).

Expression levels of NANOG were kept track of as well during the development of the S1 cells exposed to differentiation medium of cell density condition 140K cell/cm$^2$. On the second day of differentiation of the S1 cells (d4 S1), a 40±77% increase of NANOG expression was possibly observed (hence SD value is quite big). On the third day, however, expression levels decreased.

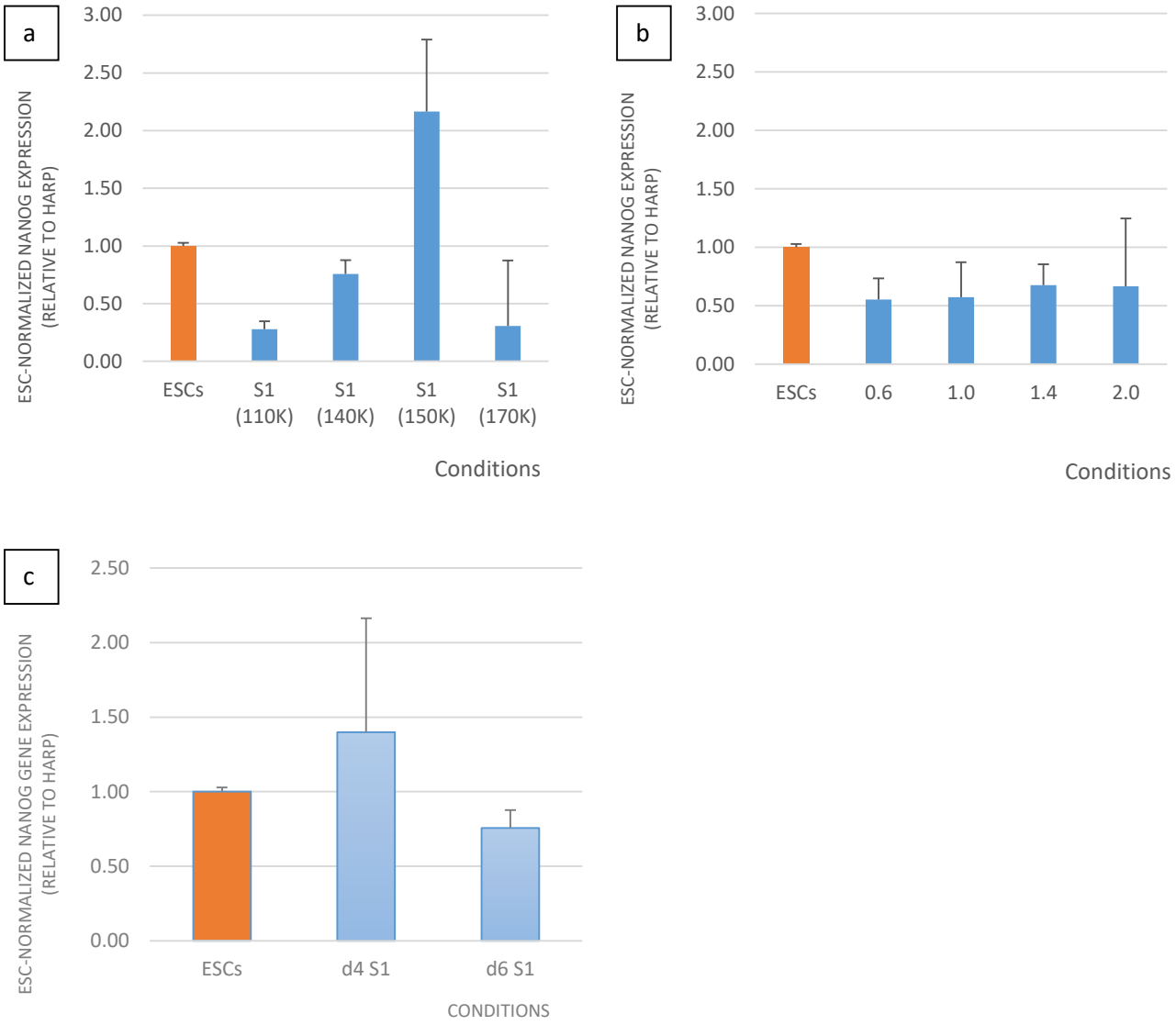



*Figure 7 - NANOG expression in S1 differentiation cells (normalized to ESCs, relative to housekeeper HARP) from (a) the Cell Density and (b) CHIR-99021 plate. (c) NANOG expression was kept track as well during S1 cell development for day 1 (d4 S1) and day 3 (d6 S1) of the differentiation. D4 S1 and d6 S1 were from the 140K condition. Data is shown as mean ±SD, with most of the conditions showing a decrease in NANOG expression.*

