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Generation of induced pluripotent stem cell-derived beta-cells in blood amino acids-like medium

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List of Abbreviations

hiPSCs	Human induced pluripotent stem cells
BALM	Blood-like amino acids medium
SC-β	Stem cell-derived β-cells
mTOR	mammalian target of rapamycin
OCT3/4	octamer-binding transcription factor 3/4
POU5F1	POU class 5 homeobox 1
DAPI	4', 6-diamidino-2-phenylindole
NANOG	homeobox protein NANOG
SOX17	SRY-box transcription factor 17
PDX1	pancreatic and duodenal homeobox 1
SOX9	SRY-box transcription factor 9
NKX6.1	homeobox protein Nkx-6.1

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Presentation Abstract

Background

Classic cell culture media do not accurately represent the availability of the nutrients in plasma. They usually contain a supraphysiological concentration of nutrients such as glucose, amino acids, etc. These high nutrients can alter the metabolism of cultured cells and induce metabolic phenotypes that do not reflect in vivo conditions. Refinement of media formulations has a potential application in the maturity modulation of stem cell-derived β cells (SC-β) generation in vitro. This has the potential importance in regenerative medicine for curative diabetes treatment. Previous reports from mice studies have shown that amino acid levels progressively decreased between embryonic day 19 and postnatal day 9. Moreover, elevated amino acid levels have been linked to the activated mammalian target of rapamycin complex 1 (mTORC1) pathway, an increase in islet cell proliferation, accompanied by a low level of maturation.

It has been demonstrated that late-stage culture in low levels of amino acids promoted the maturation of SC-β and improved their glucose-stimulated insulin secretion (GSIS) function. To the best of my knowledge, the effect of the physiological level of amino acids on the whole differentiation process has not been investigated. In this study, I asked whether SC-β cells could be derived in media that better recapitulates the composition of human plasma amino acids. And whether it would promote differentiation

Results

• Generation of induced pluripotent stem cell-derived beta-cells in blood amino acids-like medium

• Supraphysiological nutrients levels interfere with efficient differentiation through upregulation of the mTOR pathway

• Generation of induced pluripotent stem cell-derived beta-cells in blood amino acids-like medium BSA free medium.

Conclusion

The physiological level of amino acids is sufficient for the derivation of functional SC-β cells.

Chapter 1: Literature Review

1.1Physiological-like culture medium

Previous reports have shown that deprivation of nutrients can eliminate residual undifferentiated cells and potentiate differentiation. This highlights the potential of metabolic approaches for modulating differentiation efficiency and specification of initial cell fate (Dahan et al., 2019; Shiraki et al., 2013; Tohyama et al., 2013, 2016; Wu et al., 2016).

While metabolism offers a potential approach to improving the efficacy of cellular therapy, traditional cell culture media do not accurately represent the nutrients available in plasma. They usually contain supraphysiological concentrations of nutrients such as glucose, amino acids, etc. (Yao and Asayama, 2017). These high nutrients can alter the metabolism of cultured cells and induce metabolic phenotypes that do not reflect in vivo conditions (Ackermann and Tardito, 2019). Efforts in cancer research have been made to create culture media that resemble nutrient and metabolite levels found in human plasma. Such studies have shown differences in metabolic phenotype and drug efficacy compared to conventional media.

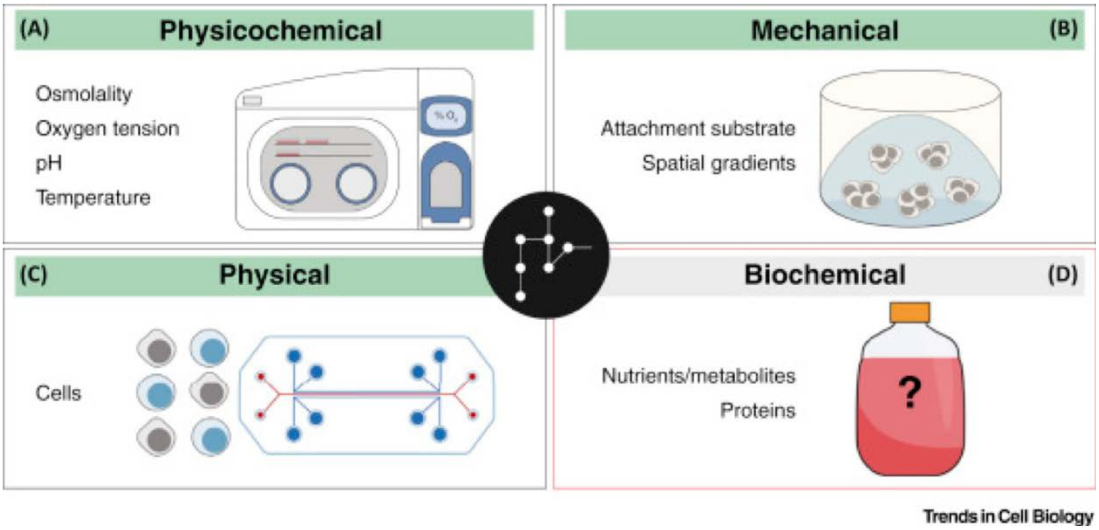

Fig 1.1. Summary of Controlled Cell Culture Conditions and Recent Efforts to Address the Modeling Capacity of in vitro Systems. Cell culture provides an artificial environment of controlled biochemical, mechanical, physical, and physicochemical conditions that influence metabolism and other cellular processes. Airtight chambers can permit cell culture under more physiologically relevant oxygen tension levels (A), 3D organoids are intended to recapitulate tissue architecture (B), and various co-culture methods and organ-on-a-chip systems may better mimic possible interactions between different cell types or tissues/organs, respectively (C). However, there has been relatively less consideration for improving the modeling capacity of in vitro biochemical conditions, which are still primarily dictated by classic media that were developed generations ago (D). medium. (Cantor, 2019).

A new culture medium for cancer cell lines that more closely mimics the in vivo environment of cancer cells is proposed (Voorde et al., 2019). The aim is to improve the accuracy of cancer cell culture models, as current culture conditions are often quite different from tumors' actual in vivo environment (figure 1.1). They explain that current culture media used for cancer cell lines are typically high in glucose and glutamine, which do not represent the in vivo tumor microenvironment. The authors argue that this creates metabolic stress on cancer cells, leading to changes in gene expression, metabolic pathways, and other cellular functions. This can lead to altered drug response and reduced reliability of preclinical studies using these models. The new culture medium more closely mimics the in vivo microenvironment of tumors. The new medium contains lower levels of glucose and glutamine and higher levels of other nutrients, such as lactate and pyruvate. The authors tested the new medium on a range of cancer cell lines. They found that it improved the metabolic fidelity of the cells, as evidenced by changes in gene expression and metabolic pathways.

They concluded that the new culture medium significantly improves cancer cell culture models and will improve the accuracy and reliability of preclinical studies. They suggest that future studies should investigate the effects of this new medium on other cancer types and explore potential clinical applications.

The effect of a physiologic medium on cellular metabolism was investigated, and uric acid was identified as an endogenous inhibitor of UMP synthase, an enzyme involved in pyrimidine nucleotide biosynthesis (Cantor et al., 2017).

The authors hypothesized that culturing cells in a medium that better reflects the in vivo environment would improve the physiological relevance of cellular studies. To test this, they developed a physiologic medium that contained levels of glucose, amino acids, and other nutrients that more closely mimicked the levels found in human plasma. They then cultured a variety of cell lines in this medium and compared their metabolism to cells cultured in traditional media.

The authors found that culturing cells in the physiologic medium led to significant changes in cellular metabolism. Specifically, they observed a shift towards increased oxidative phosphorylation and reduced glycolysis. This metabolic rewiring was associated with changes in the expression of genes involved in glucose and lipid metabolism.

Next, the authors used the physiologic medium to investigate the regulation of UMP synthase, an enzyme involved in pyrimidine nucleotide biosynthesis. They found that UMP synthase was inhibited by a metabolite present in human plasma, which they identified as uric acid. Uric acid competitively inhibited UMP synthase, leading to a reduction in pyrimidine nucleotide biosynthesis.

To confirm the physiological relevance of their findings, the authors measured uric acid levels in human plasma and found that they were within the range required for the inhibition of UMP

synthase. They also showed that reducing uric acid levels using allopurinol, a drug commonly used to treat gout, led to increased UMP synthase activity.

Finally, the authors showed that the inhibition of UMP synthase by uric acid was not limited to in vitro studies but also occurred in vivo. They measured UMP synthase activity in mice treated with allopurinol and found that it was increased compared to untreated mice.

It was demonstrated that culturing cells in a physiological medium leads to significant changes in cellular metabolism and that uric acid is an endogenous inhibitor of UMP synthase. These findings highlight the importance of considering the physiologic context when studying cellular metabolism and identifying a potential new target for the treatment of diseases associated with altered pyrimidine nucleotide biosynthesis. Another aspect of the application of physiological media is T lymphocytes. (Leney-Greene et al., 2020). T lymphocytes play a vital role in the adaptive immune system and recognize and destroy pathogens and cancer cells. In vitro experiments use various media to activate T lymphocytes, but none perfectly simulate the in vivo environment. This study aimed to investigate the use of a new human plasma-like medium (HPLM) to improve T lymphocyte activation and proliferation compared to other commonly used media.

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human donors and cultured in various media, including HPLM, RPMI-1640, AIM-V, and X-VIVO 15. T lymphocytes were then stimulated with anti-CD3 and anti-CD28 antibodies in each medium for 72 hours. The following assays were performed to evaluate T lymphocyte activation and proliferation: cell viability, flow cytometry, cytokine production, and cell counting.

Compared to RPMI-1640, AIM-V, and X-VIVO 15, HPLM significantly increased the percentage of activated CD4+ and CD8+ T lymphocytes, as well as their production of the cytokines interleukin-2 (IL-2), interferon-gamma (IFN-gamma), and tumor necrosis factor-alpha (TNF-alpha). Additionally,

HPLM showed improved T lymphocyte proliferation compared to the other media, as evidenced by increased cell counts and higher proliferation marker Ki-67. HPLM also enhanced the survival of activated T lymphocytes compared to the other media. The results of this study suggest that HPLM is a superior medium for T lymphocyte activation and proliferation compared to RPMI-1640, AIM-V, and X-VIVO 15. The improved activation and proliferation of T lymphocytes in HPLM may be due to its composition, which more closely resembles the in vivo environment. These findings have important implications for the development of new T lymphocyte-based therapies, as the use of HPLM may enhance the efficiency of T lymphocyte activation and proliferation in vitro. This study's scope is about applying physiological media to generate mature stem cell-derived β cells (SC-β)

1.2Efforts for generating mature stem cell-derived β cells (SC-β)

Diabetes mellitus is a condition of the beta cell that causes improper insulin secretion and an inability to keep blood sugar levels within a normal range. Type 1 diabetes (T1D) is caused by dysregulated autoimmune cell destruction. The hallmarks of type 2 diabetes (T2D) include cell depletion and dysfunction brought on by the prolonged stress of chronic hyperglycemia. Individuals with T1D and many patients with T2D currently depend on exogenous insulin therapy. Exogenous insulin therapy necessitates all-day injections and frequent monitoring, which lowers the quality of life and causes secondary consequences since it fails to precisely maintain normal glycemia (Powers and D'Alessio, 2011).

Patients with T1D and T2D have demonstrated accurate glycemic control and exogenous insulin independence following transplantation of the entire pancreas or purified islets of Langerhans (Kandaswamy et al., 2018). Islet transplantation's potential to be used widely is constrained by the necessity for immunosuppression and the availability of replacement tissue (Millman and Pagliuca, 2017).

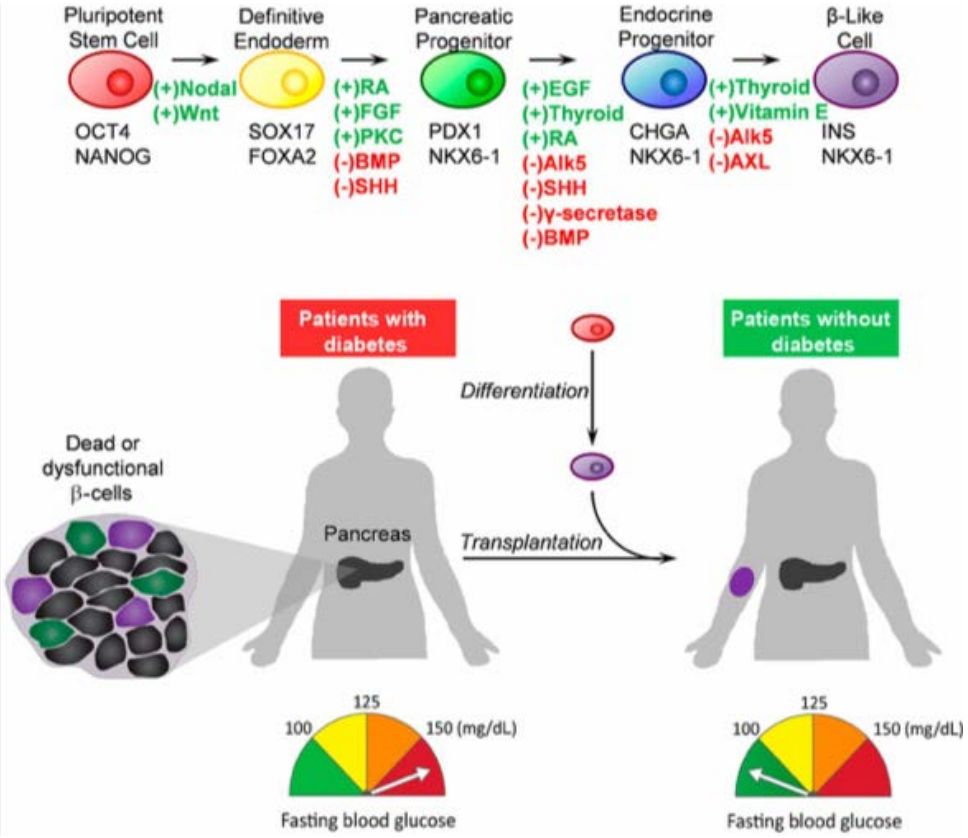

Fig. 1.2 Directed differentiation of hPSCs into β-like cells. Top: Schematic of signaling pathways that are activated [green, (+)] or inhibited [red, (-)] during stepwise differentiation of hPSCs to β-like cells that are shared by at least two differentiation protocols. Major gene expression markers for each cell type are also indicated. Bottom: Overview schematic for cellular therapy to control blood glucose in patients with diabetes. RA, retinoic acid (Millman and Pagliuca, 2017).

A promising alternative cell source for diabetes cell replacement therapy, disease modeling, and drug screening is human SC-β cells (figure 1.2) (Millman and Pagliuca, 2017). In 2014, the first in vitro differentiated SC-β cells were generated (Pagliuca et al., 2014; Rezania et al., 2014). These early SC-β cells had characteristics of mature cells, including insulin secretion in response to glucose, expression of transcription factors specific to mature cells, and in vivo function in mice weeks after transplantation.

