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Studies on the effects of short-chain fatty acids on beta cell differentiation and maturation in neonatal porcine islet-like cell clusters

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Zusammenfassung (Deutsch):

Die Zellersatztherapie ist eine realistische Option zur Behandlung und Heilung des Typ 1 Diabetes. Hierfür könnten neonatale Schweineinselzellcluster (NPICC) eine unbegrenzte Quelle darstellen. Es bestehen jedoch einige ungelöste Herausforderungen wie zum Beispiel die hohe Immunogenität und die Notwendigkeit der Isolierung einer großen Zahl von qualitativ hochwertigen Inselzellcluster. Da NPICC aus Vorläuferzellen und unreifen insulin-produzierenden Betazellen zusammengesetzt sind, sind neue Strategien für die *in vitro* Generierung von reifen Betazellen und/oder für die Steigerung der Betazellzahl notwendig, um in naher Zukunft einen Transfer der Xenotransplantation in die Klinik zu erreichen.

In der vorliegenden Studie wurden NPICC von 2-3 Tage alten Ferkeln isoliert, um die Effekte drei kurzkettigen Fettsäuren SCFA (Acetat, Propionat und Butyrat) auf die *in vitro* Inselzell-Differenzierung und Betazellfunktion mittels real-time PCR, FACS-Analyse, Immunhistologie und Glukose-stimulierter Insulinsekretion (GSIS) zu bestimmen. Butyrat induzierte eine signifikante zeit- und dosisabhängige Hochregulierung der Insulingenexpression und eine Erhöhung der Betazellzahl. Acetat und Propionat zeigten eine nur marginale Wirkung auf die Induktion eines Betazellphänotyps. Durch die Behandlung mit spezifischen Inhibitoren der SCFA-Rezeptoren, der G-Protein-gekoppelten Rezeptoren GRP41 (β-Hydroxybutyrat) und GPR43 (GPLG0974), wurde die durch Butyrat induzierte Steigerung der Insulinexpression nicht supprimiert. Die Inkubation von NPICCs mit den Klasse-I-Histon-Deacetylase (HDAC)-Inhibitoren (Mocetinostat, MS275, FK228 und RGFP996) aber nicht mit den selektiven Klasse-II-HDAC-Inhibitoren (TMP269, MC1568) hatte einen Butyrat-ähnlichen Effekt auf die Betazelldifferenzierung. Diese Daten lassen annehmen, dass der proendokrine Effekt von Butyrat hauptsächlich durch die HDAC-Hemmung vermittelt wird.

Aus den Resultaten der vorliegenden Arbeit wird gefolgert, dass Butyrat und Klasse-I-HDAC-Inhibitoren Hemmer wichtige neue Wirkstoffe darstellen, um die Mechanismen der Differenzierung von porzinen Inselzellen zu erforschen und optimierte NPICC-Präparate für neue Zellersatztherapien herzustellen.

Summary (English):

Cell replacement therapy is a realistic option for the treatment and cure of type 1 diabetes. Neonatal porcine islets cell clusters (NPICCs) are considered to represent an unlimited cell source but faces some challenges including the strong immunogenicity and the need for generation of high numbers of excellent quality NPICCs. Since NPICCs are composed of progenitor cells and immature insulin-secreting beta cells, novel strategies to improve *in vitro* generation of matured beta cells and/or to increase islet numbers is a prerequisite for the near future transfer of xenotransplantation to the clinic.

In the present study, NPICCs were isolated from 2-3 days-old piglets to evaluate the effects of three main short-chain fatty acids (SCFA), acetate, propionate and butyrate, on *in vitro* islet differentiation and beta cell function assessed by real-time quantitative PCR, FACS analysis, immunohistology and glucose-stimulated insulin secretion (GSIS). Butyrate promoted a significant time- and dose-dependent up-regulation in insulin gene expression and an increased beta cell number, whereas acetate or propionate only marginally influenced the beta cell phenotype. The treatment with specific inhibitors of SCFA receptors such as G-protein-coupled receptor GPR41 (β-hydroxybutyrate) and GPR43 (GPLG0974) did not suppress butyrate-mediated increase of insulin expression. However, the application of specific class I histone deacetylase (HDAC) inhibitors mocetinostat, MS275, FK228 and RGFP996 mimicked butyrate on beta cell differentiation, whereas selective class II HDAC inhibitors (TMP269, MC1568) displayed no effects. These data suggest that the pro-endocrine impact of butyrate is mainly mediated through its HDAC inhibitory activity.

The data of the present study suggest that butyrate and class I HDAC inhibitors are important agents to study the mechanisms of beta cell differentiation in porcine islets and to produce optimized NPICC cell products for novel cell replacement therapies.

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List of abbreviations

ADA	American Diabetes Association
ALDOB	aldolase fructose-bisphosphate B
A/A	antibiotic/antimycotic
BSA	Bovine Serum Albumin
cAMP	cyclic adenosine monophosphate
CFRD	cystic fibrosis-related diabetes mellitus
CMS	Chicago Medical School
DAG	diacylglycerol
DE	Deutsches Edelschwein
ECM	extracellular matrix
Edu	5-Ethynyl-2´deoxyuridine
EPAC	exchange factor directly activated by cAMP
ER	endoplasmic reticulum
FCS	fetal calf serum
FDA	fluorescein diacetate
FFAR2	free fatty acid receptor 2
FFAR3	free fatty acid receptor 3
FICC	fetal islet-like clusters
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCG	glucagon
GL	German Landrace
GLP-1	glucagon-like peptide-1
GPCRs	G protein-coupled receptors
GPR41	G protein-coupled receptor 41

GPR43	G protein-coupled receptor 43
GSIS	glucose stimulated insulin secretion
HAR	hyperacute rejection
HAS	human albumin serum
HDAC	histone deacetylases
HDACi	HDAC inhibitor
IBMIR	instant blood-mediated inflammatory reaction
ICC	islet-like cell cluster
IEQ	Islet equivalent
IL-6	Interleukin 6
KO	knockout
KRBH	krebs-ringer bicarbonate HEPES
MafA	MAF bZIP transcription factor A
MCT1	monocarboxylate transporter 1
MFI	median fluorescence intensity
NDC	non-digestible carbohydrates
NGN3	neurogenin 3
Nkx6.1	NK6 homeobox 1
NPICCs	neonatal porcine islet cell like clusters
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
PDX1	pancreatic and duodenal homeobox 1
PERVs	porcine endogenous retroviruses
PI	propidium iodide
PKA	protein kinase C
PKC	protein kinase C

PLC	phospholipase C
PYY	peptides tyrosine
RT-PCR	reverse transcription polymerase chain reaction
SCFAs	short-chain fatty acids
SMCT1	sodium-coupled monocarboxylate transporter 1
T1DM	type 1 diabetes mellitus
T2D	type 2 diabetes
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	tumor necrosis factor alpha
TSA	trichostatin A
WT	wild-type
WZS	Wuzhishan

1. Introduction

1.1 Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia. The disorders of glucose metabolism are caused by relative or absolute insulin deficiency, or by a defect in insulin release together with insulin resistance. Diabetes mellitus is one of the most widespread diseases in the developed world with a 2021 prevalence estimate of 10.5% worldwide and 10% (20-79 years) in Germany. The number of people with diabetes is projected to rise to 783 million by 2045, with a prevalence of 12.2% [1].

Diabetes is one of the top 10 causes of death worldwide with an estimated 6.7 million deaths directly caused by diabetes in 2021 [1]. Poor metabolic control results in many serious complications including acute complications of hypoglycemia and diabetic ketoacidosis, or long-term cardiovascular complications (myocardial infarction, stroke), kidney damage, blindness due to retinopathy and polyneuropathy. Studies have shown that adults with diabetes have a two- to threefold increased risk of heart attack and stroke [2]. Diabetes is one of the leading causes of kidney failure[3], while diabetic retinopathy is a major cause of blindness [4]. In addition, reduced blood flow and nerve damage to the foot due to chronic hyperglycemia increases the risk of foot ulcers, infection and eventual amputation [5]. According to a recent report by the IDF, diabetes causes at least $966 billion in health spending in 2021, which represents 9% of total spending on adults. Overall, the global burden of diabetes is increasing significantly, posing a serious challenge not only to public health but also to social and economic development [1].

According to the standards of the American Diabetes Association (ADA), diabetes can be classified into four categories: type 1 diabetes, type 2 diabetes, gestational diabetes and specific types of diabetes caused by other causes. Type 1 diabetes and type 2 diabetes are the most common types, while specific types of diabetes include monogenic diabetic syndrome, diabetes caused by exocrine diseases of the pancreas such as cystic fibrosis-related diabetes mellitus (CFRD) and post-

pancreatectomy diabetes mellitus, and diabetes caused by drugs or chemicals (such as the use of glucocorticoids, treatment of HIV/AIDS, or after organ transplantation) [6].

1.1.1 Type 1 Diabetes mellitus

Type 1 diabetes mellitus (T1DM) is caused by destruction of the insulin-producing beta cells of the pancreatic Langerhans islets. Although the molecular mechanisms underlying T1D pathogenesis are not completely understood, it is thought that susceptibility genes in combination with environmental factors, such as viral infections and some dietary factors, initiate and trigger a beta cell specific autoimmune process [7]. When more than 90% of the beta cells are destroyed, patients develop clinical symptoms such as extreme thirst, excessive drinking, polyuria, weakness, and unintended weight loss. The drastic loss of beta cells results in severe insulinopenia and proneness to diabetic ketoacidosis [8].

According to the report from IDF Diabetes Atlas 2021 [1], there are 1,211,900 children and adolescents living with type 1 diabetes worldwide. Europe has the highest number of children and adolescents (0–19 years) with type 1 diabetes, approximately 295,000 in total. About 31,000 children and adolescents suffered from type 1 diabetes in Germany. Germany ranks eighth in the world in terms of type 1 diabetes incidence, with a rate of 35.1 per 100,000 among children and adolescents aged under 20 years. Based on a report by the Robert Koch Institute (RKI), the incidence of type 1 diabetes per 100,000 person-years increased 1% annually [9]. Although type 1 diabetes occurs predominantly in children and adolescents, there have been few surveys on standardized estimates of the prevalence and incidence of new-onset type 1 diabetes in adults as well as overall estimates of all age groups. In Germany the national incidence of T1DM in adults was 6.1 per 100,000 person-years from 2014 to 2016 [10]. Overall, the increasing prevalence and incidence of diabetes in Germany and worldwide is a serious threat to public health and health care expenditure.