### 3.4 CHANGING CHIR-99021 CONCENRATIONS AFFECT DE GENE MARKERS EXPRESSION

To evaluate if S1 differentiation cells change expression levels of DE markers when exposed to different concentrations of GSK3β inhibitor CHIR-99021, qPCR was used to determine mRNA expression levels of CXCR4, SOX17, FOXA2 and NANOG. Using different concentrations of CHIR-99021 for S1 differentiation, DE gene markers were expressed for all conditions. S1 differentiation cells exposed to µM 0.6 CHIR-99021 had a lower expression rate compared to cells exposed to a higher concentration for all DE markers. However, errors are quite big for conditions 1.0, 1.4 and 2.0, as can be seen in Figure 8.

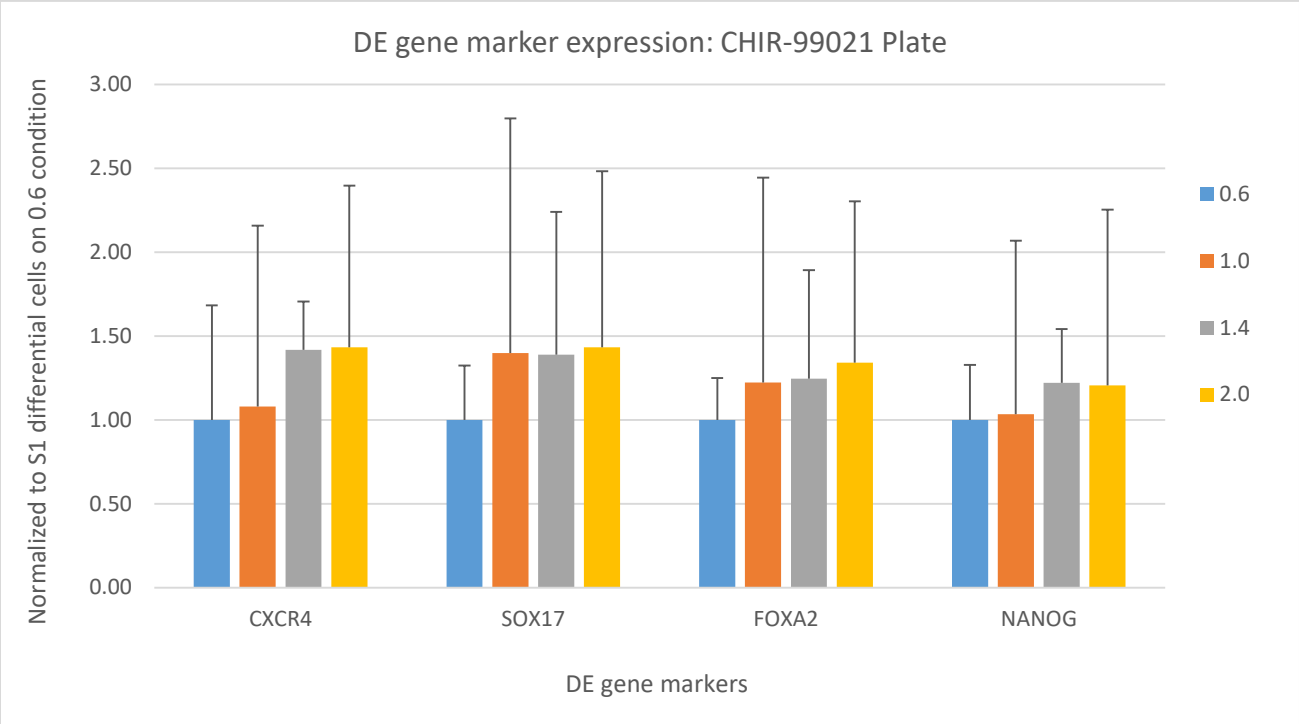



*Figure 8 - DE gene marker expression of the CHIR-99021 condition plate, normalized to S1 differentiation cells (relative to housekeeper HARP), where its progenitor cell was initially passaged with a cell density of 110K cells/cm2. Using 0.6 µM CHIR-99021 decreases DE gene marker expression compared to using higher concentrations. Data is shown as mean ±SD, with the error being really high for most conditions.*

### 3.5 UNCLEAR WHETER CXCR4 IS EXPRESSED AT CORRECT LOCATION

An attempt of doing immunostaining to evaluate the location of CXCR4 expression was done on the undifferentiated ESCs and S1 differentiation cells. However, results show no emission of PE fluorophores at all.