SC-β cells and primary adult cells nevertheless differ significantly in several important ways, including insufficient insulin secretion per cell, dysregulated glucose-stimulated insulin secretion (GSIS) dynamics, and reduced expression of key transcription factors. The functional maturity of SC- β cells has significantly advanced recently, leading to functionally enhanced SC- β cells (Nair et al., 2020; Velazco-Cruz et al., 2020).

Some of these improved SC-β cells achieve dynamic insulin secretion, characterized by first and second-phase insulin secretion. Despite being enhanced, these SC-β cells cannot meet the primary cadaveric islets' transcriptome profile and glucose response (Hogrebe et al.; Mahaddalkar et al., 2020; Nair et al., 2020; Velazco-Cruz et al., 2019).

Recent studies have shown faster in vivo glucose regulation after transplantation, increased glucose-stimulated insulin secretion, first- and second-phase insulin secretion, and enhanced differentiation efficiency. Reserachers used a suspension differentiation protocol with temporal TGF modulation, cellular cluster size control, serum-free media, endocrine enrichment without cell selection, and a simplified final stage media devoid of notable preceding factors. Velazco-Cruz et al. were the first to report robust dynamic insulin secretion of SC-β cells. The authors demonstrate that ALK5i, a type II TGF- β inhibitor, is required to specify the cell fate. However, after cell specification, TGF-β signaling permittance is necessary for SC-β cell functional maturation. The authors demonstrate that increasing static GSIS and nearly doubling the population of C-Peptide+ NKX6.1+ co-expressing SC- β cells can be achieved by resizing cellular clusters during the final stage of differentiation. This process involves partial dissociation of clusters.

Dynamic secretion assays demonstrated robust dynamic function with a distinct first phase, a stable second phase, and a return to basal levels after the end of the high glucose treatment. A shortcoming in the study was that various hPSC lines produced varying amounts of insulin secretion per cell in response to high glucose levels. These cells were transplanted, and the mice showed enhanced glucose tolerance.

Veres et al. demonstrated that, compared to unsorted and reaggregated SC-cells, enrichment of the cell population through CD49a+ cell sorting increased static GSIS (Veres et al., 2019). First and second-phase insulin secretion was seen in CD49a+ sorted cells, but when high glucose was removed, basal levels were not restored. The authors did not demonstrate if dynamic GSIS could occur in reaggregated cells. In their SC-β cell preps, the authors identified enterochromaffin-like

cells, and CD49+ sorting eliminates these cell types. The human pancreas lacks enterochromaffin cells, which are identified by TPH1 expression and release serotonin in the gastrointestinal tract. According to this study, enterochromaffin cells and β cells have a similar developmental path, which explains why they are present in cell differentiation protocols. Uncertainty remains about whether the functional benefits of CD49+ sorting are brought about by eliminating off-target cell types, such as enterochromaffin-like cells, or by some other mechanism. This study did not examine transplantation in mice. In a different study, Nair et al. used an insulin-driven GFP tag cell sorting approach and a reaggregation technique to achieve first-phase insulin production (Nair et al., 2019). The authors achieved a high β cell population with 80% C-Peptide/NKX6.1 co-expression. The authors compare un-reaggregated, unsorted cell clusters versus reaggregated, GFP-enriched cells in functional studies. The authors observe no first-phase response in the immature clusters or the reaggregated and unsorted clusters. Still, they did observe a first phase with a stimulation index of 3 and no second phase response in the GFP-sorted reaggregated clusters. Since only one insulin-driven GFP reporter cell line was used in this study, it is unclear how well this approach would translate to other cell lines and whether the low levels of insulin secretion in this cell line are attributable to genetic engineering or its genetic background in comparison to other protocols. In contrast to other improved SC-β cell differentiation methods, this differentiation protocol keeps ALK5i and T3 during the final stage of differentiation.

Therefore, the removal of these compounds may make it possible for dynamic secretion to be more robust, including the missing second phase of this protocol. These cells were transplanted, and the mice showed enhanced glucose tolerance.

By using a different differentiation method to generate SC-β cells, Hogrebe et al showed how the modulation of actin cytoskeleton polymerization regulates differentiation to endocrine and other endodermal lineages. This insight drove the development of a procedure for planar cell differentiation. Other protocols employ suspension, or pseudo-suspension air-liquid interface culture

methodologies for endocrine specification (Pagliuca et al., 2014; Velazco-Cruz et al., 2019; Veres et al.), which raises the level of technical expertise needed for SC-β cell differentiations. Using the actin-depolymerizing agent latrunculin and a new planar differentiation procedure, the authors produce SC- β cells that go through dynamic GSIS by upregulating neurogenin 3. When these SC-β cells are implanted into mice, they rapidly reverse severe preexisting diabetes at a pace comparable to that of cadaveric islets.

Hogrebe et al. used the Velazco-Cruz et al. technique to compare suspension-differentiated cells to planar differentiated cells in a controlled and parallel manner (Hogrebe et al.2020).The two methods were optimized for the HUES8 cell line, and the suspension protocols yielded larger percentages of CP+ NKX6.1+ SC-β cells. Planar and suspension-derived SC-cells exhibited similar functional properties when examined by static and dynamic GSIS as well as insulin content. The SC- β cells generated from suspension and planar were real-time PCR analysis. Diabetic mice received an equal number of SC- β cell transplants from suspension and planar sources. Planar differentiated cells were able to reverse diabetes in two weeks as opposed to five weeks with the suspension approach. This variation in the diabetes cure period is intriguing because in vitro functional and transcriptome studies did not clearly distinguish between the two methods. To understand more about the cause of this disparity, single-cell RNA sequencing and transcriptome comparisons between SC-β cells obtained from suspension and planar cultures may be used. Importantly, the Hogrebe et al. planar methodology was able to distinguish SC-β cells from a variety of pluripotent stem cell lines, with some cell lines having a function similar to that of cadaveric islets when tested with dynamic perfusion assays. HUES8 suspension and planar-generated SC- cells had comparable functionalities. However, when the planar approach was used with various cell lines, it generated cells with better functionalities.

Mahaddaklkar et al. isolated anterior definitive endoderm cells with higher pancreatic and cell potential by sorting for the CD177/NB1 glycoprotein (Mahaddalkar et al., 2020). Comparing sorted

and CD275+ definitive endoderm populations to CD177+ cells, the authors find that the latter has higher PDX1 and NKX6.1 pancreatic progenitor potential. Relative to unsorted cells, the efficiency and function of differentiation of CD177+ cells toward cells enhanced. Unsorted cells lacked both a first and second phase of insulin secretion, while CD177+ cells displayed a first-phase insulin response with no second phase (Mahaddalkar et al., 2020). The cells were not transplanted in mice. It is challenging to directly compare these studies because assays evaluating function differ in terms of technical methodology, normalization techniques, and in vivo models. Standardized static and dynamic GSIS assays that are normalized to DNA can substantially simplify the comparison of differentiating procedures while putting a little burden on investigators. It is difficult to standardize in vivo assays due to many mouse and diabetic models with varying degrees of diabetes.

Current SC-β cells lack the functional maturity of cadaveric islets despite advancements. Teams use novel technologies and methods, such as single-cell sequencing, genetic engineering, cell sorting, and drug screening, to find factors contributing to cell differentiation and function. Many adult genes may play a role in the functional maturation of SC- β cells, according to recent publications comparing gene expression between adult and fetal or juvenile islets. ERRγ has been shown to be enriched in adult mouse cells as opposed to neonatal mouse cells, and mice deficient in cell ERRγ are unable to effectively regulate their blood glucose levels (Yoshihara et al., 2016) differentiate hPSCs to resemble an immature state where many β cells genes are expressed but cannot go through in vitro GSIS. The authors noticed improvements in mitochondrial respiration and the ability of the cells to undergo in vitro GSIS when they overexpressed ERRγ in these β-like cells. It may be possible to exploit the upregulation of ERR γ to enable SC-cells to mature even further. However, because its results were limited to immature insulin-expressing endocrine cells unable to undergo in vitro GSIS with immature mitochondrial respiration, it might not apply to more advanced procedures that are metabolically more mature (Nair et al., 2019). In another study, Arda et al. identified numerous genes associated with age in β cells, including ONECUT2, MAFA, TSHZ3, SIX2, and

SIX3, and showed that islet function increases in adult vs juvenile human islets (Arda et al., 2016) Investigating whether these genes' overexpression in SC-cells can promote their functional maturation is still needed. Neurogenin 3 overexpression caused by YAP signaling inhibition has been found to promote endocrine induction. Differentiation efficiency and function are improved when verteporfin is used in differentiation procedures during endocrine induction (Rosado-olivieri et al., 2019). This work does not test dynamic functions. An earlier study that demonstrated how YAP inhibition stimulates pancreatic progenitor to differentiate into endocrine cells provides support for this work (Mamidi et al., 2018). Glycoprotein 2 has been identified as a cell surface marker useful for sorting PDX1+/NKX6.1+ pancreatic progenitors, enhancing cell differentiation efficiency. This tactic may boost the percentage of cells produced utilizing increased differentiation methods. The functional maturation of SC-β cells may be accelerated by enriching the population of cells through sorting (Nair et al., 2019; Veres et al., 2019). More thorough research is required to identify whether this improvement results from cell-cell contact, paracrine signaling, or the elimination of inhibitory cell types such as enterochromaffin cells. SC-β cell generation on a large scale is restricted by cell enrichment utilizing cell sorting. However, magnetic-activated cell sorting techniques may be promising. Another promising approach to improve the maturation of SC-β cells is throughout the nutritional environment. It has been proposed that β-cell maturation may be affected by environmental changes rather than only transcriptional factors. (Barsby and Otonkoski, 2022; Katsumoto and Grapin-Botton, 2020).

1.3 mTOR role in pluripotency and beta cell maturation

Serine/threonine protein kinase Mammalian Target of Rapamycin (mTOR) Complex 1 (mTORC1) is activated in response to particular nutrient levels, phosphorylating downstream substrates that control cell growth, proliferation, and survival (Saxton and Sabatini, 2017). mTORC1 relocalizes from the cytosol to the lysosomal membrane via an interaction with the Rag family of guanosine triphosphatases (GTPases) that is mediated by amino acids (Sancak et al., 2008). The Rag GTPases

receive signals from specific amino acid-responsive upstream regulators of mTORC1, such as lysosomal vacuolar H(+)-adenosine triphosphatase (v-ATPase), SAM sensors, leucine sensor Sestrin2, arginine sensor CASTOR1, SLC38A9, and SAMTOR (Chantranupong et al., 2016; Gu et al., 2017) Like with the amino acids arginine, leucine, and glutamine, glucose also has the ability to directly activate mTORC1 (Figure1.3) Mouse embryonic fibroblasts (MEFs) demonstrated a reduction in mTORC1 kinase activation in glucose deprivation experiments (Kalender et al., 2010). In conclusion, mTORC1 activation is mediated by a variety of different glucose and amino acid signaling pathways.

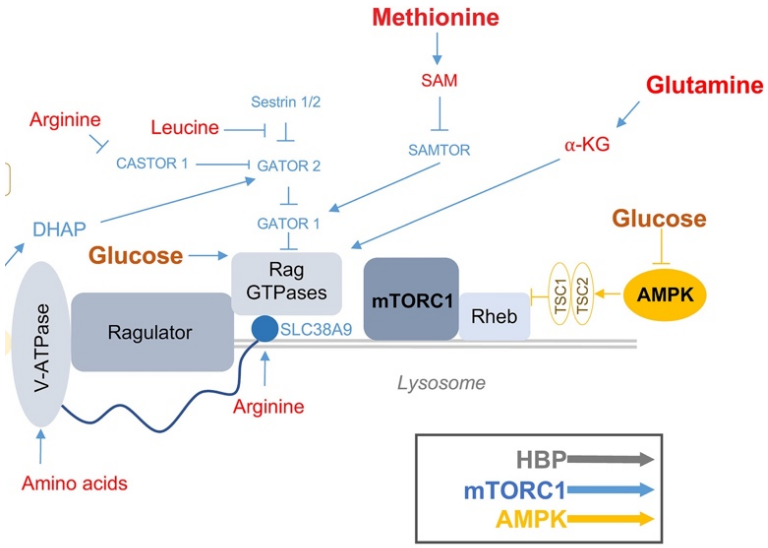

Fig.1.3 Nutrient sensing pathways impact pluripotent stem cell fate
Signaling by mTORC1 and AMPK is regulated by concentrations of glucose and amino acids at multiple sensors, with activities localized to the lysosomal membrane. These specific nutrients fuel enzyme-based alterations in the epigenome, gene transcription, translation to shape PSC fate. dihydroxyacetone phosphate, DHAP. Modified from Lu et al, 2021.

The most studied cause of mTORC1 inhibition and subsequent induction of autophagy is amino acid deficiency (Saxton and Sabatini, 2017). The maintenance of pluripotency has been shown to be positively influenced by amino acids like glutamine, methionine, and threonine (Sahay et al., 2013; Shiraki et al., 2013; Shyh-Chang et al., 2013). These amino acids may also promote pluripotency by signaling nutrient abundance to activate mTORC1. Threonine promotes pluripotency via modulating mTORC1 activity and histone methylation levels (Ryu and Han, 2011; Shyh-Chang et al., 2013;

Wang et al., 2009). OCT4, NANOG, and SOX2 expression was elevated in mESCs after threonine treatment (Ryu and Han, 2011). This result was reversed when mTORC1 signaling activity was inhibited with rapamycin, providing more evidence that nutrient-activated mTORC1 is involved in controlling pluripotency (Ryu and Han, 2011). It's possible that threonine promotes mESC self-renewal through mTORC1 activity to prevent pluripotency transcription factors from degradation through autophagy.

Mammalian beta cells are known to mature functionally postnatally and continue to grow after weaning (Stolovich-Rain et al., 2015). The necessity for the neonate to adapt to changing patterns of food consumption and composition is arguably the most important potential driver of this response. According to mouse studies, the transition from a diet high in pulsatile carbohydrates after weaning to one high in amino acids during pregnancy (and the high-fat milk diet of neonates) stimulates the maturation of postnatal beta cells .The relative activity of the energy-sensing pathways of the mechanistic target of rapamycin (mTOR) complex (mTORC1) and AMP-activated protein kinase (AMPK) shifts as a result of this change in food type, with functional maturation favoring AMPK signaling's basal activity.

On the other hand, the activation of the mTORC1 signaling is increasingly limited to times of glucose stimulation. Intriguingly, maintaining a high-fat diet into adulthood results in the retention of a more functionally immature beta cell phenotype (Jaafar et al., 2019a), while temporarily inhibiting mTOR in islets made from stem cells (SC-islets) improves functional outcomes (Helman et al., 2020), indicating that these pathway shifts are correlational to beta cell maturation.