Patients with type 1 diabetes suffer from absolute insulin dependency and require life-long daily insulin injections in combination with diet and exercise to maintain

appropriate blood glucose levels [11]. The currently available therapeutic options for T1DM to keep blood glucose levels as close to normal as possible include the following aspects: the first is intensive exogenous insulin replacement therapy with multiple daily insulin injections using long- and rapid-acting insulins, which is the standard treatment for type 1 diabetes. The second is continuous subcutaneous insulin infusion using an insulin pump with and without continuous glucose monitoring (CGM) by a subcutaneous glucose sensor. The third and most advanced are the so called semi-closed loop or hybrid-closed-loop artificial pancreas systems with automated insulin delivery using control algorithms between insulin pump and CGM systems to automatically vary insulin delivery within prespecified glucose thresholds and insulin dose limits [12, 13].

The remarkable advances in the developments of different insulin analogue and diabetes technology considerably improved diabetes management by increasing the time in range on glucose targets and preventing frequencies of severe hypoglycemia. However, artificial pancreas is costly and has some technical and psychological issues for many patients. Closed-loop systems depend on subcutaneous blood glucose measurements and s.c. insulin delivery which lead to problems with system failures, patient-associated issues such as human errors in system calibration, wrong estimation of carbohydrate intake or physical activity and the lag between blood and tissue glucose [12, 14]. At present, current developments of automated insulin delivery systems are available for only a subgroup of patients and cannot provide a full-value substitute of a pancreas organ. Due to the pharmacological properties of insulin itself, exogenous insulin delivery predisposes T1D patients to severe hypoglycemia and psychosocial stress. Many individuals with T1DM still have blood glucose levels that are above normal values which reduces quality of life and puts them at high risk for long-term complications [15].

The development of a technology that can provide physiologic blood glucose control without the requirement of frequent patient intervention would thus substantially improve the lives of subjects with T1DM. Beta cell replacement therapy can address these deficiencies in diabetes care because it has the potential to restore

physiological blood glucose regulation [16]. This includes pancreas organ transplantation, human islets transplantation, xenotransplantation and stem cell–based therapies [17].

1.2 Pancreas and human islet transplantation

Pancreas and human islet transplantation is an effective treatment for patients with type 1 diabetes, especially for those who frequently experience severe hypoglycemia. After pancreas transplantation, 5-year insulin independence rates varied among the 3 major graft types, 73% for simultaneous pancreas–kidney (SPK) transplants, 64% for pancreas-after-kidney (PAK) transplantation and 53% for pancreas transplant alone (PTA) [18]. However, pancreatic transplantation has a high risk of surgical complications, most of which are related to exocrine tissue. Diabetic patients only need pancreatic islet cells with endocrine function.

With the introduction of the Edmonton Protocol in 2000 and the revolutionary progress of islet isolation technology in the past decade, a breakthrough has been made in international islet transplantation. Islet transplantation has significant advantages over whole gland transplantation, and islet transplantation is a less risky procedure in comparison. Transplantation of human islets has a lower success rate as compared to pancreas transplantation with an insulin independency rate of 80% after 1 year and 60% after 5 years [19, 20]. However, clinical trials have shown that human islet cell transplants can significantly reduce frequency of severe hypoglycemia in patients with instable glucose metabolism and/or hypoglycemia unawareness.

The major limitation of both procedures is the shortage of organ donors with the consequence that worldwide few thousand patients with type 1 diabetes has been transplanted during the last decades. To offer this potentially life-saving therapy to the majority of diabetic patients an abundant source of beta cells is urgently needed.

1.3 History of xenotransplantation

Xenotransplantation is defined as any procedure involving the transplantation, implantation, or injection of living cells, tissues, or organs of non-human animal origin into a human recipient. It also includes any procedure in which human body fluids, cellular tissues or organs have ex vivo contact with living animal cells, tissues or organs in vitro [21]. The first xenotransplantation has been performed in humans in the early 1970s, when Professor Reemtsma, a surgeon at Tulane University in Louisiana transplanted a baboon kidney into a human patient. He performed a total of 13 chimpanzee-to-human kidney transplantations in 1963-1964. However, due to various problems such as immune rejection, none of the patients survived for more than 9 months [22]. Thomas E. Starzl performed the first chimp-to-human liver transplant in 1966 [23], and a baboon liver transplant in 1992 resulted in patient survival for 70 days [24]. Since then, xenotransplantation has become more common, with more than 100 procedures performed worldwide. The majority of these have involved kidneys or livers. However, other organ types are also investigated for use as transplantable tissue substitutes, such as heart valves, skin and islets.

In 1894, Williams and his surgeon colleague William Harsant subcutaneously implanted three freshly slaughtered and aseptically prepared sheep pancreas into the chest and abdomen of a 15-year-old severely emaciated diabetic boy [25]. However, the patient died in a diabetic coma three days later. Then James Allen and Dr. Barlow removed the pancreas from a cat and transplanted it subcutaneously to a patient in 1903. As a result, the patient died of diabetic coma two weeks later [26]. Pancreas xenotransplantation was also performed in the laboratory of C. Frugoni in 1926, when they transplanted baboon pancreases into the subvaginal membranes of two adolescent diabetic patients. After one year, no clear conclusions could be drawn, except that they needed less insulin than before [25]. After this, many researchers have performed several experimental xenotransplantations, but the results are mostly uncertain [27, 28]. The first clinical pig islet transplant was carried out by Groth in 1993. Although no improvement effect was obtained, it proved for the first time that porcine pancreatic islets could survive in

the human body under immunosuppressive conditions and produce C-peptide [29]. Since then, an increasing number of studies has been conducted in this field.

1.3.1 Pigs as promising source for islet xenotransplantation

Researchers have attempted to use different species as the source for islet transplantation. Lacy PE et al. studied islet xenograft survival and metabolism by transplanting islets from rat, hamster, and rabbit into diabetic C57BL/B6 mice [30]. In1992, some researchers also transplanted tilapia pancreatic islets into diabetic nude mice and observed function of the grafts for 50 days [31]. Piero Marchetti's research team made modifications based on the isolation technology of porcine islets and successfully transplanted isolated bovine islets under the kidney capsule of nude mice with streptozotocin-induced diabetes. The results showed that bovine islet transplantation reversed hyperglycemia in diabetic mice [32]. Early clinical organ xenotransplantation attempted to use non-human primates as organ sources because of their high similarity to humans. Non-human primates commonly used in experiments include chimpanzees, baboons and rhesus monkeys. Among them, the chimpanzee is considered to be theoretically the most suitable animal donor, but it is now listed as an endangered species and cannot be used for ethical issues. Although baboons have an advantage in numbers, they are difficult to breed under sterile conditions, and they have a long gestation period and very few offspring. There are other serious issues, such as the fact that non-primates are more likely to carry viruses that can infect humans than other animals. Therefore, researchers search for other animal sources. Pigs were subsequently found to be the most promising source as organ donor for islet xenotransplantation.

Pigs as donor animals for islet transplantation have many advantages over other animals [33]. First, the organs of pigs are similar in structure and physiology to humans and the glucose-responsiveness of porcine islets is similar to that of humans. Additionally, tissue of porcine origin, including heart valves, blood vessels, and skin, has been routinely and safely used for medical treatment for decades. Second, pigs are easier to breed and rear, with a high number of offspring in one

litter. As a common domestic animal, pigs have lived with humans for a long time. Its diseases and sources of infection are well known, which helps to reduce the risk of zoonotic diseases. Porcine insulin is 98% identical to human insulin, and it was used for decades to treat patients before the advent of recombinant human insulin [34].

1.3.2 Current status of islet xenotransplantation

The major barrier for clinical application of pig islet transplantation is their strong immunogenicity and the induction of an acute and chronic immune response of the innate and adaptive immune system.

A study published in 2005 showed that 9.5 years after a T1DM patient received alginate-microencapsulated porcine islets, some insulin positive stained cells were still present in the xenograft. The remaining porcine islets still produced tiny levels of insulin when placed at high glucose concentrations *in vitro* [35]. A long-term follow-up study by Valdes-Gonzalez R showed that these patients achieved good glycemic control after receiving neonatal porcine islet transplantation, but the residual insulin production of the transplanted porcine islets remains unclear [36]. These data suggest that encapsulated porcine islets have the potential for long-term survival in human patients. Macroencapsulated adult islets (Beta0_2 device) were successfully tested in diabetic rhesus monkeys and in one first-in-human clinical trial. Although additional insulin treatment was still required after transplantation, the insulin dose could be lowered, and porcine C-peptide secretion was consistently detected [37, 38]. Some experiments in non-human primates (NHP) provide evidence that insulin-dependent diabetes can be reversed after transplantation of non-encapsulated adult as well as neonatal porcine pancreatic islets using a strong systemic immunosuppressive protocol [39-41].

More recently, it has been shown that porcine islet grafts can normalize blood glucose levels after transplantation in diabetic rhesus monkeys for more than 1 year using an immunosuppression induction therapy with Antithymocyte globulin, cobra-venom factor and anti-TNFα antibody and a maintenance therapy including CD40/CD40L co-stimulation blockade (anti-CD154 antibody) and sirolimus [42-

44]. One animal was insulin independent for more than 900 days. However, large islet doses and intense immunosuppressive regimes were necessary to achieve insulin independency.

Recent advances in efficient genetic engineering made it possible to generate transgenic and knock-out pigs with less immunogenic tissues and organs. Hyperacute rejection is strongly reduced by elimination of carbohydrate xenoantigens such as Galα1-3Galβ, N-Glycolylneuraminic acid, and Sd (a) for which humans have natural antibodies [45-48]. Cellular rejection against porcine islets was reduced by overexpression of transgenes inhibiting co-stimulation (cytotoxic T-lymphocyte antigen 4-immunoglobulin (CTLA4-Ig), the CTLA4 analogue LEA29Y), by activation of co-inhibitiory signals (humanized PD-L1), and transgenes mediating decreased posttransplant inflammation such as human hemeoxygenase-1 (HO-1) and soluble human tumor necrosis factor-alpha receptor type I-IgG1-Fc (TNFR1-Fc) [49-55]. These data suggest that islets from genetically multi-modified pigs may reduce xenorejection to a level that can be controlled by immunosuppressive drugs that are currently used in the clinic. Clinical studies are planned to investigate this hypothesis.