### 3.6 UNCLEAR WHETER SUFFICIENT AMOUNT OF DE CELLS WERE DIFFERENTIATED

To determine what percentage of the differentiated S1 cells express DE gene markers, FACS analysis was performed. However, due to lack of proper negative controls, this cannot be determined with the obtained FACS results. As a result, CXCR4 expression of S1 differentiation cells and ESCs was analyzed through FACS without negative control. Histogram showed overlapping mean values of 7.56 for S1 differentiation cells and 8.05 for ESCs (see Figure 9). This suggest with low validity that ESCs and S1 differentiation cells were both expressing CXCR4.

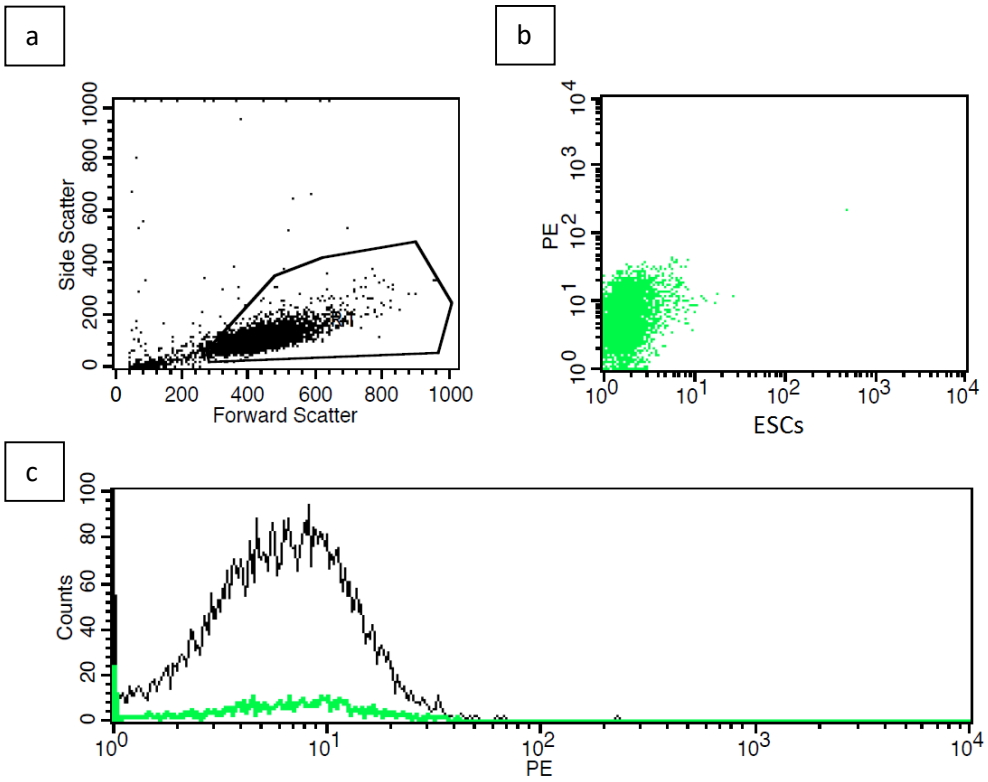



*Figure 9 – Based on (a) the forward and side scattering and (b) the representative FACS plot, it can be shown that PE intensities were measured, being characterized with middle sized cells with not many granules. However, (c) histology mean values of the S1 differentiation cells and the ESCs are overlapping, suggesting CXCR4 are being expressed in both cell lines with low validity.*

### 3.7 MORPHOLOGIC CHANGES

ESCs that underwent differentiation for three days straight with S1 Differentiation Medium changed morphology and looked different from the population of the undifferentiated cells. Cells from the S1 differentiation population were more elongated and sharper-shaped. The ESCs, however, were more oval and round-shaped.