It is well documented that mTOR signaling plays a positive role in beta cell development and function (Ardestani et al., 2017). The precise processes modulating mTOR signaling to cause beta cell development are still unknown. Beta cell-specific overexpression of a kinase-dead mTOR negatively affected function in mice (Alejandro et al., 2017), as is beta cell-specific mTOR knockout (Sinagoga et al., 2017).

1.4 Aim and objectives

Refinement of media formulations has a potential application in the maturity modulation of stem cell-derived β cells (SC-β) generation in vitro. This has the potential importance in regenerative medicine for curative diabetes treatment. Previous reports from mice studies have shown that amino acid levels progressively decreased between embryonic day 19 and postnatal day 9 (Katsumoto and Grapin-Botton, 2020; Zeng et al., 2017). Moreover, elevated amino acid levels have been linked to the activated mammalian target of rapamycin complex 1 (mTORC1) pathway, an increase in islet cell proliferation, and a low level of maturation (Barsby and Otonkoski, 2022; Jaafar et al., 2019b).

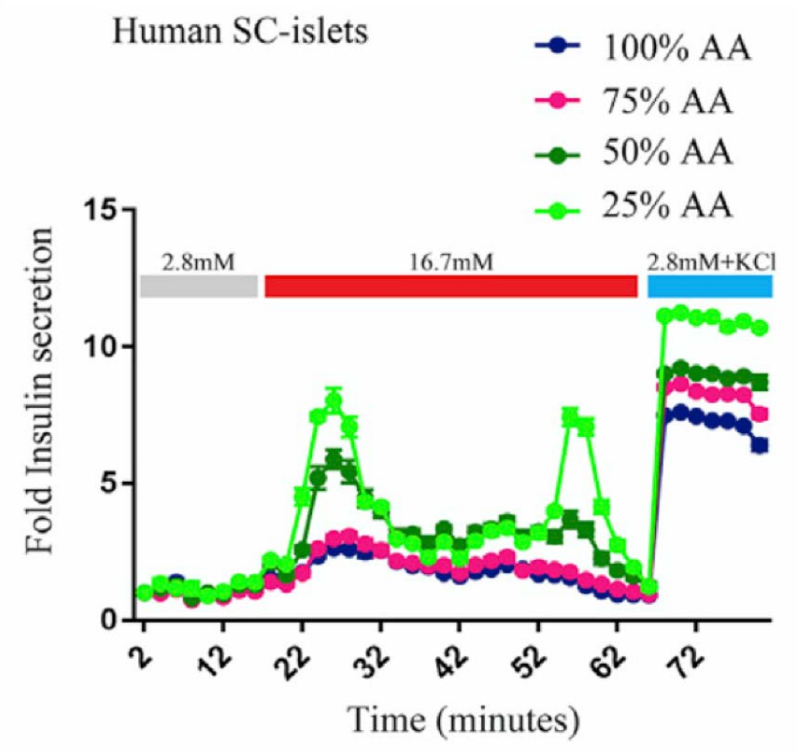

Fig. 1.3. Insulin levels secreted by SC-β cells cultured at varying amino acids conditions inn a dynamic perfusion assay in low (2.8 mM, gray line), high (16.7 mM, red) glucose concentrations, and KCl (30 mM, blue line) (Helman et al., 2020).

It was hypothesized that mimicking the postnatal change in nutrients and downregulating mTORC1 activity in vitro by reducing amino acid levels in the culture media might improve the functional maturation of SC-β (Helman et al., 2020). The authors cultured SC-β cells for two weeks in media with varying amino acid concentrations. They confirm mTORC1 suppression in low concentrations of amino acid level media by measuring p-s6, a downstream marker of the mTORC1 pathway.

Culturing SC-β cells with 25% amino acid-rich media was correlated with an increase in the average cellular insulin content. The authors have found that reducing amino acid levels to 50% or 25% of the amino acid-rich medium improved the GSIS of the cells (Figure 1.3). Similar improvement of GSIS was achieved by treating cells that were grown in an amino acid-rich medium with Torin1 (a mTORC1 inhibitor) for 48 h, confirming that amino acid reduction improves glucose responsiveness via inhibition of mTORC1 activity. While it has been demonstrated that late-stage culture at low levels of amino acids promoted the maturation of SC- and improved their glucose-stimulated insulin secretion (GSIS) function. However, to the best of my knowledge, the effect of the physiological level of amino acids on the whole differentiation process has not been investigated. In this study, I asked whether SC-β cells could be derived in media that better recapitulates the composition of human plasma amino acids. And whether it would promote differentiation.

1.5 Chapters Overview

To address these questions, I established for the first time a defined culture system to derive SC-β cells using a blood-like amino acids medium (BALM) using RPChiPS771-3G cell line. RT-PCR, immunohistochemistry,western blot analysis and GSIS, were performed to evaluate differentiation efficiency and maturation level. In **Chapter 2,** it was demonstrated that human pluripotent stem cells (hiPSCs) could be efficiently differentiated into definitive endoderm, pancreatic progenitors, endocrine progenitors, and SC-β in BALM. The differentiated cells secreted C-peptide *in vitro* in response to high glucose levels and expressed several pancreatic β-cell markers
In **Chapter 3,** it was demonstrated that supraphysiological nutrients level interferes with efficient endodermal differentiation through upregulation of the mTOR pathway. Moreover**,** In **Chapter 4, a** defined fetal bovine albumin (BSA) free differentiation system was applied for BALM-based differentiation. It was shown that human pluripotent stem cells (hiPSCs) could be efficiently differentiated into definitive endoderm, pancreatic progenitors, endocrine progenitors, and functional SC-β in BALM BSA free conditions.

Chapter 2: Generation of induced pluripotent stem cell-derived beta-cells in blood amino acids-like medium

2.1 hiPSCs are efficiently differentiated into definitive endoderm (DE) in the BALM-based medium.

The concentrations of amino acids in the media used for cell cultures, such as DMEM F12, are much higher than those found in the blood and therefore do not seem to reflect in vivo conditions. It was referred to a report to develop a chemically defined medium with amino acids and glucose at concentrations generally found in human plasma (Matsumoto et al., 2014) and tested a custom-made blood amino acids-like medium (BALM). The comparison of the formulation of BALM and DMEM F12 with the blood amino acid is shown in Table 1**.** I compared using BALM and DMEM F12-based medium and assayed for their effects on differentiation of the human-induced pluripotent stem cells (hiPSCs) RPChiPS771 cells. To examine the impact of BALM on differentiation, we assessed SOX17 and OCT3/4 expression of day 3 clusters. Immunofluorescence revealed an increase in the ratio of SOX17-positive cells cultured in BALM compared to those cultured in a DMEM F12-based medium. OCT3/4-positive cells were significantly reduced when hiPSCs were differentiated in BALM compared to DMEM F12 (Fig. 2.1 A, B). Expression analysis of the pluripotency markers (*POU5F1, NANOG*) showed significantly decreased levels in cells cultured in BALM compared to those cultured in DMEM F12-based medium. The definitive endoderm (DE) marker genes (*GATA4, SOX17*, *FOXA2*) revealed a significant increase in cells cultured in BALM compared to those in DMEM F12 (Fig. 2.1 C). The BALM-based endoderm differentiation protocol was beneficial in that potentiation of differentiation could be achieved compared to the DMEM F12-based conventional medium.

Despite BALM having been designed and developed independently, a human plasma-like medium (HPLM) (Cantor et al., 2017) was reported during our study. The composition of HPLM is also

shown in Table 1. I then tested HPLM and compared it with a BALM-based medium in the endodermal differentiation of hiPSCs (Fig. 2.1 D). *POU5F1* and *NANOG* expressions were reduced in iPSC-derived endodermal cells cultured in HPLM or BALM compared to those in DMEM F12. In contrast, expressions of DE marker genes *GATA4*, *GATA6*, *SOX17,* and *FOXA2* increased significantly in cells differentiated in BALM or HPLM compared to DMEM F12 (Fig. S2.1). The result thus cross-validates my hypothesis of physiological nutrients' role in differentiation enhancement.

Table 1. The compositions of DMEM F12, BALM, HPLM and amino acid concentration in human plasma.

Amino acids [μM]	DMEM F12	BALM	HPLM	Human Plasma
Alanine	50	400	430	375
Arginine	699	115	110	115
Asparagine	50	50	50	49
Aspartate	50	1	20	0.6
Cystine	100	30	40	54
Glutamate	50	35	80	34
Glutamine	2500	600	550	612
Glycine	250	250	300	268
Histidine	150	90	110	93
Isoleucine	416	70	70	67
Leucine	451	125	160	128
Lysine	499	225	200	214
Methionine	116	35	30	34
Phenylalanine	215	65	80	68
Proline	150	150	200	147
Serine	250	115	150	125
Threonine	449	150	140	151
Tryptophan	44	60	60	59
Tyrosine	214	80	80	78
Valine	452	250	220	251

Vitamins [μM]	DMEM F12	BALM	HPLM
Biotin	0.0143266	0.0143266	0.8
Choline chloride	64.1429	64.1429	21.5
D-Calcium pantothenate	4.6958	4.6958	1.05
Folic Acid	6.0091	6.0091	2.3
Niacinamide	16.5574	16.5574	8.2
Pyridoxine hydrochloride	9.7276	9.7276	4.9
Roboflavin	0.5824468	0.5824468	0.5
Thiamine hydrochloride	6.4392	6.4392	3
Vitamine B12	0.5016969	0.5016969	0.0037
i-Inositol	70	70	194.3
Aminobenzoate	NA	NA	7.3
Ascorbate	NA	NA	NA

Inorganic Salts [μM]	DMEM F12	BALM	HPLM
Calcium Chloride	1050.6	1050.6	2350
Cupric Sulfate	0.0052062	0.0052062	NA
Ferric Nitrate	0.0032178	0.0032178	NA
Ferric Sulfate	1.5	1.5	NA
Magnesium Chloride	301.4707	301.4707	480
Magnesium Sulfate	405.6478	405.6478	350
Potassium Chloride	4181.9	4181.9	4100
Sodium Bicarbonate	14285.7	14285.7	24000
Sodium Chloride	119704	119704	105000
Disodium Hydrogen Phosphate Anhydrous	500.1408	500.1408	NA
Disodium Hydrogen Phosphate	452.8986	452.8986	840
Zinc Sulfate	NA	NA	NA
Ammonium Chloride	NA	NA	40
Ammonium Metavanadate	NA	NA	NA
Ammonium Molybdate	NA	NA	NA
Sodium Selenite	NA	NA	NA

(continued to next page)

Table 1. Continued

Other Components [µM]	DMEM F12	BALM	HPLM
D-Glucose	16666.8	5555.6	5000
Hypoxanthine	15.0331	15.0331	10
Linoleic Acid	0.1497593	0.1497593	NA
Lipoic Acid	0.5089675	0.5089675	NA
Phenol Red	21.5197	21.5197	14
Putrescine 2HCl	0.5028869	0.5028869	NA
Na Pyruvate	500	500	50
Thymidine	1.6515	1.6515	NA
2-hydroxybutyrate	NA	NA	50
3-hydroxybutyrate	NA	NA	50
4-hydroxyproline	NA	NA	20
Acetate	NA	NA	40
Acetone	NA	NA	60
Acetylglycine	NA	NA	90
Alpha-aminobutyrate	NA	NA	20
Betaine	NA	NA	70
Carnitine	NA	NA	40
Citrate	NA	NA	130
Citrulline	NA	NA	40
Creatine	NA	NA	40
Creatinine	NA	NA	75
Formate	NA	NA	50
Fructose	NA	NA	40
Galactose	NA	NA	60
Glutathione	NA	NA	25
Glycerol	NA	NA	120
Hypoxanthine	NA	NA	10
Lactate	NA	NA	1600
Malonate	NA	NA	10
Ornithine	NA	NA	70
Succinate	NA	NA	20
Taurine	NA	NA	90
Urea	NA	NA	5000
Uric acid	NA	NA	350

The compositions of DMEM F12 and BALM are shown. For human plasma, only the amino aci
concentrations are shown (Matsumoto et al, 2014).

Table 1. The compositions of DMEM F12, BALM, HPLM and amino acid concentrationsin human plasma

Fig. 2.1

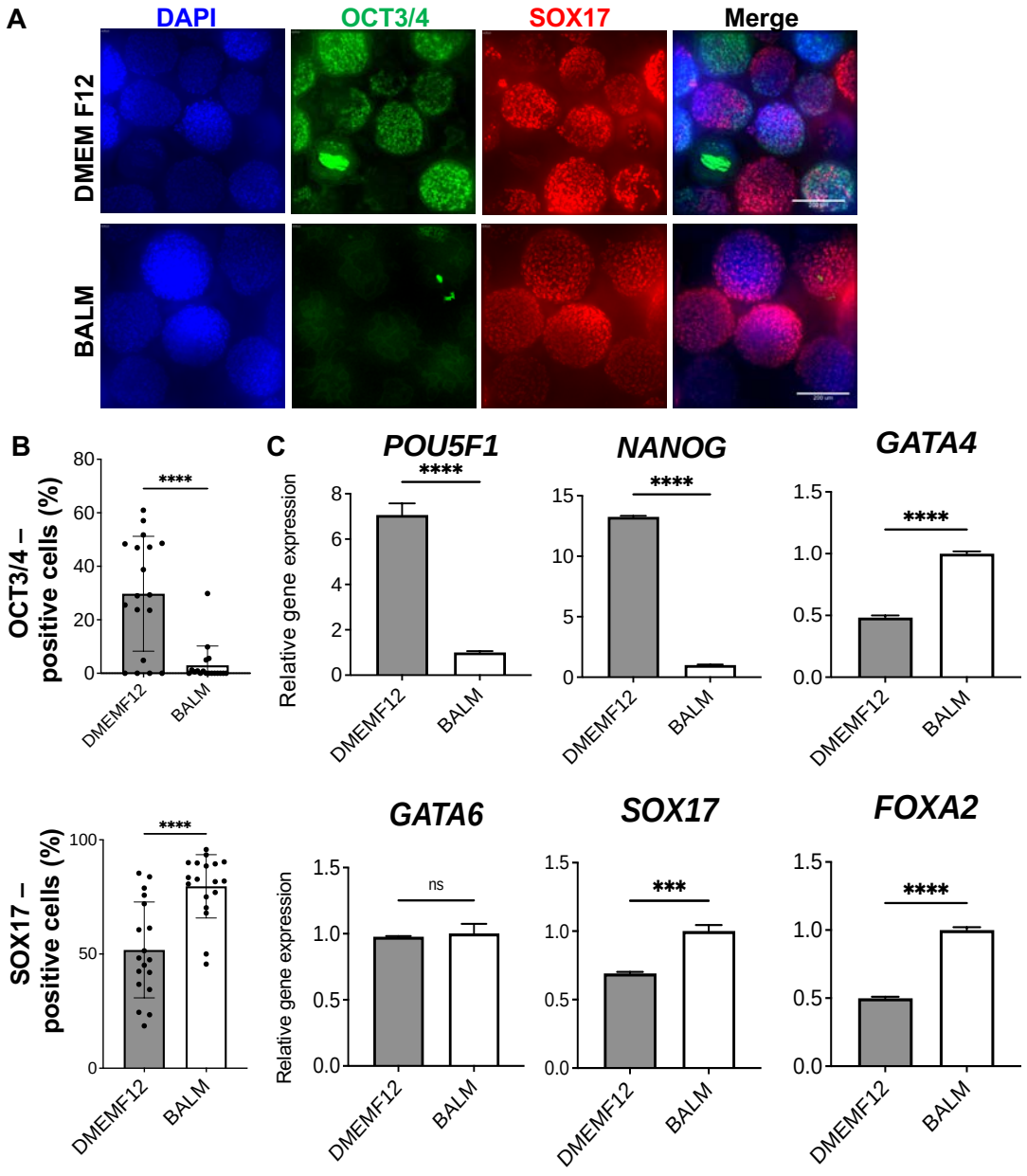

Fig. 2. BALM improved definitive endoderm (DE) differentiation efficiency compared to conventional DMEM F12 media

In vitro differentiation of hiPSCs cells into DE cells in BALM medium

(A) Immunocytochemical analysis of hiPSCs-derived cell clusters cultured in DMEM F12 (upper) or BALM (lower) based media on differentiation day 3, stained with DE marker SOX17 (red), pluripotent marker OCT3/4 (green), and nuclei (DAPI, blue). Representative images are shown. Scale bars, 200 µm. (B) Quantification of OCT3/4 (upper) and SOX17 (lower)-positive day 3 cell clusters cultured in DMEM F12 or BALM-based medium. Y axis, the % positivity of immunostainings. Each dot represents one cluster. (C) Expression of marker genes for pluripotency (*NANOG*, *POU5F1*) and DE (*GATA4*, *GATA6*, *SOX17*, *FOXA2*) in iPSC derived day 3 clusters cultured in DMEM F12 or BALM, analyzed by real-time PCR analyses. Y axis, fold changes compared to those cultured in BALM (=1).