Another major obstacle to bring islet xenotransplantation to the clinic is the transmission of porcine pathogens to humans, particularly porcine endogenous retroviruses (PERVs). PERV-A and PERV-B are integrated in the genome of all pigs, but PERV-C is present in only some pigs. PERV-A and-B and PERV-A/C recombinants are capable of infecting human cells in vitro. However, there is no report on the transmission of PERVs to human individuals working closely with pigs or to patients in clinical trials transplanting pig tissues or cells [56]. Therefore, the risk of a PERV infection by transplantation of porcine tissues/cells is very low und very unlikely but cannot be completely excluded [57].

To minimize the risk, selection of PERV-C-free animals and pigs with low PERV-A and PERV-B expression are recommended. In August 2017, researchers in the labs of George Church and Yang at Harvard University inactivated 62 PERV genes in pig cells using the CRISPR-cas9 system, a cutting-edge gene editing

technique. They cloned embryos from these fetal cells and generated healthy PERV-inactivated pigs by somatic cell nuclear transfer [58].

The first clinical trial on safety and efficacy of free porcine islet xenotransplantation has started in 2013 (NCT03162237) in China. Ten patients have been successfully transplanted without pathogen transmission [59]. Another clinical trial on pig islet transplantation is planned in South-Korea 2022/2023.

1.3.3 Selection of the optimal donor pig

1.3.3.1 Selection of pig strain

The quality and quantity of islet yield after isolation is related to the breed, age, gender and size of the donor pig. Several studies have demonstrated that the islet capsule, size and yield of different porcine donors are quite different. The selection of pig breeds for xenotransplantation is mainly based on the following aspects: peri-insular matrix, islet morphology and islet yield. The expression of extracellular matrix (ECM) proteins in islet capsules largely influences the quality and yield of the islets [60]. ECM plays an important role in providing mechanical and physiological support, acting as a protective shield [61]. The isolation of islets from pig strains with high expression of ECM proteins results in improved islet yield and quality. In comparison to other breeds studied, such as Hampshire, Duroc, German Landrace (GL), Deutsches Edelschwein (DE), and hybrid pigs, GL pigs had the highest overall expression of ECM protein. In addition, aged pigs expressed more ECM proteins than younger pigs [62]. According to several studies, pancreases containing round, well-demarcated and large-sized (>100 µm diameter) islets can provide the greatest potential for achieving significant isolation outcome [63]. Pancreases from adult Chicago Medical School (CMS) miniature pigs contain larger islets and higher islets yields than market pigs or other miniature pigs [64]. Jiang et al. found that the anatomical structure of the pancreas of the Chinese Wuzhishan (WZS) miniature pigs is more similar to that of human pancreas, and the yield of islets obtained from this strain is much higher than that

of some market pigs, which provides another viable source of islets for xenotransplantation [65]. When compared to well-researched pig breeds, the market-raised breeds have shown lower yields of islet [66]. At present, there is still no real consensus on the optimal pig strain that reproducibly provides a high number of viable and functional pancreatic islets required for preclinical/clinical xenotransplantation.

1.3.3.2 The optimal age of the donor pig

Age of the donor pigs is one of the major factors affecting islet isolation output. Porcine islets can be acquired at four different life stages: embryonic, fetal, neonatal and adult. Islets from each stage have their own advantages and disadvantages for islet xenotransplantation. Embryonic pancreatic tissues mainly show insulin-positive beta cells, and exocrine tissues will not proliferate after transplantation. Therefore, the immune response and inflammatory complications caused by xenotransplantation are less prominent [67]. The disadvantage of embryonic pancreas is that it takes a long time to differentiate and mature, and the yield is particularly poor, indicating that a large number of pigs would be required for the transplantation of one patient.

Fetal and neonatal porcine pancreatic islets are easy to separate without purification process. Immaturity may be the main disadvantage of islet-like cell clusters (ICCs) as a xenograft. It takes 8-12 weeks for ICCs to mature before they can take over normal islet function *in vivo* [68]. In addition, the yield of porcine fetal islet-like clusters (FICC) per pancreas is low. Freshly isolated neonatal porcine islet-like cluster (NPICCs) are mainly composed of pancreatic endocrine cells (~35%) and epithelial cells (~57%) which are also considered to be endocrine precursor cells [69]. Neonatal pancreatic islets are easy to separate, and the costs of the isolation process is low. They show higher resistance to ischemic and inflammatory damage during the separation process, which makes the recovery of the cells more effective [70]. Additionally, they are supposed to be less immunogenic than adult porcine islets. Although NPICCs need a certain amount of time to secrete insulin after transplantation, their maturation time (>4 weeks) is significantly shorter than that of fetal pancreatic islet cell clusters. Furthermore,

maintenance of neonatal pigs is both easier and cheaper as compared to adult pigs, as they are only maintained for few days after birth. The long-term reversal of diabetes after transplantation of wild-type (wt) NPICCs into immunosuppressed non-human primates has been demonstrated [35, 39, 71, 72], emphasizing the use of neonatal pigs as an alternate source of islet grafts for medical properties in the future.

The major advantages of using adult pigs as donor animals is that the islet yield is substantial, and that the isolated islets are mature and functional, capable of secreting insulin in vitro and in vivo [73, 74]. On the other hand, the cost raising pigs until late adulthood is very high, and its islets are fragile, difficult to separate and more susceptible to ischemic and hypoxic damage. The following table summarizes the comparison of islets isolated from pigs of different ages.

Table 1. Comparison of islets isolated from pigs of different ages*

Characteristics	Embryonic	Fetal	Neonatal	Adult
Islet size (µm)	<50	80	50 ~ 150 (90%)	100 ~ 200 (>30%)
Composition (beta cells % of islet cells)	Undeveloped cells	Immature precursor cells. 10% beta cells after culture	25% beta cells after culture	>70% beta cells after culture
Isolation	No necessary	Simple	Simple	Difficult
Gradient purification	No	No	No	Yes
Proliferation in vivo	Yes	Yes	Yes	Little
In vivo functioning	Delayed 4 to 6 months	Delayed >2 months	Delayed >1 month	Within hours
Gal expression	High	High	High	Low
Islet yield/pancreas (IEQs)	Not applicable	~8,000	25,000–50,000	200,000–500,000
Tumorigenicity	Possible	Low	Low	None
Risk of pathogen transmission	Low	Low	Low	Low
Cost	Low	Low	Low	High

*Table modified from Nagaraju et al [75], Liu et al [62], and Maheswaran et al[33].

1.3.4 Optimization of neonatal porcine islet-like clusters culture system

High quality and high numbers of porcine islets are required for transplantation experiments. The 4-7 days duration of culture to separate pancreatic exocrine cells from endocrine/ductal neonatal islet-like clusters (NPICC), the loss of NPICC during the cultivation and the limited maturation (glucose-responsive insulin secretion) of the resulting NPICCs are major obstacles that hinder their use for xenotransplantation. The endocrine part of the pancreas of neonatal pigs is composed of immature beta cells and progenitor cells. NPICCs still needs the extended period of *in vitro* culture to obtain more mature and functional pancreatic islets clusters for transplantation [76]. Studies have shown that more mature and functional islets are produced in the process of *in vitro* culture and that it takes less time to reverse hyperglycemia after transplantation of cultured NPICCs than fresh islets or islets cultured *in vitro* for only a few days. However, at the same time, a significant amount of islet cell mass is lost during *in vitro* culture [76-78]. An Australian research team isolated neonatal islet-like clusters from 1- to 3-day-old piglets and cultured them for up to 27 days after isolation. They observed that clusters cultured *in vitro* for 12, 19 and 27 days restored diabetic mice to normal blood glucose levels by days 46, 32 and 35, respectively. In contrast, standard 6-days culture took an average of 63 days to restore normoglycemia in 35% of mice treated [79].

Over the last years, some research teams have made significant efforts to improve the culture conditions of NPICCs, including recovering from islet isolation techniques and accelerating *in vitro* maturation, or adding additional supplements during the culture process. Exendin-4 is a peptide agonist of glucagon-like peptide (GLP) receptor. Exendin-4 added to the culture media of NPIs can boost endocrine cell differentiation, improve glucose-stimulated insulin secretion, and enhance the ability to restore hyperglycemia after transplantation into diabetic mice [80]. Some labs added high concentrations of glucose (> 10 mM) to the culture medium to simulate a diabetic environment [53, 80, 81], and some research groups used human serum [82] or porcine serum [76, 79] or added bovine or human serum albumin (HSA) under serum-free conditions to cultivate NPICCs [83, 84]. Additional supplements, such as nicotinamide and L-glutamine, have

been demonstrated to be beneficial to the survival of NPICCs [85]. So far, there has been no agreement on the optimal medium composition for NPICCs cultivation.

1.4 Short-Chain Fatty Acids (SCFAs)

1.4.1 The production of SCFAs

SCFAs are a category of saturated fatty acids with fewer than six carbon atoms, which are the main metabolites generated by bacterial anaerobic fermentation of non-digestible carbohydrates (NDC) such as dietary fiber, resistant starch, and oligosaccharides in the colon [86]. Furthermore, roughly 1% of the gut flora utilizes amino acids to create SCFAs. This method of synthesis usually occurs in the distal region of the large intestine, where carbohydrates are almost exhausted [87]. The most abundant SCFAs are acetate (C2), propionate (C3), and butyrate (C4), accounting for 90-95% of SCFA in the colon [88]. SCFAs are rapidly absorbed by colonic cells mainly through active transport mediated by transmembrane H^+ gradient-coupled monocarboxylate transporter 1 (MCT1) and sodium-coupled monocarboxylate transporter 1 (SMCT1). A part of the undissociated SCFAs is also absorbed by the colon through passive diffusion [89]. The SCFAs, especially butyrate, are reabsorbed to supply energy to the colonic cells. The SCFAs that are not metabolized by colon cells enter the portal circulation of the liver through the basolateral membrane to provide energy substrate for hepatocytes [90].