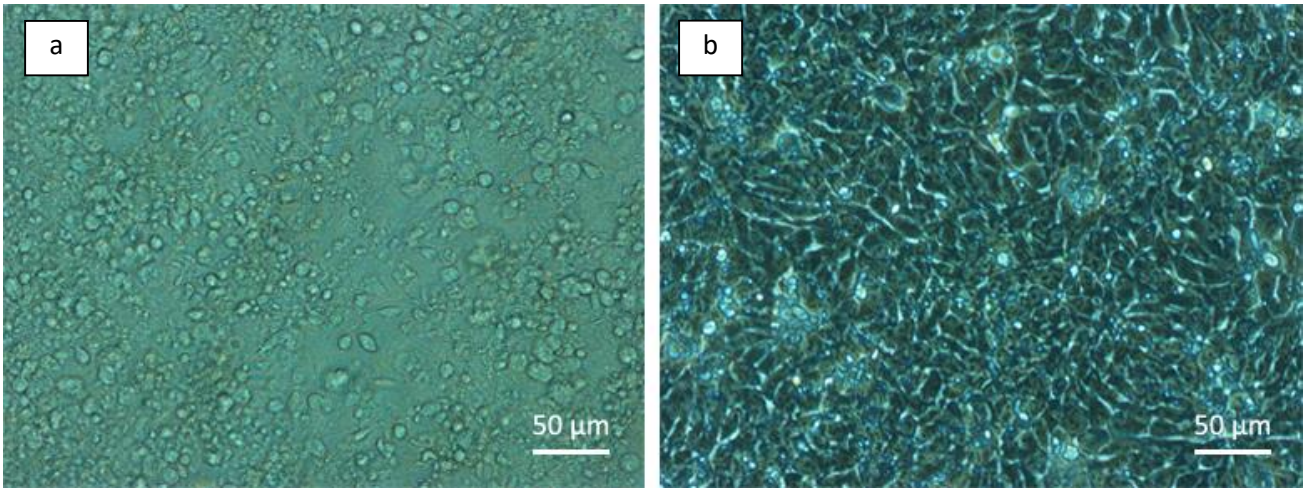



*Figure 10 - (a) Undifferentiated ESCs (P13+54) and (b) S1 differentiated cells differ in morphology. ESCs are more round/oval-shaped, whereas S1 differentiation cells were more elongated on day 6 of the experiment. A 200x magnification is shown.*

# 4 DISCUSSION

In the last decades, multiple studies have been working on solutions for long-term treatment of type 1 diabetes, with islet transplantation to be the most promising technique. However, there is a huge demand for cadaveric pancreata donors, which cannot be met. Alternative methods have been found in the regenerative medicine research field, with the most advanced developments being *in vitro* pluripotent stem cell derived β-cells differentiation. In this study, the proven protocol by Rezania et al. [22, 28-30] for developing insulin-producing β-cells was evaluated only up to stage 1: differentiation of definitive endoderm from embryonic stem cells. This study shows development of S1 differentiation cells, which successfully expressed the definitive endoderm gene markers CXCR4, SOX17, FOXA2 and NANOG. To be more precise, increase in expression of CXCR4 was observed compared with undifferentiated ESCs. Furthermore, the S1 differentiation cells had a decrease of NANOG expression levels, suggesting the cells are migrating to a less pluripotent state.

### *The Cell Density conditions*

According to the Cell Density plate, S1 differentiation cells were expressing relatively more DE gene markers when its progenitors were passaged on the conditions 140K and 150K cells/cm$^2$. Before the E8 Medium was replaced with S1 Differentiation Medium (with 100 ng/ml GDF-8) for the first time, the confluency of these progenitors was 85-95% and 85-90% respectively. In fact, only cell populations from these two conditions were living around this confluency: conditions under 140K and above 150K had different confluences. Interestingly, Rezania et al. [22] described similar confluency (~90%) after 48 hours of passaging the ESCs, from where S1 differentiation can be initiated. Therefore, our study supports the confluency condition of the proven protocol. However, the proven protocol mentioned passaging cell densities between 130K to 150K cells/cm$^2$. This study has not evaluated the 130K condition, which is still open for future investigation.

### *Increase of CXCR4 expression*

Just as how the proven protocol describes [30], our S1 differentiated cells expresses significantly more CXCR4 (at least 13 times more) compared to ESCs with all of our conditions, especially the cells from the 140K, 150K and 1.4 condition. The other conditions of the CHIR-99021 plate have big error bars, suggesting the obtained data is not trustworthy.

### *Depletion of NANOG expression*

Our results suggest decrease in NANOG expression with S1 differentiated cells. In contrary, the differentiated cells from the 150K condition had an increase of NANOG expression. However, its possible error is relatively high compared to other conditions. Since NANOG expression is decreased at the 140K and 170K condition, there is a possibility that the 150K condition actually should express less NANOG, suggesting possible technical mistakes during qPCR preparations. This should be evaluated in the future.