Data are expressed as mean ± SD. N= 3 (biological replicates). Differences between groups were analyzed by Student' 's t-test; significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$.

Fig. S2.1

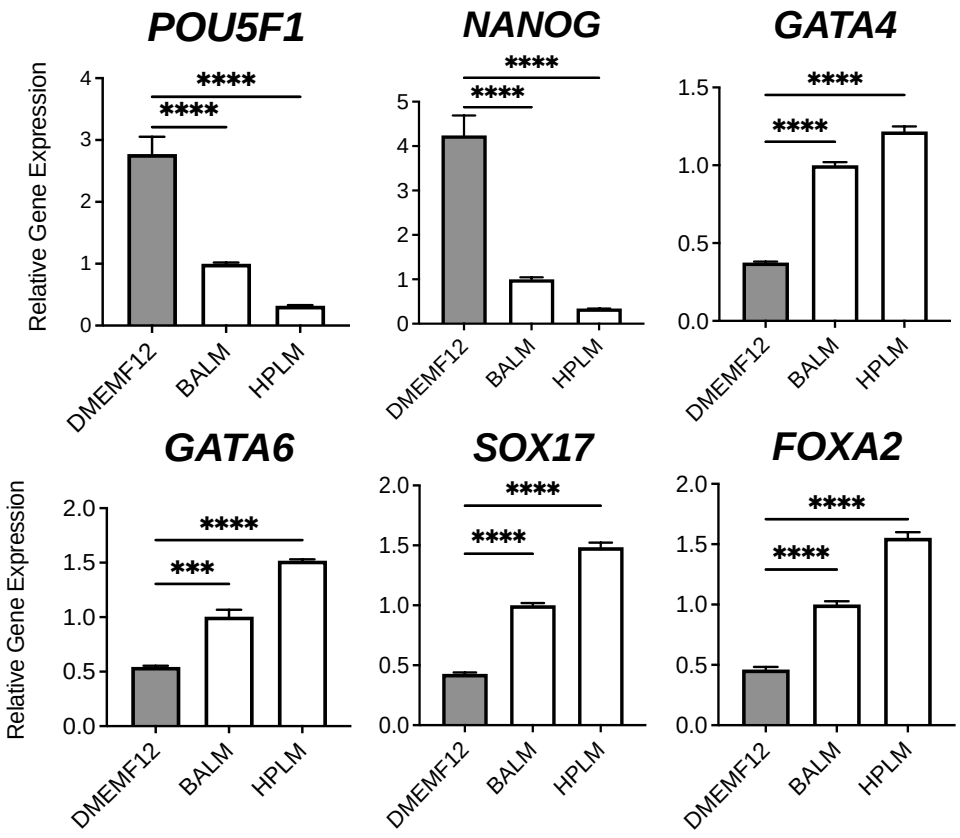

Fig. S2.1. *In vitro* differentiation of hiPSCs into DE cells in BALM or HPLM-based medium is potentiated compared to those cultured in DMEM F12-based medium.
Real-time PCR analyses of hiPSCs-derived cell clusters on differentiation day 3, analyzed for marker gene expressions for pluripotency (*NANOG*, *POU5F1*) and DE (*GATA4*, *GATA6*, *SOX17*, *FOXA2*). N= 3 (biological replicates). Y axis, fold-changes compared to those cultured in BALM-based medium (=1).
Data are expressed as mean ± SD. Differences between groups were analyzed by one-way ANOVA ; Significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$.

2.2 hiPSCs differentiated in the BALM-based medium can give rise to pancreatic progenitor cells, endocrine progenitor cells, and endocrine cells.

Next, to determine whether the culture in a BALM-based medium permits differentiation into PDX1+ pancreatic progenitor (PP). hiPSC-derived DE cells were differentiated into PP cells (day 9.) Differentiation of hiPSC in BALM-based medium yielded (76.0% ± 7.6%) PDX1+ PP cells and

(42.7% ± 7.4%) SOX9+ cells (Fig. 2.2 A, B). Further differentiation of PP cells into endocrine progenitor (EP) cells yielded approximately (46.0% ± 10.4%) PDX1/ NKX6.1 double-positive cells on day 15 (Fig. 2.2 C, D). hiPSC-derived EP cells (day 15) expressed pancreatic progenitor marker genes, *PDX1*, *SOX9*, and endocrine progenitor marker genes, *NGN3* and *NKX6.1* (Fig. 2.2 E). The higher expression levels of these genes in the hiPSC-derived EP cells than those found in human islets suggest their immature nature. Differentiation in BALM-based medium yielded INS+ endocrine cells (EC) on day 21, and the majority were INS single-positive cells (Fig. 2.2 F). The results show that hiPSC-derived DE can be efficiently differentiated into the PP, EP, and EC cells under the BALM-based medium.

Fig. 2.2

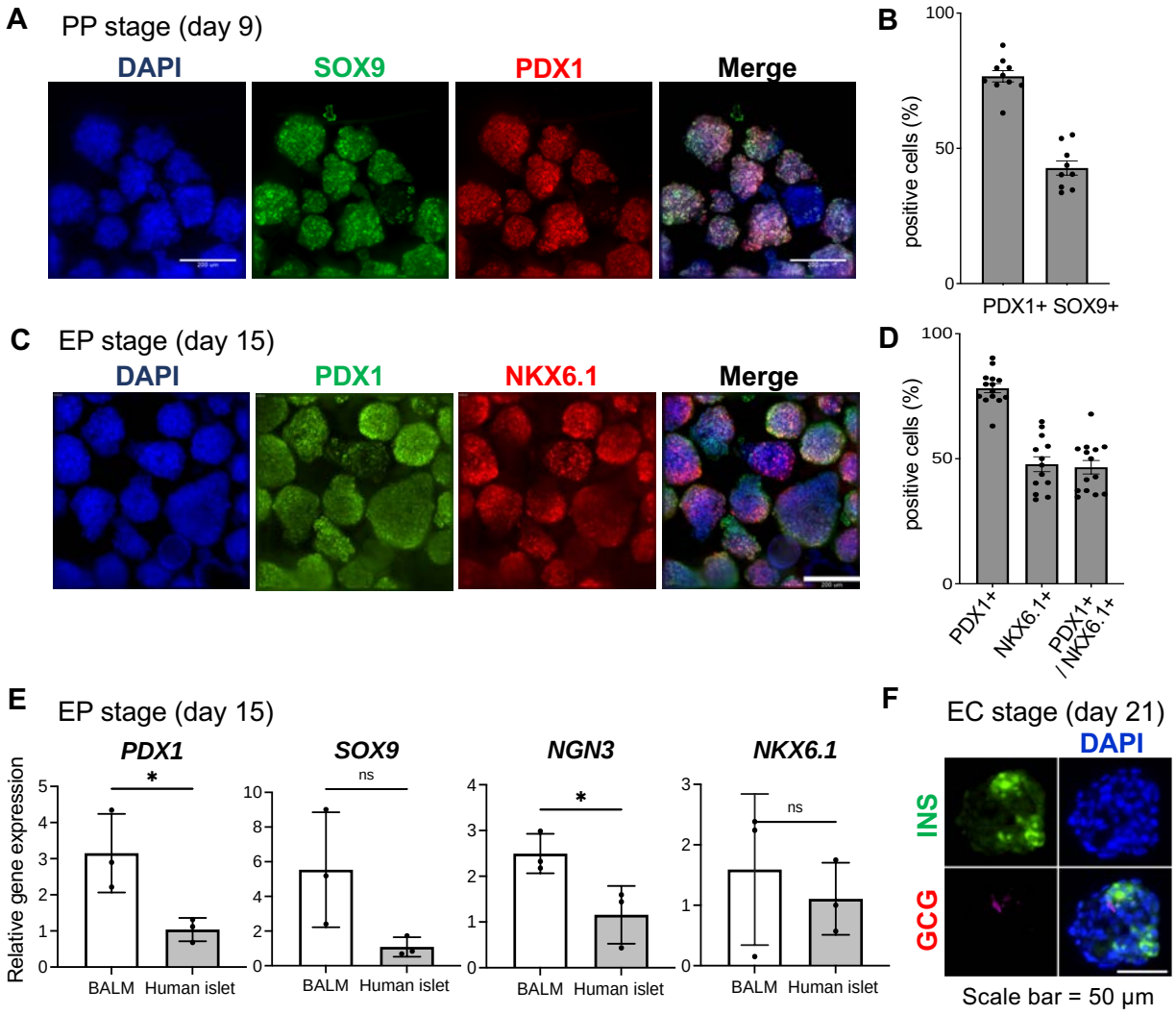

Fig. 2.2 Differentiation in BALM-based medium can give rise to pancreatic progenitor cells, endocrine progenitor cells and endocrine cells.

(A, B) Immunocytochemical analysis of stage 3 hiPSC-derived PP cells generated with BALM-based medium on day 9. Cell clusters were stained for PP marker PDX1 (red), SOX9 (green), and nuclei (DAPI, blue). (C, D) Immunocytochemical analysis of stage 4 EP on day 15. Cell clusters were stained for EP marker NKX6.1 (red), PDX1 (green), and nuclei (DAPI, blue). (A, C) Representative images are shown. Scale bars, 200 µm. (B, D) Each dot represents one cluster. (E) Real-time PCR analyses of pancreatic differentiation marker genes on differentiation day 15. Y axis, fold changes compared to human islets (=1). (F) Immunocytochemical analysis of stage 5 EC on day 21. Cell clusters were stained for EC marker INS (green), GCG (red), and nuclei (DAPI, blue). Scale bar, 50µm. Data are expressed as mean ± SD. N= 3 (biological replicates). Differences between groups were analyzed by Student's t-test; significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$

2.3 BALM-based medium enables the derivation of glucose-responsive SC-β Cells

Next to examine the differentiation into insulin-expressing SC-β cells. Immunostaining of SC-β cells on differentiation day 33 revealed (40.7% ± 4.9%) INS+ cells, (39.2% ± 4.3%) NKX6.1+ cells, and (18.9% ± 5.0%) INS/NKX6.1 double-positive cells (Fig. 2.3 A, 3B). I examined the expression of several β cells and islet markers in SC-β cells and compared them with cadaveric human islets. BALM generated SC-β cells exhibited higher *PDX1*, *MAFB*, *CHGA*, *SST*, *PCSK1*, *GCK*, and *ISL1* levels than human islets. *INS* and *MAFA* expressions were lower than those of the human islets. *GCG*, *NKX6.1*, *ABCC8*, *PCSK2*, and *SLC30A8* expressions were comparable with human islets. Gene expressions are presented as fold-changes compared to human islets (human islet = 1) (Fig. 2.3 C). The results are consistent with previously published results of stage 6 cells where the expressions of several β cell markers were equal to or greater than human islets. However, other markers remain low (Velazco-Cruz et al., 2019).

I tested the function of stage 6 cells generated in a BALM-based medium using a static GSIS assay. The SC-β cells secreted insulin when challenged with high glucose (Fig. 2.3 D). The data suggest that BALM-based differentiation is suitable for pancreatic beta-cell differentiation.