1.4.2 SCFAs and energy metabolism

The three SCFAs acetate (C2), propionate (C3), and butyrate (C4) have been shown to play an important regulatory role in human health and disease processes through their effects on regulating cell proliferation and differentiation, apoptosis, gut microbiome composition, inflammatory responses, metabolism of glucose and lipids, insulin resistance, nutrient uptake and other mechanisms [91-

94]. Multiple mechanisms are involved in the effects of SCFA on insulin resistance, insulin secretion, and glucolipid metabolism. A recent study showed that SCFAs reduce visceral and hepatic fat by increasing energy expenditure and fat oxidation [95, 96]. Another clinical study has proven that dietary supplementation with SCFAs improved beta cell function and stimulated insulin secretion [97]. Additionally, short-chain fatty acids, especially propionate and butyrate, can significantly reduce the expression of inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) in liver and adipocytes, thereby reducing hepatic steatosis and inflammation [98]. Glucagon-like peptide-1 (GLP1) decreases plasma glucose levels by directly regulating insulin and glucagon secretion, as well as improving insulin resistance in peripheral target organs through GLP1 receptors. The ability of SCFAs to increase GLP-1 secretion has been demonstrated in both animal models and human beings [99-101]. A study investigating at the role of the human gut microbiome in obesity showed that bacteria that produce butyrate are reduced in the guts of obese people [102]. Another study on metagenomic analysis of samples from Chinese type 2 diabetic patients found that patients with diabetes showed dysbiosis of gut flora, especially the significant reduction of butyrate-producing bacteria (F prausnitzii, Roseburia, etc.) [103]. Similar findings were made in patients with T1D. Murri *et al.* found that children with T1D have significantly different fecal microbial compositions than healthy children and that blood glucose levels in diabetic children were linked to alterations in the phylum bacteroides thick-walled phylum ratio. Furthermore, diabetic children have considerably less lactate-and butyrate-producing, and mucin-degrading bacteria than healthy children, all of which are necessary for preserving intestinal integrity [104]. Researchers have also observed that supplementation of butyrate in the diet of obese mouse model reduced high-fat diet-induced obesity and insulin resistance [105]. In the same way, dietary supplementation with propionate or butyrate in obese and diabetic mice, respectively, induced intestinal gluconeogenesis [106]. In summary, the existing studies found that SCFAs, especially butyrate play an important role in the regulation of energy metabolism. **Figure 1** summarizes the effects of short-chain fatty acids on host metabolism [107].

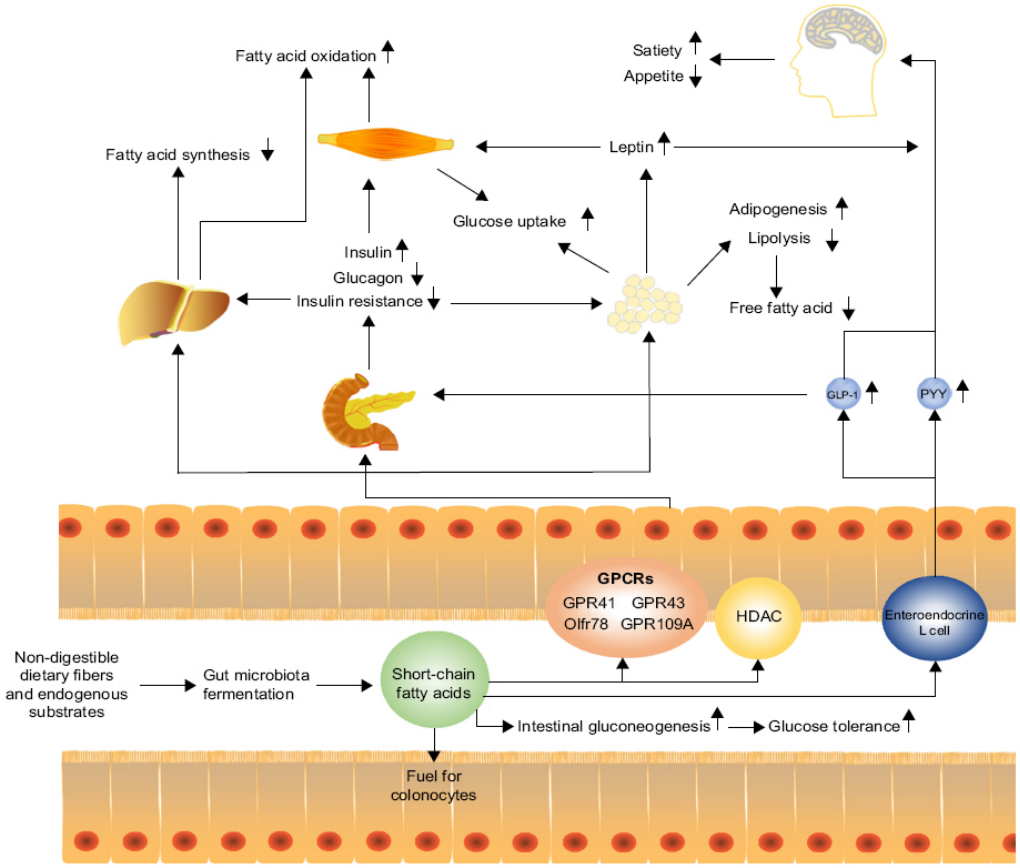


Figure 1. The effects of short-chain fatty acids on host metabolism [107]. SCFAs generated by intestinal flora digesting indigestible dietary fiber serve mostly as fuel for colonic cells, but they also increase intestinal glucose metabolism, thereby enhancing glucose tolerance. In addition, SCFAs can stimulate the release of anorexigenic hormones PYY and GLP-1 from enteroendocrine L cells; GLP1 operates on the brain to promote satiety and suppress appetite; as well as on the pancreas to increase insulin secretion, decrease glucagon secretion, and improve insulin resistance. SCFAs also affect the liver and adipose to reduce fatty acid synthesis and promote fatty acid oxidation in the liver. They also promote adipogenesis and limit lipolysis in adipose tissue, resulting in a reduction in free fatty acids.

1.4.3 SCFAs and G-protein coupled receptors

It is widely established that SCFAs' capacity to modulate physiological processes depends on two major pathways. The first mechanism of SCFA action is through binding to G protein-coupled receptors (GPCRs) to activate signaling cascades. The second is to inhibit the activity of histone deacetylases (HDACs) which are involved in the regulation of gene expression.

GPCRs are a large family of membrane receptor proteins found on the cell surface that transduce extracellular signals into intracellular responses. GPCRs are widely found in eukaryotic organisms and play a key role in regulating cellular processes such as cell growth, differentiation, motility, secretion, and gene transcription. The major GPCRs activated by SCFAs are G protein-coupled receptor 41 [GPR41 or free fatty acid receptor 3 (FFA3)], GPR43 (also named FFA2) and GPR109A (also known as hydroxycarboxylic acid receptor 2 or HCAR2) [108]. A study of differences in the potency and selectivity of SCFAs to activate their receptor ligands showed, the ability of SCFAs to activate GPR43 is ranked as acetate=propionate>butyrate, whereas for GPR43 is propionate=butyrate>acetate [109]. The importance of short-chain fatty acid receptors in metabolism has been proven in many studies. Upregulation of GPR43/FFA2 expression in colon cells has been demonstrated to enhance the levels of the anorectic hormones peptides tyrosine (PYY) and GLP-1 in mice [110]. SCFAs also inhibits lipid accumulation in insulin-stimulated adipocytes via GPR43 signaling [111]. It has also been reported that SCFA-induced decrease in GLP-1 secretion and impaired glucose tolerance in the GPR43 and GPR41-deficient mice [98]. Eliana et al.'s study on the pathogenesis of type 1 diabetes showed that GPR43 protects against damage to islet beta cells by inflammatory factors [112].

1.4.4 SCFAs-mediated HDACs inhibition

Histones promote or inhibit the interconversion of chromatin structures through acetylation or deacetylation. In general, acetylation of histones facilitates the dissociation of DNA from histone octamers and the relaxation of nucleosome structure, thus allowing various transcription factors and co-transcription factors to bind specifically to DNA binding sites and activate gene transcription. In contrast, histone deacetylase (HDAC) catalyzes its inverse reaction histone deacetylation, causing dense chromatin coiling and inhibition of gene transcription [113]. Histone acetylation not only promotes gene transcription but also affects DNA replication and repair. As HDACs deacetylate histones, inhibiting their activity or expression can boost gene transcription by increasing histone acetylation [114]. The HDAC family includes four subfamilies: class I (HDAC1, 2, 3, 8), class II

(HDAC 4, 5, 6, 7, 9, 10), class III (SIRT1-7) and class IV (HDAC11) [115]. HDACs exhibit diverse functional activities, including the regulation of multiple metabolic pathways. It has been observed that class I and II HDACs modulate the differentiation of pancreatic islet cells and adipocytes. For example, inhibition of HDAC3 enhances insulin signaling and glucose uptake in white adipocytes of mice on high-fat diet. Moreover, knockdown of HDAC5 or HDAC9 genes increased pancreatic beta cell mass and insulin secretion [116]. SCFAs are well known for their ability to suppress the HDAC activity. After translocation into cells via SMCT1, SCFAs could directly occupy the active site of HDACs and lead to inhibition [114]. whereby this effect is not mediated by G protein-coupled receptors. Butyrate as an endogenous HDAC inhibitor (HDACi) was shown to be the most effective as compared to other SCFAs [117]. Mounting evidence suggests that butyrate-mediated inhibition of HDAC, impedes cell proliferation and induces cell differentiation or death in cancer. In addition to its potency as anticancer drug, butyrate also has anti-inflammatory properties mediated partly by the inhibition of HDAC [90]. In the insulin-resistant L6 myocytes, butyrate promoted the expression of Insulin receptor substrate-1 (IRS-1) by enhancement of histone acetylation at the promoter region, suggesting a direct mechanism by which butyrate increases insulin sensitivity via inhibition of HDAC [118].