### *The CHIR-99021 plate*

The CHIR-99021 plate was designed to determine optimal CHIR-99021 concentrations to be used for definitive endoderm differentiation. Unfortunately, as can be seen in Figure 8 (p. 16), data from all

concentration conditions show big possible errors. Therefore, no accurate information can be obtained. However, a pattern of lowering expressions of DE gene markers with condition 0.6 can be observed, suggesting it is possible that using lower concentrations of CHIR-99021 supports relatively lower expressions of CXCR4, SOX17, FOXA2 and NANOG. Since condition 1.0, 1.4 and 2.0 are, according to our data, expressing around the same levels, there is probably no additional value of increasing the CHIR-99021 concentration than using the proposed 1.0 µM by Rezania et al. [22]. By increasing the concentration of this GSK3β inhibitor, it will only increase the chances of differentiation of mesodermal cells [37]. To proof this hypothesis, this experiment should be repeated (possibly the big error bars are a result of poor qPCR preparations) with definitive endodermal and mesodermal primers. In addition, ESCs should be passaged with a cell density of 140K and 150K cells/$cm^2$ as well to evaluate if the same results are obtained as with cell densities higher than 170K cells/$cm^2$.

*FACS analysis and immunocytochemistry*

Rezania et al. [22] described efficient S1 differentiation is achieved if the percentage of total population expressing CXCR4 and FOXA2 is around 98-99% (with ~40% expressing OCT4 and ~75% SOX17) when following their protocol. However, if >90% of the populations are CXCR4-positive cells, differentiation to S2 is acceptable [29]. FACS analysis was planned for testing expressions of target genes CXCR4 and SOX17, however, due to unavailability of negative isotype control and unexpected cell death of the new negative control ESCs (this stresses the importance of using live/dead assay staining as well), FACS results only suggests that ESCs and S1 differentiation cells were probably expressing CXCR4.

The results from the immunocytochemistry experiment were quite unexpected: no fluorescence was observed. As a result, it is not clear if the genes are being expressed at the correct location. However, qPCR analysis showed high expression levels of CXCR4 (especially with condition 140K and 150K). Therefore, our immunostaining protocol should be improved by either changing the dilution ratio of the antibody or the components of the used buffers.

*Uncertainty of actual DE differentiation*

In general, our study succeeded in developing cells that express DE gene markers, however we cannot conclude with absolute certainty that definitive endoderm was differentiated due to the fact that the exact absolute amount of expression levels of DE gene markers is not known (despite the fact we were able to observe cell morphological changes). Idealistically, qPCR analysis should be done with positive controls using cDNA from definitive endodermal cells. Only when differentiation of definitive endoderm has been proved, the research on the generation of S2 differentiation cells from S1 differentiation cells can be recommended.

To improve validity of this study, relative DE gene marker expressions could be compared with literature. According to Hoveizi et al. [36], expression levels of SOX17 should be relatively higher than FOXA2, a requirement which our cells met (error bar for condition 150K for FOXA2 expression is quite high, which means lower expressions than SOX17 possibly occurred). However, since no p-values have been calculated due to lack of expertise and time, there is no suggestive indication if the S1 differentiated cells are actually expressing more SOX17 than FOXA2.

# 5 CONCLUSION

This study has shown successful development of S1 differentiation cells from embryonic stem cells that were able to express all the definitive endoderm gene markers. This includes an increase in CXCR4 and a decrease in NANOG expression. In addition, our results indicate expressions of SOX17 is relative higher than FOXA2 between the S1 differentiation cells. To achieve these cell characteristics, it is important to passage the ESCs with a cell density between 140K and 150K cells/$cm^2$ on day 1 of the experiment. After 48 hours, confluency should be between 85% and 95%, following the use of 1.0 µM CHIR-99021 and 100 ng/ml GDF-8 for differentiation. On the next day, the concentration of CHIR-99021 used was 0.1 µM instead.

Based on our results, it is not sure whether definitive endodermal cells have been differentiated: in qPCR analysis there was no positive control (cDNA of definitive endoderm), which means it is unknown if our cells express the same absolute amount of DE gene markers. In addition, the exact location of expression of the target genes, as well as the percentage of differentiated cell populations to express DE gene markers, are unknown. Despite the fact that further research is required on these problems, our results evaluated a promising protocol for insulin-producing β-cells regeneration that could treat T1D patients and provide improved support towards T2D patients.

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