Fig. 2.3

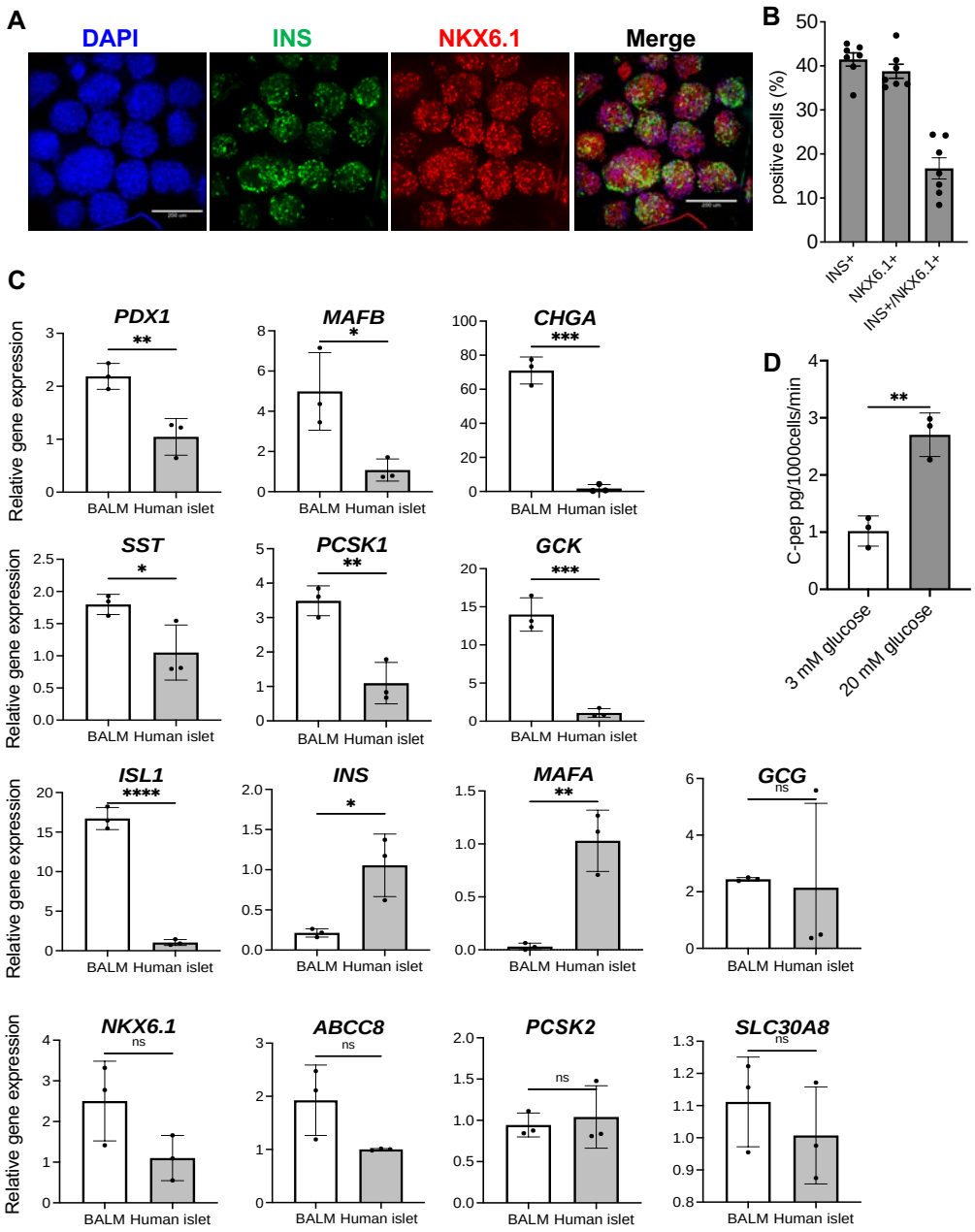

Fig. 2.3 BALM-based medium enables derivation of SC-β Cells

(A, B) Immunocytochemical analysis of stage 6 SC-β generated with BALM-based medium on day 33. Cell clusters were stained for SC-β marker NKX6.1 (red), INS (green), and nuclei (DAPI, blue). Representative images are shown. Scale bar, 200 μm. (B) Y axis, the % positivity of staining. Each dot represents one cluster. (C) Real-time PCR analyses of pancreatic endocrine differentiation marker genes on day 33. Y axis, fold changes compared to human islets (=1). (D) Glucose-stimulated insulin secretion of SC-β cells differentiated by culturing in a BALM-based medium. Data are expressed as mean ± SD. N= 3 (biological replicates). Differences between groups were analyzed by Student's t-test; significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$

2.4 Discussion

The presence of undifferentiated cells that might interfere with the function of the desired cell types or even be carcinogenic remains a challenging issue with hiPSCs-derived therapeutic products. Improving the efficiency of the generation of glucose-responsive cell populations while minimizing undesired cell types during in vitro differentiation is crucial for the clinical translation of SC-β cells toward curative diabetes treatment (Nair et al., 2020). Exploiting metabolic dependence by eliminating or restricting nutrient availability is emerging as a novel cancer therapeutic module (Nair et al., 2020; Pavlova and Thompson, 2016; Wang et al., 2019).

Many of the conventional cell culture media are established to sustain continuous cancer cell proliferation in vitro. Yet, their formulation does not reflect the nutritional environment of our body fluid. Recent reports show that culturing cells in media with supraphysiological concentrations of nutrients leads to recapitulating more cancer-cell-like metabolism. In cancer research, efforts have been undertaken to develop culture media that mimic the amounts of nutrients and metabolites in human plasma. Compared to traditional media, such investigations have revealed changes in metabolic phenotype and therapeutic efficacy (Cantor et al., 2017; Leney-Greene et al., 2020; Rossiter et al., 2021; Voorde et al., 2019). Refinement of media formulations can potentially improve the efficiency of the in vitro generation of pancreatic β-cells from hiPSCs. The metabolic signature of hiPSCs is highly similar to cancer cells. Both depend on aerobic glycolysis, increased amino acid uptake, and glutamine oxidation as sources of energy expenditure and biomass production (Intlekofer and Finley, 2019).

This study shows that by refining the nutrient composition of BALM-based medium, we successfully promoted hiPSCs differentiation into the DE. The physiological-like culture medium formulation significantly reduced the undifferentiated cell population. Moreover, pluripotency markers (*POU5F1, NANOG*) expression was considerably lower in cells cultured in BALM than in

those cultivated in DMEM F12-based medium. When comparing cells grown in BALM to those cultured in DMEM F12, the DE marker genes (*SOX17*, *FOXA2*, *GATA4*) exhibited a significant increase in cells cultured in a BALM-based medium. These findings indicate that supraphysiological concentrations of nutrients suppressed DE differentiation. The use of physiological levels of nutrients can lead to modulating cell function.

My finding may have potential translational application in developing hPSCs models for studying metabolic regulation of pancreatic β-cell differentiation (Balboa et al., 2019; Tyurin-Kuzmin et al., 2020). In conclusion, I developed a specified culture system to generate SC-β cells using a blood amino acids-like medium (BALM). BALM-based medium successfully converts hiPSCs into the definitive endoderm, pancreatic progenitors, endocrine progenitors, and SC-β.

Chapter 3: Relationship between supraphysiological levels amino acids and endoderm differentiation efficiency

3.1 Supraphysiological nutrients levels interfere with efficient differentiation through upregulation of the mTOR pathway

Given the higher supraphysiological level of glucose and amino acids of DMEM/F12 compared to BALM, therefore, to understand the underlying mechanisms, we examined the activity of the nutrient-sensing mTOR pathway. hiPSCs cultured in BALM showed inhibition of the mTOR pathway as indicated by a decrease in the Phospho-S6 Ribosomal Protein (P-S6), a downstream target of mTOR widely used to monitor mTOR. hiPSCs cultured under mTOR inhibition (+ Rapamycin) revealed an increase in the protein level of the DE marker SOX17 (Fig. 3.1 A, B). This result correlated with that the SOX17+ cells increased in iPSCs differentiated under BALM-based medium or hiPSCs treated with mTOR inhibitor rapamycin, compared to those cultured in DMEM/F12 (Fig. 3.1 C, D).

The result suggests that the hiPSCs differentiated in the BALM-based medium show an enhanced endoderm differentiation through downregulation of the mTOR pathway. Expression analysis

revealed that *SOX17* expression was upregulated when hiPSCs were cultured with the mTOR inhibitor or BALM-based medium compared to those cultured in DMEM/F12 (Fig. 3.1 E)

The result suggests that the BALM medium potentiates endoderm differentiation of hiPSCs through downregulation of the mTOR pathway.

Fig. 3.1

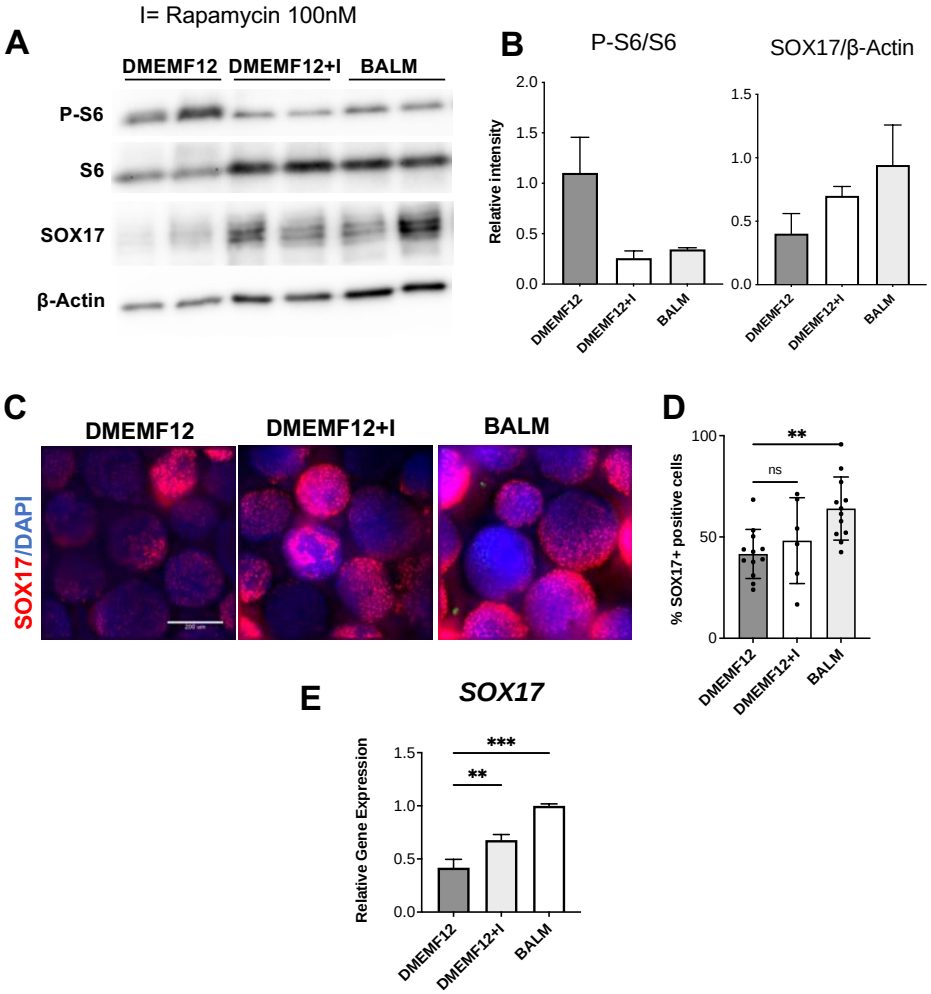

Figure 3.1. Supraphysiological nutrients level interfere with efficient differentiation through upregulation of the mTOR pathway

(A, B) Western blot analysis of P-S6 and total S6. β-Actin was a loading control (A). Y-axis represents the relative intensities of the band measured with Image J (B). (C, D) Immunocytochemical analysis of the hiPSCs-derived DE cells cultured under DMEM/F12 (DMEMF12), BALM-based medium, or DMEM/F12 medium + mTOR inhibitor Rapamycin (DMEMF12 + I). SOX17 (red). Counterstained with DAPI. Scale bar: 200 µm. (E) RT-PCR expression of marker genes for DE SOX17. Data are expressed as mean ± SD. N= 3 (biological replicates). Differences between groups were analyzed by one way ANOVA; significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$.

3.2 Supraphysiological arginine and methionine levels interfere with efficient differentiation

Among growth factors that were being used for the cell culture system, amino acids are also well studied for ES/iPS cell culture settings. It is known that these cells have a unique amino acid metabolism, and the amino acids in the cell culture are key components in order to maintain pluripotency. It was reported that deprivation of methionine or both arginine and proline from cell culture had been reported to impair the pluripotency of ES cells, resulting in downregulation of NANOG and OCT4 (He et al., 2018; Shiraki et al., 2013)

The concentrations of these amino acids in the media used for cell cultures, such as DMEM F12, are much higher than those found in the blood and therefore do not seem to reflect in vivo conditions. Methionine and arginine concentration at DMEMF12 are 115 µM, 699 µM respectively, where BALM concentration values mimic that of plasma and are 35 µM and 116 µM respectively. I compared using BALM supplemented with methionine and arginine up to DMEMF12 and DMEM F12-based medium and assayed for their effects on differentiation of the human-induced pluripotent stem cells (hiPSCs) RPChiPS771 cells. To examine the impact of other amino acids on differentiation, I assessed SOX17 and OCT3/4 expression of day 3 clusters. Immunofluorescence revealed a significant increase in OCT3/4-positive cells derived in DMEM F12 and BALM + arginine (Arg) +methionine (Met) compared to BALM (Fig. 2.1 A, B). Expression analysis of the pluripotency marker *OCT* (*POU5F1*) showed significantly increased levels in cells cultured in DMEM F12, and BALM+ arginine (Arg) +methionine (Met) compared to BALM. The definitive endoderm (DE) marker genes (*GATA4*, *SOX17*, *FOXA2*) revealed a significant decrease in cells cultured in BALM+ arginine (Arg) +methionine (Met) compared to BALM (Fig. 2.1 C). I examined the activity of the nutrient-sensing mTOR pathway. hiPSCs cultured in BALM+ arginine (Arg) + methionine (Met) showed upregulation of the mTOR pathway as indicated by an increase in the Phospho-S6 Ribosomal Protein (P-S6), a downstream target of mTOR widely used to monitor

mTOR compared to BALM or BALM with additional single amino acid arginine or methionine. These results may suggest that supraphysiological level of arginine and methionine may have a synergistic effect that interferes with proper definitive endodermal differentiation.