1.4.5 Roles of SCFAs and its receptors on pancreatic beta cells

SCFAs and their GPCRs were designated to affect glucose stimulated insulin secretion (GSIS), beta cell mass, and alleviate insulin resistance. However, the findings are contradictory as to whether these effects are stimulatory or inhibitory [119]. A series of studies have reported beneficial effects of SCFAs on islet beta cells. The study performed in 2015 showed that GPR43 knockout (KO) mice exhibited reduced beta cell function and mass after 14 weeks on high-fat diet. Furthermore, a specific agonist of GPR43 increased insulin secretion of isolated islets in vitro [120]. In the same year another group reported that although islet expression of GPR43 was increased in a state of insulin resistance, GPR43-mediated GSIS was not significantly altered by high-fat diet [121]. Another study focusing on GPR43 showed that loss of this receptor leads to impaired islet beta

cell mass and beta cell survival [122]. This is contradictory to the research of Tang's group, which showed that GPR43/GPR41 gene deficient mice on high-fat diet exhibit increased insulin secretion and improved glucose tolerance as compared to normal mice [123]. According to some reports about the effect of SCFAs on the islet function, GSIS was rapidly and significantly increased after adding acetate, propionate, or butyrate in both perfused humans and mouse islets [124]. The mechanism by which SCFAs regulate insulin secretion through their receptors may involve the following (**Figure 2**): after activation of GPR43/GPR41 receptors by SCFAs, GPR43 is coupled to $G\alpha_{i/o}$ and $G\alpha_{q/11}$ G proteins, whereas GPR41 is only coupled to $G\alpha_{i/o}$ proteins. When GPR43 is activated, the ligand primarily binds to the $G\alpha_{q/11}$ subunit, activating the phospholipase C (PLC)/diacylglycerol (DAG)/protein kinase C (PKC) pathway, which prompts the endoplasmic reticulum (ER) to releases calcium ion and hence enhances insulin release. In contrast, in case of GPR43/GPR41 binds to the $G\alpha_{i/o}$ subunit, cyclic adenosine monophosphate (cAMP) levels decrease and block protein kinase A (PKA) and EPAC (exchange factor directly activated by cAMP) -mediated insulin release [125]. Therefore, although no definitive conclusions have been drawn, current evidence suggests that SCFAs have a protective effect on beta cells.

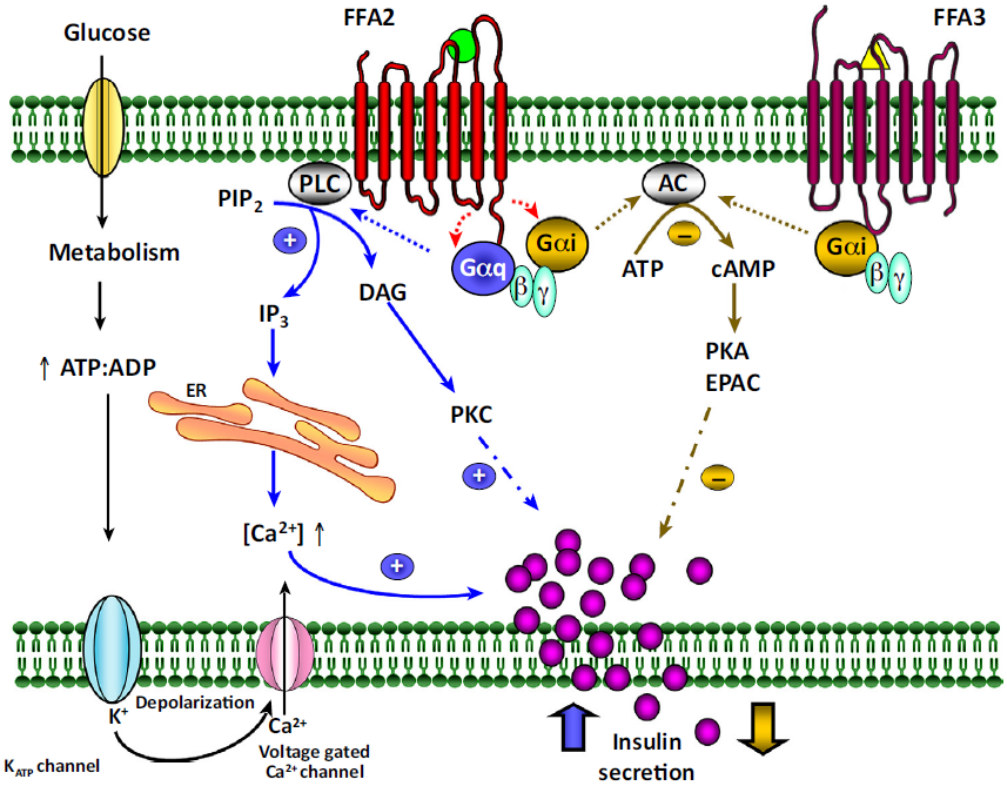


Figure 2. Modulation of insulin secretion through FFA2 (GPR43) and FFA3 (GPR41) by SCFAs[125]. SCFAs can activate the PLC-DAG-IP3 signaling cascade and boost glucose-stimulated insulin secretion via FFA2 receptors, while they can also bind to FFA3 to decrease cAMP concentration and reduce insulin secretion.

1.5 Aims of the project

Islet transplantation represents an effective alternative treatment for patients with Type 1 diabetes mellitus suffering from instability of glucose values under insulin injection therapy. Since there is a severe shortage of human pancreatic donors to meet the clinical demands, alternative cell sources are needed for beta cell replacement therapies. Previous studies reported that porcine islets, especially neonatal porcine islet-like clusters (NPICC), are a potential alternative cell source for beta cell replacement therapies. However, NPICCs are immature and require short-term *in vitro* culture pre-transplantation. The aim of the present study was to optimize conditions of NPICC culture to improve endocrine differentiation and maturation. Because short-chain fatty acids (SCFA) are reported to stimulate insulin secretion and improve beta cell function in human islets, their time- and concentration-dependent effects on differentiation of progenitor cells and maturation of beta cells were analyzed in NPICCs by quantitative RT-PCR, FACS, immunohistochemistry and glucose stimulated insulin secretion (GSIS). In order to elucidate the mechanisms involved in SFCA signaling, NPICCs were treated with SCFA receptor agonist and antagonists. Moreover, several specific inhibitors of histone deacetylases were used to investigate whether HDAC inhibition mimic butyrate induced maturation and differentiation of isolated NPICCs. The identification of novel critical factors for improvement of *in vitro* maturation of high quality NPICCs is very important to provide islet cells that are most appropriate for cell therapy.

2. Material and Methods

2.1 Materials

2.1.1 Chemicals and consumables

2.1.1.1 Chemicals and kits

Name	Catalog	Company
Acetic acid	# A6283	Merck, Germany
Agarose standard	# 3810.3	Carl Roth, Germany
Antibiotic-Antimycotic (A/A)	# 15240062	Gibco, Thermo Fisher Scientific, USA
AR420626	# SML1339	Sigma-Aldrich, Germany
Bovine serum albumin (BSA)	# 1071145401	Roche, Germany
β-hydroxybutyrate	# 166898	Sigma-Aldrich, Germany
$CaCl_2$•$2H_2O$	# 223506	Merck, Germany
Ciprobay Infusion solution	# 15GA064P3	Fresenius Kabi, Germany
Citric acid	# 251275	Merck, Germany
Click-iT EdU Alexa Fluor 488 Imaging Kit	# C10337	Thermo Fisher Scientific, USA
DAPI	# H-1200	Vector Laboratories, USA
D (+)-Glucose monohydrate	# 6780.1	Carl Roth, Germany
Dimethyl sulfoxide (DMSO)	# D2650	Sigma-Aldrich, Germany
100 bp DNA Ladder	# 15628019	Thermo Fisher Scientific, USA
Donkey serum	# D9663	Sigma-Aldrich, Germany
EDTA disodium salt solution	# 2854	Sigma-Aldrich, Germany
Entinostat (MS-275)	# S1053	Selleckchem, USA
Ethidium bromide	# H5041	Promega, Germany
Ethanol, absolute	# 5054	Carl Roth, Germany
Exendin-4	# E7144	Sigma-Aldrich, Germany

4% paraformaldehyde	# J61899.AK	Thermo Fisher Scientific, USA
Fetal bovine serum (FBS)	# 26140079	Gibco, Thermo Fisher Scientific, USA
FFK288 (Romidepsin)	# S3020	Selleckchem, USA
Fluorescein diacetate	# F7378	Sigma-Aldrich, Germany
Goat serum	# 31872	Thermo Fisher Scientific, USA
GoScript™ reverse transcriptase	# A5001	Promega, USA
GLPG0974	# SML2443	Sigma-Aldrich, Germany
Hams F -10 medium	# 11550043	Gibco, Thermo Fisher Scientific, USA
Hanks balanced salt solution (HBSS)	# 14175095	Gibco, Thermo Fisher Scientific, USA
HDAC activity fluorometric assay kit	# 50034	BPS Bioscience, USA
HEPES	# 6763.3	Carl Roth, Germany
HistoGel	# HG-4000012	Thermo Fisher Scientific, USA
Human serum albumin	# A1V084AC	Takeda, Germany
Hydrochloric acid solution	# H9892	Merck, Germany
In situ HDAC activity fluorometric assay Kit	# K339	BioVison, USA
3-Isobutyl-1-methylxanthine (IBMX)	# I5879	Sigma-Aldrich, Germany
KCl	# 58221	Merck, Germany
KH_2PO_4	# P0662	Merck, Germany
Liberase™ DL research grade	# 05466202001	Sigma-Aldrich, Germany
MC1568	# S1484	Selleckchem, USA
Mocetinostat (MGCD0103)	# S1122	Selleckchem, USA
$MgSO_4$	# T888.2	Carl Roth, Germany
NaCl	# S9888	Merck, Germany
$NaHCO_3$	# S5761	Merck, Germany
Na_2HPO_4	# S9763	Merck, Germany