Fig. 3.2

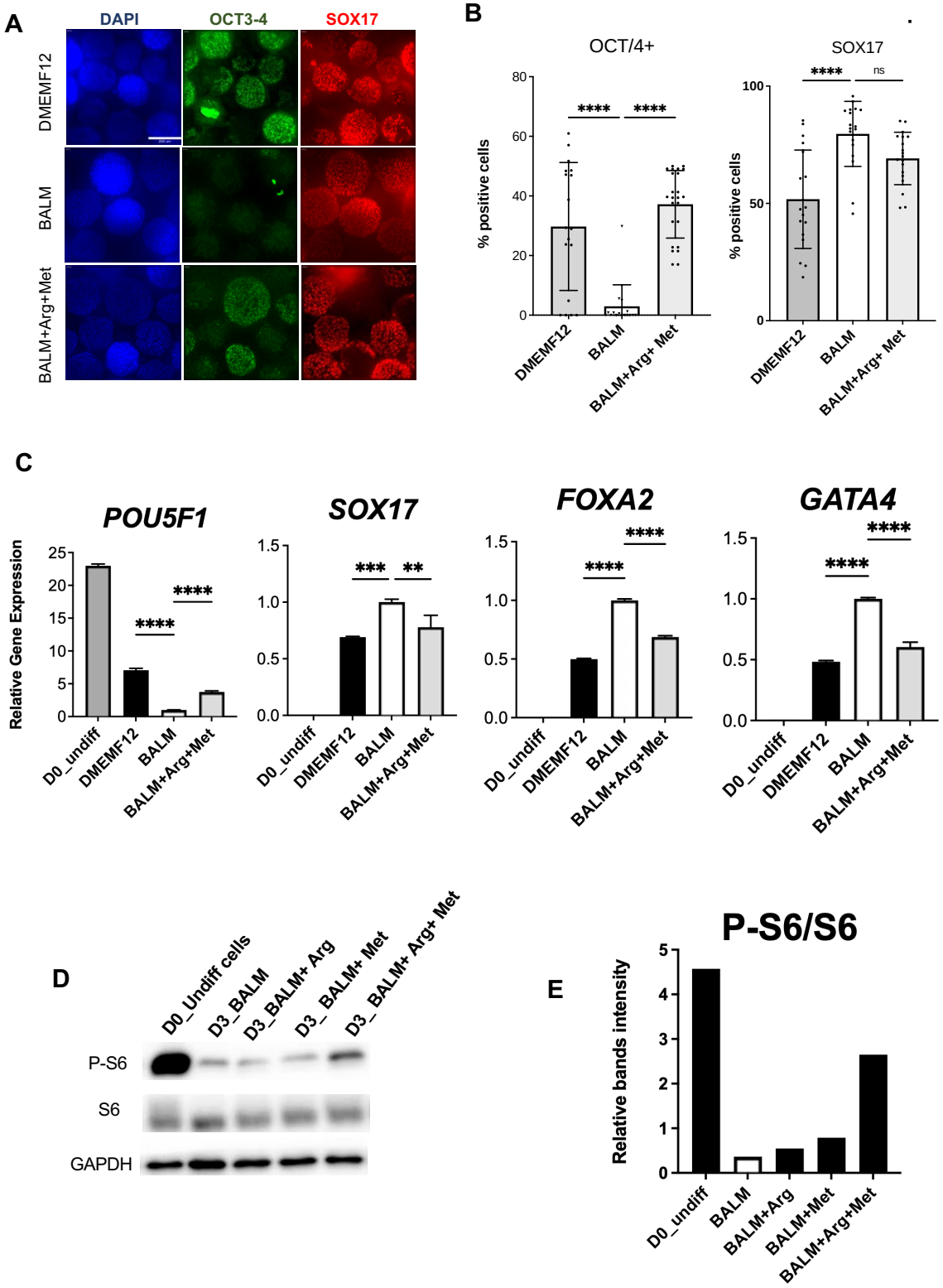

Figure 3.2 Conventional medium has supraphysiological of arginine and methionine that interfere with efficient differentiation
(A) Immunocytochemical analysis of hiPSCs-derived cell clusters cultured in DMEM F12 (upper) or BALM (lower) or BALM+ arginine (Arg)+ methionine (Met) up to levels of that of DMEMF12 media on differentiation day 3, stained with DE marker SOX17 (red), pluripotent marker OCT3/4 (green), and nuclei (DAPI, blue). Representative images are shown. Scale bars, 200 μm. (B) Quantification of OCT3/4 (upper) and SOX17 (lower)-positive day 3 cell. Y axis, the % positivity of immunostainings. Each dot represents one cluster. (C) Expression of marker gene for pluripotency (*POU5F1*) and DE (*GATA4*, *SOX17*, *FOXA2*) in iPSC derived day 3 clusters cultured in DMEM F12 or BALM or BALM+ arginine (Arg)+ methionine (Met), analyzed by real-time PCR analyses. Y axis, fold changes compared to those cultured in BALM (=1). (D, E) Western blot analysis of P-S6 and total S6 of day3 cells. GAPDH was a loading control (D). Y-axis represents the relative intensities of the band measured with Image J (E). Data are expressed as mean ± SD. N= 3 (biological replicates). Differences between groups were analyzed by one-way ANOVA; significances are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$.

3.3 Distinct metabolite profiles in hiPSCs cultured between physiological and supraphysiological nutrient-containing media

The metabolic profiles of the hiPSCs cultured in the supraphysiological and physiological nutrients were explored. We used MCDB as a supraphysiological medium since MCDB is a widely used DMEM/F12-based medium for differentiating SC-β cells. HPLM was used instead of BALM since HPLM is a commercially available media. The hiPSC culture supernatants before and during DE differentiation were sampled and measured daily by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Since the medium contains amino acids and other metabolites that cells consume to accompany cell growth, the initial amounts of the metabolites existing in the fresh medium were subtracted from our measurements and normalized to the area of the cells and 24h. A plus value represents the excretion from the cells, and a minus value represents the uptake (consumption) by the cells (relative value / cm^2 / 24h).

The metabolite changes are as follows: hiPSCs excrete Alanine and Ornithine in MDCB, whereas they intake Alanine and Ornithine when cultured in an HPLM-based medium. hiPSCs uptake lower levels of many amino acids, such as serine, threonine, lysine, arginine, valine, methionine, tyrosine, isoleucine, leucine, and phenylalanine, when they are cultured in HPLM, compared to in MCDB-based medium. On the other hand, there are no changes in the excretion of Lactic acid between

hiPSCs cultured in HPLM and MCDB-based medium, suggesting the activities of glycolysis are similar between the two conditions.

It was concluded that the hiPSCs cultured in physiological nutrients behave thriftily and are metabolically suppressed. The hiPSCs utilize lesser amino acids when cultured in physiological nutrients than those cultured under supraphysiological nutrients.

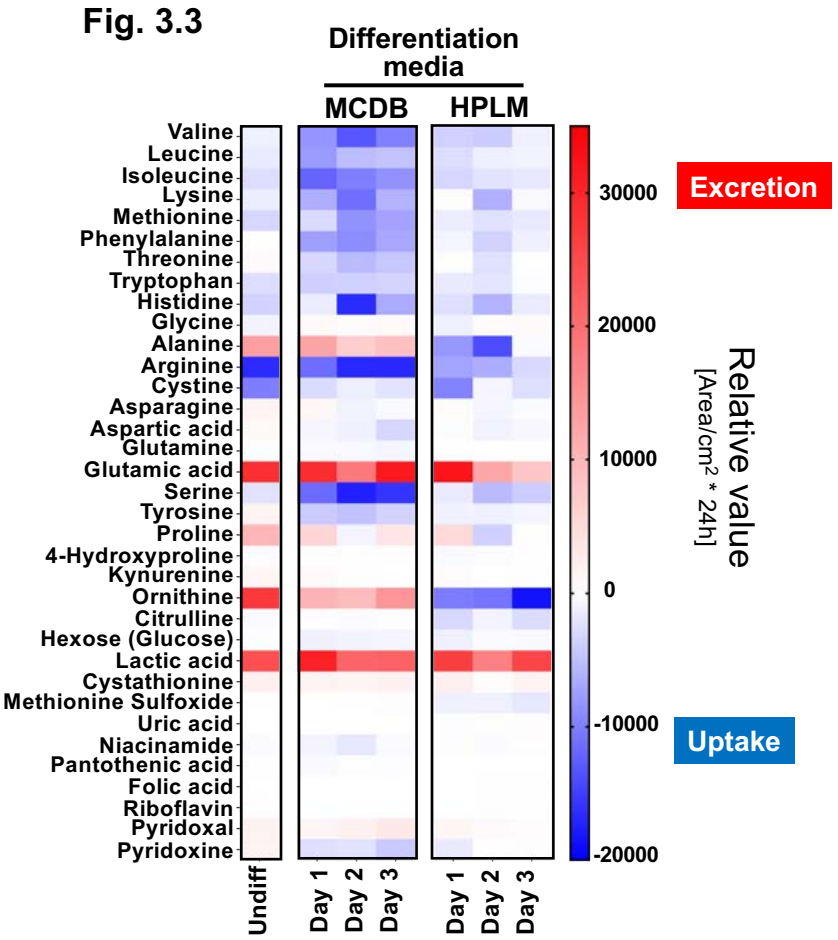

Figure 3.3. Distinct metabolite profiles between hiPSCs cultured in physiological (HPLM) and supra-physiological (MCDB) medium.
Differentiated hiPSCs cultured in HPLM or MCDB medium (a DMEM/F12-based) on days 1, 2, and 3 were sampled and analyzed for metabolites. The metabolite profiles are shown in a heatmap. Blue: uptake. Red: excretion of metabolites. Supernatants from undifferentiated iPSCs (Undiff) were used as controls. Data are expressed as mean N= 3 (biological replicates).

3.4 Discussion

Generating therapeutic modalities from hiPSCs is challenging due to the presence of undifferentiated cells that can interfere with desired cell types or even be carcinogenic (Mayhew and Wells, 2011). To develop effective diabetes treatments using SC-β cells, it is crucial to improve the generation of glucose-responsive cell populations while minimizing the unwanted cell types during differentiation in vitro. A new cancer treatment approach is to exploit metabolic dependence by restricting nutrient availability (Voorde et al., 2019).

Conventional cell culture media are designed to sustain continuous cancer cell growth in vitro, but recent studies show that using a physiological media composition that mimics the nutritional environment of serum can result in cells with a more cancer-cell-like metabolism (Kate Shannon ., 2016). Efforts have been made to develop culture media that mimic human plasma's nutrient and metabolite levels, resulting in changes in metabolic phenotype and therapeutic efficacy. Improving the nutrient composition of BALM-based medium can enhance the in vitro generation of pancreatic β-cells from hiPSCs by reducing the population of undifferentiated cells without affecting cell numbers. This study suggests that using physiological levels of nutrients can modulate cell function, as hiPSCs' metabolic signature is similar to cancer cells.

I elucidated the causes underlying phenotypic differences in cells cultured in BALM and DMEM-F12. hiPSCs cells cultured in BALM showed potentiated endoderm differentiation, correlated with downregulation of the mTOR pathway. Our results are consistent with the previous report that showed that the activated mTORC1 pathway sustains pluripotency and negatively affects differentiation (Yu and Cui, 2016; Zhou et al., 2009).

The effects of HPLM and MCDB1on cell metabolism were compared. The glucose and lactate exchange rates in hiPSCs cultured between the two mediums were not significantly different, suggesting it had little effect on glycolysis. In contrast, we found a correlation between the

availability and consumption of several amino acids. A positive correlation was found between amino acid uptake and excretion. In particular, MCDB1 cultured hiPSCs released alanine but consumed it in HPLM, which is more concentrated.

Together, these findings reveal that supplementing the medium with amino acids different from those present in serum resulted in corresponding changes in their uptake/excretion and metabolism. The results show that the supraphysiological concentrations of nutrients typically found in conventional media skew the metabolism of hiPSCs. The results agreed with previously reported results in cancer cells (Cantor et al., 2017; Voorde et al., 2019). Our finding may have potential translational application in developing hPSCs models for studying metabolic regulation of pancreatic β-cell differentiation (Balboa et al., 2019; Tyurin-Kuzmin et al., 2020).

In conclusion, we developed a specified culture system to generate SC-β cells using a blood-like amino acid medium (BALM). Through metabolite analysis of the culture media, we revealed that hiPSCs changed their metabolism to adapt to physiological levels of nutrients and became thriftily to utilize lesser nutrients. BALM may convert hiPSCs into the definitive endoderm, pancreatic progenitors, endocrine progenitors, and SC-β. It would be interesting to study the long-term metabolic changes of the hiPSCs in the future.

Chapter 4: Generation of induced pluripotent stem cell-derived beta-cells in blood amino acids-like medium BSA-free medium

4.1 hiPSCs are efficiently differentiated into definitive endoderm (DE) in the BALM-based medium BSA free

Gradual adaptation of hiPSCs for culture from AK02N to E8 BSA-free medium was followed as seen in the scheme (Fig. 4.1 A). In addition, hiPSCs cells grown under BSA-free condition maintained their self-renewal capacity and pluripotency, as confirmed by expression analysis of the pluripotency markers (*POU5F1, NANOG*) in E8 compared to those cultured in AK02N medium,

BSA free condition also exhibited a distinctive morphology of sharp-edged, flat, and tightly packed colony structures (Fig. 4.1 B, C).

To examine the impact of BSA-free BALM condition on differentiation, I assessed SOX17 and OCT3/4 expression of day 3 clusters. Immunofluorescence revealed an increase in the ratio of SOX17-positive cells cultured in BALM, low OCT3/4-positive cells (Fig. 4.1 D, E). The BALM BSA free-based endoderm differentiation protocol was beneficial in that potentiation of differentiation.

Fig. 4.1

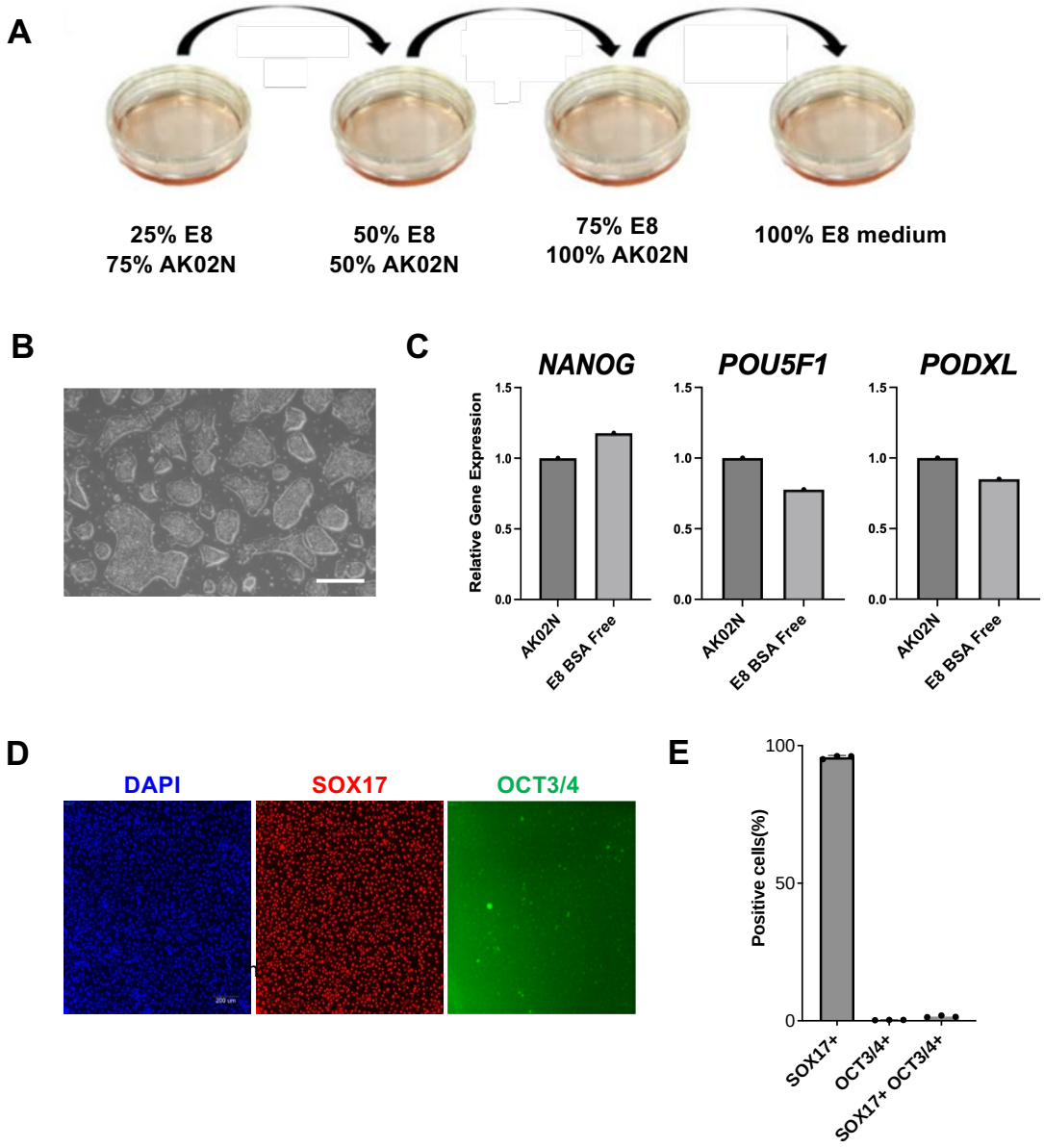

Fig. 4.1. *In vitro* DE differentiation of hiPSCs cells cultured in BALM BSA free medium
(A) Schematic representation of adaptation of hiPSCs to E8 BSA fee culture.
(B) hiPSCs cultured in E8 BSA fee culture maintained compact colony structure (C) Expression of marker genes for pluripotency (*NANOG*, *POU5F1*) and hiPSC cultured in E8 or AK02N, analyzed by real-time PCR analyses (D) Immunocytochemical analysis of hiPSCs-derived cell clusters cultured on differentiation day 3, stained with DE marker SOX17 (red), pluripotent marker OCT3/4 (green), and nuclei (DAPI, blue). Representative images are shown. Scale bars, 200 μm. (E) Quantification of OCT3/4 (upper) and SOX17 (lower)-positive day 3 cells. Data are expressed as mean ± SD. N= 3 (biological replicates). (C, N=1)

4.2 hiPSCs are efficiently differentiated into pancreatic progenitor cells and endocrine progenitor cells in the BALM- BSA free-based medium

Further differentiation of into endocrine progenitor (EP) cells yielded approximately (37.4% ± 3.9%) PDX1/ NKX6.1 double-positive cells on day 15 (Fig. 4.2 A, B). The results show that hiPSC-derived DE can be efficiently differentiated into the PP, EP cells under the BALM-based medium and BSA-free medium. Next, I examined the differentiation into insulin-expressing SC-β cells. Immunostaining of SC-β cells on differentiation day 31 revealed (69.4% ±2.2 %) INS+ cells, (52.3% ± 2.4%) NKX6.1+ cells, and (27.3% ± %) INS/NKX6.1 double-positive cells (Fig. 4.2 C.D). The function of stage 6 cells generated in a BALM- BSA free based medium using a static GSIS assay. The SC-β cells secreted insulin when challenged with high glucose (Fig. 4.2 E). The data suggest that BSA-free BALM-based differentiation is suitable for pancreatic beta-cell differentiation

Fig 4.2

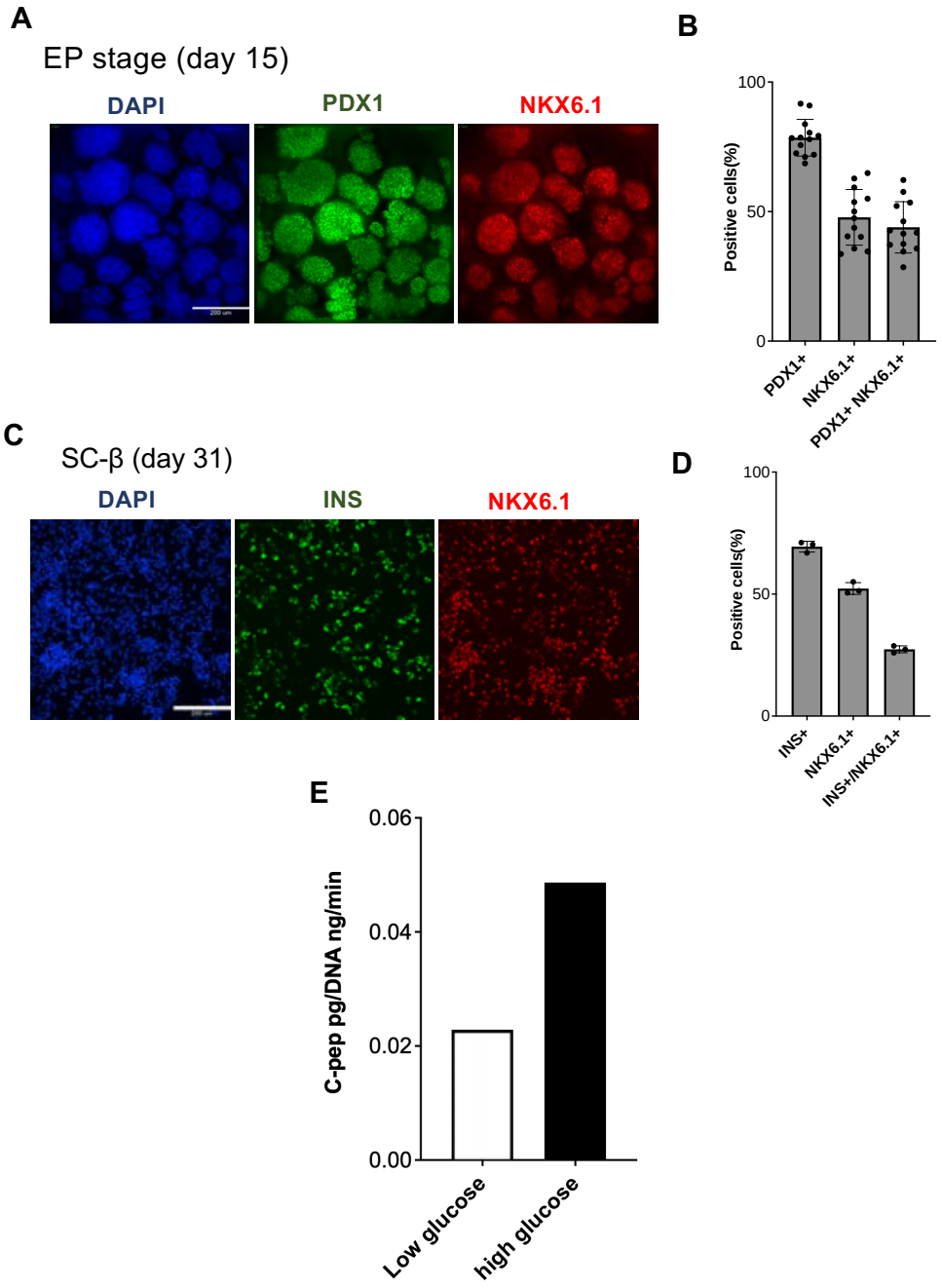

Fig. 4.2 Differentiation in BALM-based medium can give rise to endocrine progenitor cells and endocrine cells.

(A, B) Immunocytochemical analysis of stage 4 EP on day 15. Cell were stained for EP marker NKX6.1 (red), PDX1 (green), and nuclei (DAPI, blue). (A) Representative images are shown. Scale bars, 200 μm. (C, D) Immunocytochemical analysis of stage 6 SC-β generated with BALM-based medium. Cell were stained for SC-β marker NKX6.1 (red), INS (green), and nuclei (DAPI, blue). Representative images are shown. Scale bar, 200 μm. (D) Y axis, the % positivity of staining. (E) Glucose-stimulated insulin secretion of SC-β cells

differentiated by culturing in BALM BSA free-based medium. Data are expressed as mean ± SD. N= 3 (biological replicates) (GSIS results N=1)

4.3 Discussion

Albumin-free cell culture refers to a type of cell culture where the growth media used to culture the cells does not contain any albumin. Albumin is a protein that is often used in cell culture media as a supplement because it can bind to various nutrients and growth factors, thereby increasing their stability and availability to the cells. However, there are some situations where the use of albumin in cell culture media may be undesirable (Francis, 2010).

For example, some cell types may be sensitive to the presence of albumin in the media, and the protein may interfere with experimental outcomes. Additionally, albumin can introduce variability into cell culture experiments because the concentration and quality of albumin can vary from batch to batch.(van der Valk et al., 2010)

Albumin-free cell culture media is typically formulated using alternative protein sources, such as transferrin or bovine serum albumin (BSA)-free formulations. These media are designed to support the growth and survival of cells in the absence of albumin (Chen et al., 2011).

Researchers may choose to use albumin-free cell culture media in a variety of applications, including drug discovery, toxicology, and biomanufacturing. By eliminating albumin from the media, they can reduce experimental variability and ensure the reproducibility of their results (Burridge et al., 2015; Pei et al., 2017; Qu et al., 2017). I developed a chemically defined bovine serum albumin-free specified culture system to generate SC-β cells using a blood amino acids-like medium (BALM). BALM-based medium successfully converts hiPSCs into the definitive endoderm, pancreatic progenitors, endocrine progenitors, and functional SC-β.

Chapter 5: Conclusion

5.1 Conclusion

Classic cell culture media do not accurately represent the availability of the nutrients in plasma. They usually contain a supraphysiological concentration of nutrients such as glucose, amino acids, etc. These high nutrients can alter the metabolism of cultured cells and induce metabolic phenotypes that do not reflect in vivo conditions. Refinement of media formulations has a potential application in the maturity modulation of stem cell-derived β cells (SC-β) generation in vitro. This has the potential importance in regenerative medicine for curative diabetes treatment. Previous reports from mice studies have shown that amino acid levels progressively decreased between embryonic day 19 and postnatal day 9. Moreover, elevated amino acid levels have been linked to the activated mammalian target of rapamycin complex 1 (mTORC1) pathway, an increase in islet cell proliferation, and a low level of maturation.

It has been demonstrated that late-stage culture in low levels of amino acids promoted the maturation of SC-β and improved their glucose-stimulated insulin secretion (GSIS) function. To the best of our knowledge, the effect of the physiological level of amino acids on the whole differentiation process has not been investigated.

In this study, I asked whether SC-β cells could be derived in media that better recapitulates the composition of human plasma amino acids. And whether it would promote differentiation.

To address these questions, I established for the first time a defined culture system to derive SC-β cells using a blood-like amino acids medium (BALM) using RPChiPS771-3G cell line. RT-PCR, immunohistochemistry, western blot analysis and GSIS, were performed to evaluate differentiation efficiency and maturation level.

I demonstrated that supraphysiological nutrients level interferes with efficient endodermal differentiation through upregulation of the mTOR pathway. Moreover**,** human pluripotent stem cells (hPSCs) can be efficiently differentiated into definitive endoderm, pancreatic progenitors, endocrine

progenitors, and SC-β in BALM. The differentiated cells secreted C-peptide *in vitro* in response to high glucose levels and expressed several pancreatic β-cell markers. Therefore, I conclude that the physiological level of amino acids is sufficient for the derivation of functional SC-β cells.

Materials and Methods

Ethics Approval

The use of hiPSCs was approved by the institutional ethical committee involving human materials by the Tokyo Institute of Technology.

Maintenance culture of human iPS cells

RPChiPS771 human induced pluripotent stem cells (iPSCs; REPROCELL Inc.) were maintained in StemFit AK02N medium (Ajinomoto Co. Ltd) on 100 mm CellBIND cell culture dishes (Corning 3296) precoated with vitronectin (Thermofisher A31804). Cell passages were performed as follows. hiPSCs were washed, dissociated with TrypLE™ Select (Gibco), and resuspended in StemFit AK02N medium supplemented with 10 μM ROCK inhibitor (Y-27632; Wako) and replated at 1-2 x 10^6 cells per dish. For BSA-free culture, cells were cultured in Essential 8™ basal medium Thermo Fisher Scientific supplemented with Essential 8™ supplement.

Endodermal differentiation of hiPSCs

For spheroid formation, RPChiPS771 human iPSCs were harvested, dissociated, and replated at a density of $5x10^6$cells /well onto 6-well suspension culture plates (Greiner Bio One#657185) in AK02N medium supplemented with 10 μM ROCK inhibitor, and cultured at 37°C for 24 hours (h) to form sphere on a rotating orbital shaker at 95 RPMs. Endodermal differentiation was initialized 24 h post-seeding by changing with differentiation medium. (Fig.1) Custom-made BALM, DMEM F12, and HPLM were used for differentiation (Institute of peptide research Co. Ltd)

Pancreatic differentiation of hiPSCs

Pancreatic differentiation was preformed following a previously reported protocol with modifications (Velazco-Cruz et al., 2019). RPChiPS771 human iPSCs were harvested, dissociated, and replated at a density of $5x10^6$ cells /well onto 6-well suspension culture plates in BALM-based medium supplemented with differentiation factors as follows. Stage 1 (3 days): S1 medium + 100 ng/ml Activin A (Cell guidance system GFH6-1000) + 3 μM Chir99021 (Wako 034-23103) ＋10 μM Y27632 for 1 day. S1 medium + 100 ng/ml Activin A for 2 days. Stage 2 (3 days): S2 medium + 50 ng/ml KGF (KGF Wako 116-00811). Stage 3 (2 day): S3 medium + 50 ng/ml KGF + 200 nM LDN193189 (WAKO SML0559-5MG) + 500 nM PdBU (LC Laboratories P-4833) + 2 μM Retinoic Acid (Stemgent 04-0021) + 0.25 μM Sant1 (Sigma S4572) + 10 μM Y27632. Stage 4 (5-6 days): S4 medium + 5 ng/mL Activin A + 50 ng/mL KGF + 0.1 μM Retinoic Acid + 0.25 μM SANT1 + 10 μM Y27632. Stage 5 (8 days): S5-1 medium (4 days) + 10 μM ALK5i II (TOCRIS 3742) + 20 ng/mL Betacellulin (PrepoTech 100-50) + 0.1 μM Retinoic Acid + 1 μM T3 (Sigma T6397 + 1 μM XXI (Sigma; 595790)+ 10 μM Heparin+ 0.25 μM SANT1＋10 μM Y27632. S5-2 (4 days) medium + 10 μM ALK5i II (TOCRIS 3742) + 20 ng/mL Betacellulin (PrepoTech 100-50) + 0.1 μM Retinoic Acid +1 μM T3 (Sigma T6397) + 1 μM XXI (Sigma; 595790) +10 μM Heparin＋10 μM Y27632. Stage 6 (14 days): On the first day of stage 6, the reaggregation of cell spheres was performed. Cell spheres were washed in PBS and incubated in TrypLE Select Enzyme (Thermo Fisher Scientific #12563029) for 10 min at 37C. Spheres were mechanically dissociated using a P1000 pipette. Cells were washed with S6 medium + Y27632 (10 μM), resuspended in S6 medium + Y27632, then passed through a 100-μm CellStrainer (pluriSelect) to remove any residual undissociated clusters. The dissociated single cells were counted and seeded into a 6-well suspension culture plate (Greiner Bio One #657185). Differentiation media formulations used were the following. S1 medium: BALM supplemented with 1% Penicillin-Streptomycin solution (PS; Nacalai Tesque), 1%(v/v) B27 insulin (-) (Life Technologies A1895601), Recombinant IGF1 100 ng/ml (Oriental Yeast). S2 medium:

BALM supplemented with 1% PS, 1%(v/v) B27 (Life Technologies 17504044), S3 medium: BALM supplemented with 1% PS, 1%(v/v) B27 (Life Technologies 17504044), S4 medium: BALM supplemented with 1% PS, 1%(v/v) B27 minus vitamin A (Life Technologies 12587010), S5 medium: BALM supplemented with 1% PS, 1%(v/v) (B27 minus vitamin A, 3.6 g/L glucose (Otsuka Pharmaceutical Factory, Inc.). S6 medium: BALM supplemented with 1% PS, 2%(w/v) fatty acid-free (FAF)-BSA (Proliant Biologicals 68700-100G), 0.44 g/L glucose, 1μM ZnSO4 (Sigma; Z0251-100G), 10 μM Heparin, 0.1% Trace Elements A (Corning; 25-021-CI), and 0.1% Trace Elements B (Corning; 25-022-CI).