NaOH	# 567530	Merck, Germany
Nicotinamide	# 128275000	Sigma-Aldrich, Germany
Paraffin	# 8002-74-2	Sigma-Aldrich, Germany
Pierce™ BCA protein assay kit	# 23227	Thermo Fisher Scientific, USA
Phosphate buffered saline (PBS) solution	# 10010023	Gibco, Thermo Fisher Scientific, USA
Propidium Iodide	# P4170	Sigma-Aldrich, Germany
Porcine insulin ELISA kit	# 10-1200-01	Mercodia, Sweden
Porcine serum	# P9783	Sigma-Aldrich, Germany
Quant-iT Pico-Green dsDNA assay kit	# P7581	Invitrogen, USA
ReliaPrep™ RNA cell miniprep system	# Z6011	Promega, USA
RGFP996	# S7229	Selleckchem, USA
RPMI 1640 medium	# P04-18500	PAN-Biotech, Germany
Santacruzamate A	# S7595	Selleckchem, USA
Sodium acetate	# S2889	Sigma-Aldrich, Germany
Sodium butyrate	# 303410	Sigma-Aldrich, Germany
Sodium propionate	# P5436	Sigma-Aldrich, Germany
SsoFast™ EvaGreen® supermix	# 1725201	Bio-Rad, USA
TMP269	# S7324	Selleckchem, USA
Trichostatin A (TSA)	# T8552	Sigma-Aldrich, Germany
Triton X-100	# 93443	Sigma-Aldrich, Germany
Trypan blue	# 15250061	Thermo Fisher Scientific, USA
TrypLE™ Express enzyme (1x), ohne Phenolrot	# 12604013	Gibco, Thermo Fisher Scientific, USA
Tween® 20	# P9416	Merck Millipore, Germany
WST-1	# 5015944001	Sigma-Aldrich, Germany
Xylene	# A11358	Thermo Fisher Scientific, USA

2.1.1.2 Consumables

Name	Company
Adhesion Microscope Slides	Epredia, Germany
Cell culture treated culture dish (100 x 20 mm)	Corning, USA
Cover slips (24 x 40 mm)	MENZEL-GLASER, Germany
12/24/96-well culture plates	Corning, USA
Centrifuge tubes (15 ml, 50 ml)	Corning, USA
Eppendorf Tubes (0.5 ml, 1 ml, 2 ml)	SARSTEDT, Germany
FEATHER® Microtome blades	pfm medical, UK
Parafilm® M	Parafilm, USA
PCR strip of 8	SARSTEDT, Germany
PCR reaction 96-well plates	SARSTEDT, Germany
Pipette, steril (1 ml, 2 ml, 5 ml, 10 ml, 25 ml, 50 ml)	SARSTEDT, Germany
Pipette tips (10 µl, 100 µl, 200 µl, 1000 µl)	SARSTEDT, Germany
30 µm Embedding cassettes	Epredia, Germany
Pre-separation filter	Miltenyi Biotec, Germany
Superfrost Plus slides	Thermo Fisher Scientific, USA

2.1.2 General devices

Name	Company
Agarose gel electrophoresis chamber	Bio-Rad, USA
Analytic balance	Sartorius Mechatronics, Germany
BD Accuri™ C6 Plus Flow Cytometer	BD Biosciences
Centrifuges	Eppendorf, Germany
Drying and heating oven	Memmert, Germany
Electric Pipette Controller	BrandTech Scientific, USA

Eppendorf Pipette Research plus (2.5 µl, 10 µl, 100 µl, 200 µl, 1000 µl)	Eppendorf, Germany
FLUOstar® Omega plate Reader	BMG LABTECH, Germany
FlowJo software version 10.4	TreeStar, USA
Gel Doc Imaging system	Intas Science imaging, Germany
Heraeus Laminar flow hood	Thermo Fisher Scientific, USA
Heracell CO_2 Incubator	Thermo Fisher Scientific, USA
LEITZ Fluovert Microscopes	Leica, Germany
MaxPro-Max3000P Real-time PCR system	Stratagene, USA
Microtome	Thermo Fisher Scientific, USA
Magnetic stirring hotplate	Heidolph, Germany
pH meter	WTW Series, Germany
SFX 150 Digitaler Sonifier	Branson, Germany
Tecan Magellan V 6.5 Genios ELISA Reader	Tecan Systems, USA
Tissue Embedding Center EC350	Kallifronas SA, Athens
Tissue Floatation Bath	Thermo Fisher Scientific, USA
T100 Thermal Cycler	Bio-Rad, Germany
Ultrasonic Probe Sonicator	Ultra Autosonic, Germany
Vortex-gene	Scientific Industries, USA
Water bath	Memmert, Germany
Xpose Spectrophotometer	Trinean, Belgium
96-well Plate Shaker	Thomas Scientific, USA

2.1.3 Buffers and solutions

Solutions	Composition
2 mM Acetic acid buffer	Add 12 mg of acetic acid to 100 ml of distilled water (dH_2O), 0.25 g BSA
BL + TG buffer	Add 32.5 µl 1-Thioglycerol to 3.25ml of BL

	buffer
0.1 M Citrate buffer	Add 25.7 g sodium citrate dihydrate and 2.4 g Citric Acid to 1 L of dH_2O
Tris-buffered saline (TBS) (10 X)	90 g NaCl
	60.5 g Tris-base
	1000 ml dH_2O
	Adjust the pH to 7.5
TBST	Add 1 ml of Tween 20 per liter of 1 X TBS.
Phosphate-buffered saline (PBS) (10 X)	80 g NaCl
	2 g KCl
	14.4 g Na_2HPO_4
	2.4g KH_2PO_4
	1000 ml distilled water
	Adjust the pH to 7.4
10 mM Na-citrate	2.94 g Trisodium citrate (dehydrate)
	1000 ml distilled water
	Adjust the pH to 6.0 with 1 N HCl
0.5 M EDTA	186.1 g of disodium EDTA•$2H_2O$
	1000 ml distilled water
	Adjust the pH to 8.0 with NaOH
1M Tris-Cl	121.1 g of Tris base
	Dissolve 800 ml distilled water.
	Adjust pH to 8.0 with concentrated HCl. Bring final volume to 1 liter with dH_2O.
Tris-EDTA (pH 8.0)	1 ml 1 M Tris-Cl (pH 8.0)
	0.2 ml 0.5 M EDTA (pH 8.0)
	98.8 ml distilled water
Krebs-Ringer bicarbonate HEPES (KRBH) buffer	128 mM NaCl (0.7490 g)
	5 mM KCl (0.0373 g)
	2.7 mM $CaCl_2$•$2H_2O$ (0.0397 g)
	1.2 mM KH_2PO_4 (0.0163 g)

	1 mM (0.0120 g) 10 mM HEPES (0.2380 g) 1 mM Na_2HPO_4 (0.0141 g) 5 mM $NaHCO_3$ (0.0420 g) 0.1% BSA (0.1 g) 100 ml distilled water, adjust pH to 7.4 Filtering and sterilizing the buffer before use
KRBH + 2.8 mM Glucose	Add 100 µl 50% Glucose in 100 ml KRBH
KRBH + 20 mM Glucose	Add 720 µl 50% Glucose in 100 ml KRBH

2.1.4 Antibodies

2.1.4.1 Primary antibodies

Name	Catalog	Company
Rat anti-mouse-PE CD16/CD32 (clone 2.4G2)	# 553145	BD Biosciences
Mouse anti-insulin AF647 (clone T56-107)	# 565689	BD Biosciences
Mouse anti-glucagon-PE (clone U16-850)	# 565860	BD Biosciences
Mouse anti-somatostatin AF488 (clone U24-354)	# 566032	BD Biosciences
Mouse anti-Pdx-1-PE (clone 658A5)	# 562161	BD Biosciences
Mouse anti-Nkx6.1-AF647 (clone R11-560)	# 563338	BD Biosciences
Mouse anti Nkx6.1-PE (clone R11-560)	# 563023	BD Biosciences
Mouse anti-cytokeratin (CK)-7-FITC (clone REA935)	# 130-115-446	Miltenyi, Germany

Polyclonal guinea pig anti-insulin	# A0564	Agilent-Dako, Germany
Rabbit anti-glucagon	# MABN238	Merck Millipore, Germany
Rabbit anti-PDX1	# ab47267	Abcam, USA

2.1.4.2 Secondary antibodies

Name	Catalog	Company
Alexa Fluor® 594 AffiniPure Donkey Anti-Guinea Pig IgG (H+L)	# NC0452490	Jackson Immuno Research Labs, USA
Goat anti-Guinea Pig IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, FITC	# A18776	Invitrogen, Thermo Fisher Scientific, USA
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	# A-11034	Invitrogen, Thermo Fisher Scientific, USA
Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555	# A32732	Invitrogen, Thermo Fisher Scientific, USA

2.1.5 Primers

Table 2. Primer sequences used for quantitative real time-PCR

Gene	Primer Sequence 5'-3'	Genbank Accession No.
INS	Forward: CAGGCCTTCGTGAACCAG	XM_021081278.1
	Reverse: CTTGGGCGTGTAGAAGAAGC	
GCG	Forward: GAATTCATTGCTTGGCTGGT	XM_005671883.3
	Reverse: CATCTGAGAAGGAGCCATCAG	
PDX1	Forward: GTGGAAAAAGGAGGAGGACA	NM_001141984.3

	Reverse: CAGCTCCTCTCCCGAGGT	
MafA	Forward: GGAGCTGGCGATGGGTG	XM_021090536.1
	Reverse: GGTGGCTCCTTCTTCACCTC	
Nkx6.1	Forward: GCCTACCCCGTTTCAGTAGC	XM_021101796.1
	Reverse: GGGTGGACTCTGCATCACTC	
NGN3	Forward: GCCTGCGTCTCAGCTGAACTT	XM_021072424.1
	Reverse: AGCCAGAGGCAGGAGGAACAA	
ALDOB	Forward: ATTTGGAGGGCACTCTGTTG	XM_021066854.1
	Reverse: AGGTTGATAGCATTGAGGTTGAG	
GPR43	Forward: TCATGGGTTTCGGCTTCTACAG	EU122439.1
	Reverse: GTACTGAACGATGAACACGACG	
GPR41	Forward: ACTACTTCTCATCCTCGGGGTT	JX566879.1
	Reverse: CTCCACTTCGCTCTTCTTCAGT	
GAPDH	Forward: GTCGGTTGTGGATCTGACCT	XM_021091114.1
	Reverse: GTCCTCAGTGTAGCCCAGGA	

INS: insulin; *GCG*: glucagon; *PDX1*: pancreatic and duodenal homeobox 1; *NGN3*: Neurogenin 3; *Nkx6.1*: NK6 Homeobox 1; *ALDOB*: aldolase fructose-bisphosphate B; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase; *GPR43*: G-Protein Coupled receptor 43; *GPR4*1: G-Protein Coupled receptor 41; *MafA*: MAF bZIP transcription factor A

2.2 Animals

Pancreata were obtained from healthy 2 - 3 days-old German landrace hybrid piglets provided by the Moorversuchsgut, Lehrstuhl für Molekulare Tierzucht und Biotechnologie der LMU München. All animal experiments were approved by the responsible animal welfare authority (Regierung von Oberbayern, Munich, Germany) and were conducted in accordance with Directive 2010/63/EU and German animal protection laws.