BSA free pancreatic differentiation of hiPSCs

Pancreatic differentiation was preformed following a previously reported protocol with modifications (Velazco-Cruz et al., 2019). RPChiPS771 human iPSCs were harvested, dissociated, and replated at a density of $5x10^6$ cells /well onto 6-well suspension culture plates in BALM-based medium supplemented with differentiation factors as follows. Stage 1 (3 days): S1 medium + 100 ng/ml Activin A (Cell guidance system GFH6-1000) + 3 μM Chir99021 (Wako 034-23103) ＋10 μM Y27632 for 1 day. S1 medium + 100 ng/ml Activin A for 2 days. Stage 2 (3 days): S2 medium + 50 ng/ml KGF (KGF Wako 116-00811). Stage 3 (2 day): S3 medium + 50 ng/ml KGF + 200 nM LDN193189 (WAKO SML0559-5MG) + 500 nM PdBU (LC Laboratories P-4833) + 2 μM Retinoic Acid (Stemgent 04-0021) + 0.25 μM Sant1 (Sigma S4572) + 10 μM Y27632. Stage 4 (5-6 days): S4 medium + 5 ng/mL Activin A + 50 ng/mL KGF + 0.1 μM Retinoic Acid + 0.25 μM SANT1 + 10 μM Y27632. Stage 5 (8 days): S5-1 medium (4 days) + 10 μM ALK5i II (TOCRIS 3742) + 20 ng/mL Betacellulin (PrepoTech 100-50) + 0.1 μM Retinoic Acid + 1 μM T3 (Sigma T6397 + 1 μM XXI (Sigma; 595790)+ 10 μM Heparin+ 0.25 μM SANT1＋10 μM Y27632. S5-2 (4 days) medium + 10 μM ALK5i II (TOCRIS 3742) + 20 ng/mL Betacellulin (PrepoTech 100-50) + 0.1 μM Retinoic Acid +1 μM T3 (Sigma T6397) + 1 μM XXI (Sigma; 595790) +10 μM Heparin＋10 μM Y27632. Stage 6 (14 days):

On the first day of stage 6, reaggregation of cell spheres was performed. Cell spheres were washed in PBS and incubated in TrypLE Select Enzyme (Thermo Fisher Scientific #12563029) for 10 min at 37C. Spheres were mechanically dissociated using a P1000 pipette. Cells were washed with S6 medium + Y27632 (10 μM), resuspended in S6 medium + Y27632, then passed through a 100-μm CellStrainer (pluriSelect) to remove any residual undissociated clusters. The dissociated single cells were counted and seeded into a 6-well suspension culture plate (Greiner Bio One #657185). Differentiation media formulations used were the following. S1 medium: BALM supplemented with 1% Penicillin-Streptomycin solution (PS; Nacalai Tesque), TeSR™-E5 20X Supplement (STEMCELL Technologies). S2 medium: BALM supplemented with 1% PS, TeSR™-E6 20X Supplement (STEMCELL Technologies)), S3 medium: BALM supplemented with 1% PS, TeSR™-E6 20X Supplement (STEMCELL Technologies), S4 medium: BALM supplemented with 1% PS, TeSR™-E6 20X Supplement (STEMCELL Technologies), S5 medium: BALM supplemented with 1% PS, TeSR™-E6 20X Supplement (STEMCELL Technologies), 3.6 g/L glucose (Otsuka Pharmaceutical Factory, Inc.). S6 medium: BALM supplemented with 1% PS, TeSR™-E6 20X Supplement (STEMCELL Technologies), 0.44 g/L glucose, 1μM ZnSO4 (Sigma; Z0251-100G), 10 μM Heparin, 0.1% Trace Elements A (Corning; 25-021-CI), and 0.1% Trace Elements B (Corning; 25-022-CI).

Real-time PCR analysis

RNA was extracted from human iPS cells using the RNeasy mini-kit, or All prep (DNA/RNA) Mini Kit (Qiagen, Germany) and then treated with DNaseI (Qiagen). 200 ng RNA was reverse-transcribed using PrimeScript™ RT Master Mix (Takara, Japan). For real-time PCR analysis, mRNA expressions were quantified with SyberGreen on a StepOne Plus (Applied Biosystems, Foster City, CA).

The PCR conditions were as follows: Initial denaturation at 95°C for 30 sec, denaturation at 95°C for 5 sec, annealing and extension at 60°C for 30 sec, for up to 40 cycles. Target mRNA levels were expressed as arbitrary units and were determined using the ΔΔ CT method.

Primer sequences used for the detection of endoderm and pancreatic differentiation and internal controls are listed in Table S2.

For human islet cDNA 3 batches of first strand cDNA from non-diabetic human islets (COSMO BIO Co. Ltd.): HIcDNA149, HIcDNA171T, and HIcDNA171) were used.

Gene	Primer Sequence (Forward)	Primer Sequence (Reverse)
ACTB	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
FOXA2	CGTCCGACTGGAGCAGCTACTAT	ATGTACGTGTTCATGCCGTTCA
GATA4	TGGTCAGATGGCAGCCAGAG	TGCTTCGAATTCGTGTTGCAG
GATA6	GCAATAATTCCATTCCCATGAC	AGCCCATCTTGACCCGAATA
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA
NANOG	TCCAACATCCTGAACCTCAGCTA	TGCGTCACACCATTGCTATTCTT
POU5F1	TGAAGCTGGAGAAGGAGAAGCTG	GCAGATGGTCGTTTGGCTGA
SOX17	CTGCAGGCCAGAAGCAGTGTTA	CCCAAACTGTTCAAGTGGCAGA
INS	GAAGCGGCATTGTGGAAC	CTGGTTCAAGGGCTTTATTCCATC
ISL1	ACAAAGTTACCAGCCACCTTGGA	TCATGCCTCAATAGGACTGGCTAC
PCSK1	CTCACCTCACCTGGATGATCACTAA	CCAGCATTGTAGGTGACTGGAGAC
NGN3	GCGAGTTGGCACTGAGCAAG	CCGAGTTGAGGTTGTGCATTC
PCSK2	CAATGCACATCCATTCCCAAG	AAGGTGCTCCAGGCTAAGCTC
SST	CCCAGACTCCGTCAGTTTC	GGACAGATCTTCAGGTTGGAG
NKX6.1	GAGGGCTCGTTTGGCCTATTC	ATCTCGGCAGCGTGCTTCT
SOX9	GGAGATGAAATCTGTTCTGGGAATG	TTGAAGGTTAACTGCTGGTGTTCTG
CHGA	TCCCTGTGAACAGCCCTATGAATAA	AAAGTGTGTCGGAGATGACCTCAA
MAFB	TTGTAACCAGAATCACCCTGAGGTC	CCAGGGTCAGGGATGGCTAA
GCK	ATGACCGGCACTGCTGAGA	AGCCCTTGGTCCAGTTGAGAA
GCG	CTTGCCGCCAGGGACTTTA	ACGTGGCTAGCAGGTGATGTTG
ABCC8	TCACACTTTGCCGAAACCGTAG	ATCTGTTGGCAGCTGTGAGGA
PDX1	ACTCCACCTTGGGACCTGTTTAGA	CGAGTAAGAATGGCTTTATGGCAGA
MAFA	TTGAGCCAGGTCTAACTTCTTTCCA	AAGGTGGGAACGGAGAACCAC

Immunocytochemistry

hiPSC-derived cell clusters were fixed with 4% paraformaldehyde (Nacalai Tesque) in phosphate-buffered saline (PBS), permeabilized with 1% Triton X-100, and blocked with 20% Blocking One (Nacalai Tesque 03953-66) / PBS-T (0.1% Tween-20 in PBS) for 1-2 h. After washing the cells in

PBS-T three times, cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Roche Diagnostics). Images were captured using an ImageXpress Micro scanning system (Molecular Devices, Japan). Quantitative analysis of positive cells versus total cells (DAPI-positive cells) was performed using the MetaXpress cellular image analysis software (Molecular Devices). The primary and secondary antibodies used are summarized in Table S2.

Table S2. Primary and seconary antibodies used for immunocytochemical analyses.

Primary Antibody	Source	Product Code	Dilution
Goat anti-SOX17	R&D Systems, Inc.	AF1924	1:100
Mouse anti-OCT3/4	Santa Cruz Biotechnology, Inc.	sc-5279	1:100
Goat anti-PDX1	R&D Systems, Inc.	AF2419	1:100
Goat anti-SOX9	EMD Milipore Cop., USA	AB5535	1:100
Rabbit anti-MAFA	Abcam	Ab26405	1:100
Mouse anti-NKX6.1	Developmental Studies Hybridoma Bank	F55A1-c	1:100
Guinea Pig anti-INSULIN	Dako Cytomation Japan	A0562	1:10
Secondary Antibody	**Source**	**Product Code**	**Dilution**
Alexa 568 donkey anti-goat IgG	Invitrogen	A11057	1:1000
Alexa 647 donkey anti-goat IgG	Jackson ImmunoResearch Inc.	124186	1:1000
Alexa 488 donkey anti-goat IgG	Jackson ImmunoResearch Inc.	124477	1:1000
Alexa 488 donkey anti-mouse IgG	Jackson ImmunoResearch Inc.	715-546-150	1:1000
Alexa 568 donkey anti-mouse IgG	BIOTIUM	CF568	1:1000
Alexa 488 donkey anti-guinea pig IgG	Jackson ImmunoResearch Inc.	115-605-164	1:1000
Alexa 647 donkey anti-mouse IgG	Jackson ImmunoResearch Inc.	706-546-148	1:1000

Glucose-stimulated insulin secretion (GSIS) assay

C-peptide release and C-peptide content assay were performed as described previously with minor modifications (Velazco-Cruz et al., 2019). Briefly, differentiated cells were loaded on Transwell (Corning #3415). Plates were allowed to fast for 60 minutes in 3 mM glucose HKRB (HEPES Krebs-Ringer Bicarbonate buffer) with 0.2% BSA, followed by another wash step and replacement of KRB with low, high glucose at 37°C on an orbital shaker (95 rpm). The collected culture supernatant was sampled and stored at -80°C until analysis. Cells were lysed, and RNA and DNA were purified using AllPrep DNA/RNA Micro Kit (Qiagen). C-peptide secretion was measured using

a human C-peptide ELISA Kit (Mercodia) and a human C-peptide AlphaLISA kit (PerkinElmer). The amount of C-peptide was normalized to total DNA contents in the corresponding cell lysate. DNA contents corresponding to 1000 cells were used for normalization. Fresh HKRB was prepared for each experiment.

Quantification and statistical analysis

Individual data are shown or expressed as the mean ± SD (standard deviation). Each experiment was conducted with biological replicates (N= 3) and repeated multiple times. Differences between groups were analyzed by Student' 's t-tests; or by one-way ANOVA. differences are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or $^{****}p < 0.0001$.

Western blot analysis

Total protein extraction was performed with M-PER™ Mammalian Protein Extraction Reagent (Thermo Scientific 78501) with 1% Phosphatase Inhibitor Cocktail (Ncalai tesque 07574-01) and Protease Inhibitor Cocktail for Use with Mammalian Cell and Tissue Extracts (Ncalai tesque 25955-11). Proteins were separated in 10% SDS-PAGE with Mini-PROTEAN Electrophoresis System (BioRad) and transferred to a PVDF (Millipore) membrane by Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). The Membranes were then blocked with 5% BSA (Ncalai tesque 01859-47) in TBS-T for 1 h and later incubated with primary antibodies with rabbit anti-β-Actin antibody horseradish peroxidase (HRP) conjugated (Cell Signaling Technology (CST) 12620) at 1:1000 dilutions, mouse anti- S6 Ribosomal Protein (CST 2317) at 1:1000 dilution, or mouse anti- Phospho-S6 Ribosomal Protein (CST 5364) at 1:1000 dilution overnight at 4°C. Membranes were washed with TBS-T and incubated with HRP-linked secondary antibody for 2 h at room temperature at 20,000 dilutions. Chemiluminescence band detection was performed by Immobilon Forte Western HRP Substrate

(Millipore). Fusion solo S (M&S instruments) was used for protein band visualization. Image J was used for the quantification of the protein bands.

Metabolite analysis

Undifferentiated hiPSCs were replated at 5 $\times 10^6$ cells/well into 6-well-plate and cultured for 24hr in AK02N and then cultured in MCDB (as a control) or HPLM supplemented with differentiation factors Stage 1 (3 days): S1 media + 100 ng/ml Activin A (GF-001-3000L, Api) + 3 μM Chir99021 for 1 day. S1 media + 100 ng/ml Activin A for 2 days. The media were collected every day. Metabolites measurements were performed using ultra-high-performance liquid chromatography equipped with tandem mass spectrometry, LCMS-8050 (HPLC-MS/MS; Shimadzu Corp.) utilizing LC/MS/MS Method Package for Cell Culture Profiling Ver. 2. Separation was achieved using a Discovery HS F5 HPLC column (567507-U, Supelco). Briefly, fresh medium or culture supernatants were deproteinized using 62.5% acetonitrile and diluted 100 times with distilled water (11307-76, Cica). Each sample was injected, and concentrations of the targeted metabolites in the samples were determined by relative quantification using the area ratio obtained from the integrated peak areas of targeted compounds divided by that of the internal standard. The excretion or intake of the metabolites by the cells is obtained by subtracting the value with the reference (initially contained in the fresh medium) and normalizing it to the area occupied by the cells and to 24 h (relative value / cm^2 / 24h). Plus values indicate excretion, and minus values indicate intake of the metabolites by the cells.

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Achievements

• **Publications**

1. Marwa Ali, Yusuke Kato, Nobuaki Shiraki, Shoen Kume. Generation of induced pluripotent stem cell-derived beta-cells in blood amino acids-like medium. Biology open (in press)

• **International Conference**

(Poster Presentation)

1. Marwa Ali *, Yusuke Kato, Nobuaki Shiraki, Shoen Kume

Title: Generation of induced pluripotent Stem Cell-Derived Beta cells in Blood Amino Acids-Like Medium
Conference Name: 22nd International congress of nutrition
Venue: Tokyo international forum, Tokyo, Japan
Date: 6th - 11th December 2022
Poster Number: PAB(T1)-98