2.3 Methods

2.3.1 Isolation of neonatal porcine islet-like clusters

The pancreases were shipped on sterile HBSS plus 1% Antibiotic/Antimycotic (A/A) solution on ice within 1 to 2 hours after removal. Porcine pancreatic islets were isolated by collagenase digestion according to previously published protocols [85]. The individual steps of the procedure are described in the following sections in detail.

Pancreases were transferred into a Petri dish with Povidone-iodine for 2 seconds and rapidly transfered into a Petri dish filed with 5 ml HBSS + A/A. After washing the pancreas with cold HBSS + A/A buffer, lymph nodes and non-pancreatic tissue were dissected. Pancreas was minced into about 1 mm^3 pieces, followed by digestion with 1.2 mg/ml collagenase Liberase DL Research Grade, and the tissue fragments were gently stirred and incubated at 37°C for 15 minutes to produce a cell suspension. After the first digestion, cell suspensions were passed through a 500 µm wire mesh to remove any clumps and undigested tissue. The filtered cell suspensions were transferred from Petri dish to a 50 ml centrifuge tube. Cells were collected by centrifugation at 1000 rpm for 3 minutes, followed by washing with HBSS + A/A three times and RPMI 1640 medium once. Finally, cells containing exogenous pancreatic cells and NPICCs were cultured in 10 cm cell culture dishes with 20 ml of basic medium, composed of RPMI 1640 medium, 2% human albumin serum, 10 mmol/L nicotinamide, 20 nM exendin-4 and 1% A/A at 37°C in a Lab humidified CO_2 incubator. The medium was changed daily for the first four days and every two days thereafter to remove single cells and dead cells and to enrich cell clusters. The islet isolation process is illustrated in the following figure.

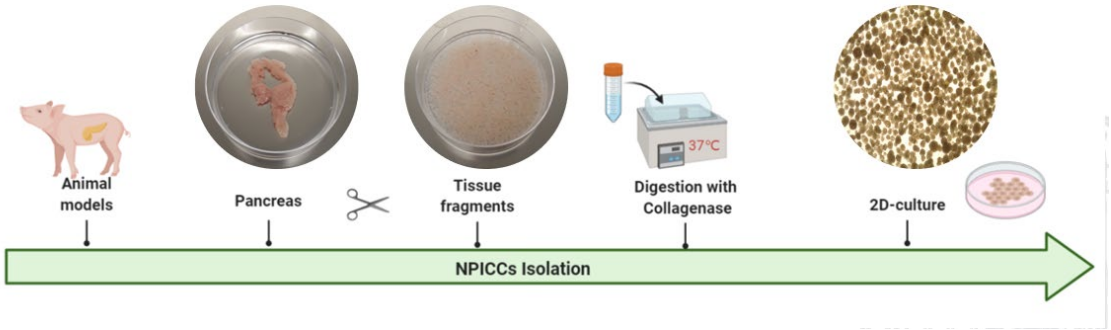


Figure 3. Overview of the procedure for the isolation of NPICCs. The pancreas was removed from neonatal pigs and transferred to the cell culture hood in cold sterile buffer. After the pancreas was cut into pieces, collagenase was added for tissue digestion in a 37°C water bath. After the digestion, the residual enzyme was washed out, and the cell suspension was cultured in vitro.

2.3.2 Test of NPICCs culture media

To compare the basic medium used in our laboratory with media used by other research groups (Ham´s F12 and RPMI1640 supplemented with either human serum albumin (HAS) or Bovine Serum Albumin (BSA) together with IBMX or exendin-4). NPICCs were cultured in different media in 10 cm dishes for 5 days. The detailed composition of the different media is shown in **Table 3**. After 5 days of culture, cells were collected for RNA extraction to determine the expression levels of genes related to islet beta cell differentiation and maturation.

Table 3. The composition of the different media used for NPICC isolation

Name	Composition
Basic medium	RPMI 1640 + 2% HSA+ Antibiotic-Antimycotic + 10 mM nicotinamide plus 20 nM exendin-4
RPMI 1640 + HSA + IBMX	RPMI 1640 + 2% HSA+ Antibiotic-Antimycotic + 10 mM nicotinamide plus 50 µM IBMX
Ham's F10 + BSA + IBMX	Ham's F10 + 0.5% BSA + Antibiotic-Antimycotic + 10 mM nicotinamide plus 50 µM IBMX
RPMI 1640 + BSA	RPMI+ 0.5% BSA + Antibiotic-Antimycotic + 10 mM nicotinamide plus 20 nM exenatide-4
Ham's F10 + HSA	Ham's F10 + 2% HSA + Antibiotic-Antimycotic + 10 mM nicotinamide + 20 nM exendin-4

2.3.3 Islet quantification and islet equivalent (IEQ) calculation

The number of islets is usually expressed in islet equivalent (IEQ), which is the international common unit for islet cell count. An IEQ is equivalent to an islet with a diameter of 150 μm [126]. The diameter of each islet was manually recorded under a stereomicroscope and converted to islet diameter categories with those less than 50 μm not being counted and those greater than 350 μm no longer being subdivided. The calculation of IEQ from the islet size is described in **Table 4**. For islets with irregular shape, two diameter measurements were taken at different locations on the islet, and the average value was used as the final diameter measurement.

Table 4. Calculation the total actual islet (total AI) and the total islet equivalents (total IEQ)

Islet Diameter Range (μm)	Islet Particle Number (AI)	IEQ Conversion Factor	IEQ per Range
50 – 100		x 0.167	
101 – 150		x 0.648	
151 – 200		x 1.685	
201 – 250		x 3.500	
251 – 300		x 6.315	
301 – 350		x 10.352	
>350		x 15.833	
∑ AI		∑ IEQ	
Dilution Factor [(ml total volume / μl sample volume) **x** 1000]			
Total AI = ∑ AI X Dilution Factor			
Total IEQ = ∑ IEQ X Dilution Factor			

2.3.4 Treatment of NPICCs with SCFAs and HDAC inhibitors

After three days culture, NPICCs were counted and 2000 IEQ per well was seeded into 24-well plates followed by incubation with 0, 100, 500, 1000, 2000 μM sodium acetate, sodium butyrate or sodium propionate for one, two, four, six, and eight days, respectively.

In the experiments with HDAC inhibitors, NPICCs were incubated separately with different HDAC inhibitors to investigate the effect of HDAC inhibitors on the differentiation and maturation of NPICCs. The details of the inhibitors used are shown in **Table 5**. After the treatment, NPICCs were collected for subsequent experiments such as qPCR, flow cytometry, and immunofluorescence staining.

Table 5. HDAC inhibitors used in the experiments

Inhibitors	Characteristics	Concentration	Incubation time
Trichostatin A	selective class I and II HDAC inhibitor	1 μM	6 days
Mocetinostat	class I HDAC inhibitor (HDAC1, 2, 3 and 11)	1 μM	
MS275	HDAC 1, 3 - inhibitor	1 μM	
TMP269	class IIa inhibitor	1 μM	
MC1568	class IIa inhibitor	1 μM	
Santacruzamate A	HDAC 2 inhibitor	1 μM	
RGFP966	HDAC 3 inhibitor	1 μM	
FK228	HDAC 1 and 2 inhibitor	10 nM	

2.3.5 Islet viability and islet yield

Islet viability of each group was determined by propidium iodide (PI) / fluorescein diacetate (FDA) dye staining. PI was dissolved in PBS to get a stock solution of 750 μM and FDA stock solution was prepared in acetone (24 μM).

NPICCs were harvested and washed by PBS. 400 μl well-mixed total islet suspension was added to the 48-well plate in duplicates. Then, 8 μl FDA solution was quickly added to the islet suspension first, followed by 8 μl of 750 μM PI. Cells were gently swirled to mix and incubated in the dark for 15 min. The preparation was assessed immediately using the fluorescent microscope (viable cells:

green cytoplasmic staining, dead cells: red nuclear staining). Islet yield and the number of living cells were expressed as the percentage compared to basal medium.

2.3.6 Measurement of glucose stimulated insulin secretion (GSIS)

NPICCs were cultured in basic medium or from day 3 to day 9 in basic medium with 1000 µM butyrate or 1 µM mocetinostat. NPICCs of similar size and shape were hand-picked to achieve as close as possible a 100% purity. Krebs-Ringer bicarbonate HEPES (KRBH) solution was warmed to 37°C in incubator 37°C, 5% CO_2. NPICCs were washed with KRBH with low glucose (2.8 mM). Then islets were transfered into a new 12 well-plate and pre-incubated in 1 ml of low glucose (2.8 mM) KRBH at 37°C, 5% CO_2 for 1 hour. After that, the islets were divided into two groups. One group continued to incubate in the low glucose (2.8 mM) KRBH for 1 hour and in the other group 6.2 µl of 50% glucose was added to reach a concentration of 20.0 mM glucose in KRBH for 1 hour. At the end of experiment, islets were gathered into the center of the well by rotating the plate in the same direction to collect 500 µl supernatant from the edge of the well from the low and high glucose-stimulated insulin secretion sample. Samples were stored at -80°C until insulin measurement. Islets were harvested by centrifugation at 1000 rpm for 8 minutes for measurement of cellular insulin concentration (low glucose stimulation) and DNA content (high glucose stimulation).

2.3.7 Insulin content measurement

Islets from the low glucose stimulation group in the GSIS experiment were washed with PBS twice and centrifuged at 1000 rpm for 8 min. At the end of centrifugation, supernatant was removed and 500 µl of 2 mM acetic acid buffer was added to the islet pellets before the samples were sonicated on ice for 1 min (five pulses at 1Joule on ice for 30 s by using Branson SFX 150 Digitaler Sonifier). After sonication, the sample was centrifuged for 15 min at 800×g at 4°C. Lastly, 500 µl of supernatant was collected for measurement of total insulin content.

2.3.8 DNA content measurement

Islets from the high glucose group in the GSIS experiment were washed with PBS once and citrate-buffer once. Then cells were centrifuged at 1000 rpm for 8 min. Supernatant was removed and 500 µl of citrate-buffer was added to the islet pellets followed by centrifugation at 200×g in 4°C for 10 min. Lastly, cell pellet was stored at -20°C until assayed of DNA content.

Total DNA content was measured using a Quant-iT PicoGreen dsDNA Assay kit. DNA standards ranged from 10 to 1000 ng/ml. NPICCs pellets were dissolved in 25 µl 1X TE (Tris-EDTA). The working buffer was prepared by diluting the high sensitivity reagent 1: 200 in the high sensitivity buffer. 5 µl DNA samples were incubated with 195 µl working buffer for 2 to 5 minutes at room temperature, protected from light. Samples and standards were plated in 96-black-well plates and read by using the FLUOstar® Omega Plate Reader. Sample concentrations were determined from the standard curve. The excitation and emission wavelengths were 480 nm and 520 nm, respectively.

2.3.9 Insulin measurement

Porcine Insulin ELISA kit (Mercodia) was used to measure total insulin content and insulin concentration in the supernatants following manufacturer instructions. Sample concentrations were determined from the standard curve including 2.3 to 173 mU/l insulin. 25 µl each of Calibrators, controls and samples were pipetted into appropriate wells. Then, 100 µl of enzyme conjugate 1X solution was added into each well and incubated on a plate shaker with the speed of 700–900 rpm for 2 hours at room temperature. After the incubation, plates were washed 6 times with 350 µl wash buffer 1X solution per well. Then 200 µl substrate 3,3',5,5'-Tetramethylbenzidine (TMB) was added into each well and incubated for 15 minutes at room temperature without shaking. Finally, 50 µl stop solution was used to stop the reaction. Plates were placed on the shaker for approximately 5 seconds to ensure mixing. Plates were read using the FLUOstar® Omega Plate Reader at absorbance of 450 nm and insulin concentrations were calculated from the standard curve.

2.3.10 Flow cytometry analysis

At day 3 after isolation, NPICCs were cultivated with or without 1000 µM butyrate for both 3 days and 6 days. Islets clusters were dissociated into single cells by treatment with 1x TrypLE express enzyme for 5 min, which were subsequently washed with PBS + 10% fetal bovine serum (FBS) and filtered through a 30 µm pre-separation filter. Then cells were fixed and permeabilized by using an intracellular staining buffer set and incubated with Fc-Block (anti-mouse CD16/CD32) for 10 minutes at room temperature to reduce nonspecific binding. Intracellular staining was performed using the fluorochrome-labeled antibodies, the details of the antibodies and the dilutions are shown in the **Table 6**. Mouse anti-insulin AF647 (clone T56-107), mouse anti-glucagon-PE (clone U16-850), mouse anti-somatostatin AF488 (clone U24-3545), mouse anti-Pdx-1-PE (clone 658A5), mouse anti-Pdx-1-AF488 (clone 658A5), mouse anti-Nkx6.1-AF647 (clone R11-560), mouse anti Nkx6.1-PE (clone R11-560), mouse anti-cytokeratin (CK)-7-FITC (clone REA935) and isotype control antibodies. All antibodies were pre-tested for appropriate dilution and for specificity using isotype control antibodies (**Figure 4**). Antibodies were incubated at 4°C for 30 min followed by two washing steps with permeabilization buffer. The positive threshold was determined based on the fluorescence-specific isotype control for each marker. Flow cytometry data were acquired on a BD Accuri™ C6 flow cytometer and analyzed using FlowJo software version 10.4.

Table 6. Details of antibodies used for flow cytometry analysis

Name	Dilution used
Mouse anti-insulin AF647 (clone T56-107)	1: 30, 30 min, 4°C
Mouse anti-glucagon-PE (clone U16-850)	1: 30, 30 min, 4°C
Mouse anti-somatostatin AF488 (clone U24-354)	1: 50, 30 min, 4°C
Mouse anti-Pdx-1-PE (clone 658A5)	1: 50, 30 min, 4°C
Mouse anti-Nkx6.1-AF647	1: 50, 30 min, 4°C
Mouse anti Nkx6.1-PE (clone R11-560)	1: 50, 30 min, 4°C
Mouse anti-cytokeratin (CK)-7-FITC (clone REA935)	1: 50, 30 min, 4°C

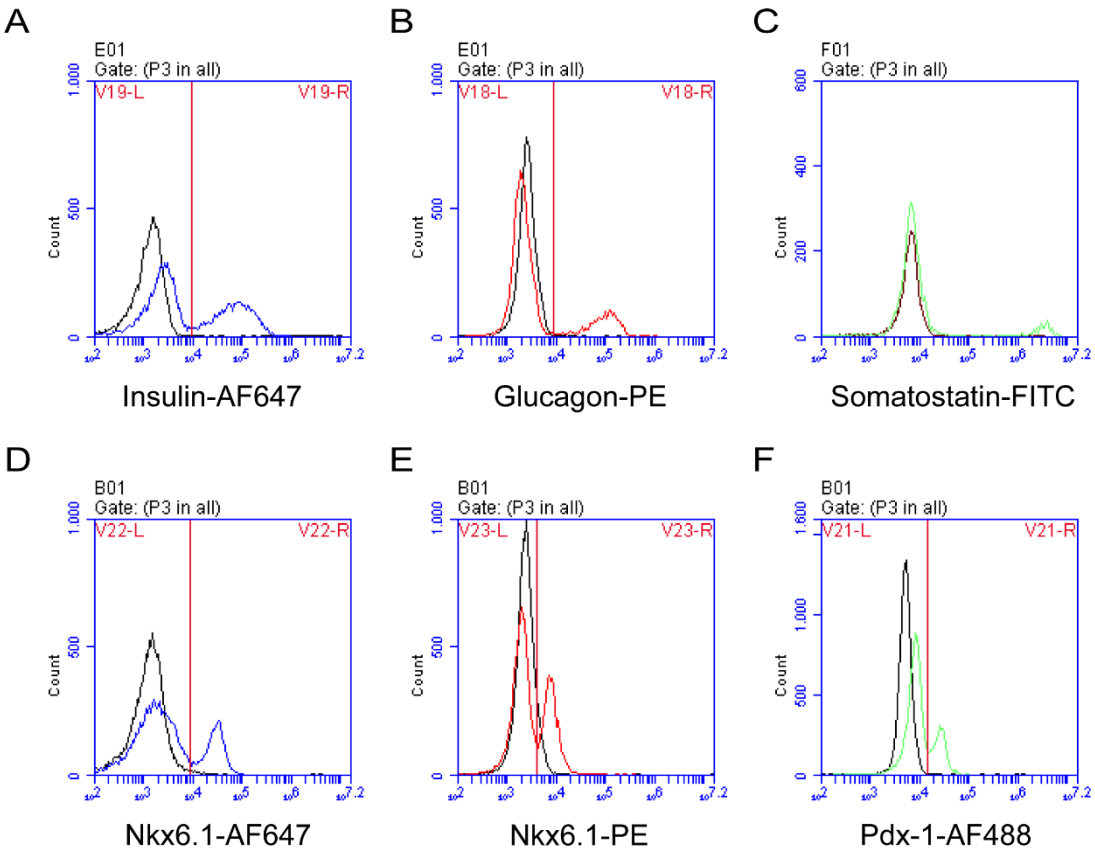


Figure 4. Representative flow cytometry of dispersed NPICCs stained with antibodies to insulin, glucagon, somatostatin, Nkx6.1, Pdx-1 and isotype control antibodies by flow cytometry analysis. The open gray histograms indicate staining with isotype control, and the color histograms indicate staining with corresponding antibodies: insulin-AF647 (**A**), glucagon-PE (**B**), somatostatin-FITC (**C**), Nkx6.1-AF647 (**D**), Nkx6.1-PE (**E**), Pdx-1-AF488 (**F**).

2.3.11 Edu-proliferation assay

5-Ethynyl-2´deoxyuridine (Edu) labeling was performed using the Click-iT EdU Alexa Fluor 488 Imaging Kit. Briefly, NPICCs were cultured in B-IC medium in 6-well plates for 6 days in the presence of 1000 µM butyrate. 10 µM EdU was added to the medium during the last 72 h of the 6 days incubation period. Then islets were embedded in Histogel and paraffin, sectioned at 4 µm and mounted on Superfrost Plus slides. EdU incorporation was detected following the manufacturer's instructions. Tissues were permeabilized with 0.5% Triton X-100 for 20 min and incubated with the Click-iT reaction cocktail for 30 minutes at room temperature. Slices were stained with polyclonal guinea pig anti-insulin for 1 hour, and secondary antibody Alexa Fluor® 594 AffiniPure Donkey Anti-Guinea Pig IgG (H+L) for 45 minutes. Images were taken and quantified by Leica DM2500 microscope and Image J software (http://rsb.info.nih.gov/ij/download.html). Proliferation was quantified by counting of at least 1000 cells for each islet from six independent NPICCs preparation.

2.3.12 WST-1 proliferation assay

NPICCs were treated for five days with different concentrations of SCFAs. Then, 100 IEQ were transferred to 96-well plates and cultured overnight. Islets incubation with 1% Triton X-100 was used as dead cell control. Cell proliferation was assayed by WST-1 assay. 10 µl/well WST-1 reagent was added and incubated for 4 h at 37°C in a humidified incubator with 5% CO_2. Finally, plates were placed for 1 min on a shaker before measurement. Absorbance (450 nm) of the samples against a background control as blank was detected by using an ELISA microplate reader.

2.3.13 *In vitro* HDAC assay

The HDAC inhibitory potential of acetate, propionate and butyrate was assessed in a cell-free experimental model. The inhibitory effects of SCFAs were compared with trichostatin A (TSA), a well-known non-specific HDAC inhibitor. The analysis

was performed using the HDAC activity fluorometric assay kit. Briefly, HDAC was incubated with 0, 100, 500, 1000, 2000 μM acetate, propionate or butyrate on a 96-well plate for 1 hour. Then 50 μl HDAC substrate was added to each well, and the plates were incubated at 37°C for 30 min. Finally, developer solution was added for an additional 15 minutes at room temperature. The fluorescence was read by the FLUOstar® Omega Plate Reader at excitation and emission wavelengths of 485 nm and 528 nm. All the measures were performed in duplicates or triplicate.

2.3.14 *In situ* HDAC assay

The ability of butyrate to inhibit activity of HDACs in NPICCs was measured by using *In situ* HDAC Activity Fluorometric Assay Kit, following the manufacturer's protocol. Briefly, NPICCs were treated with 1000 μM butyrate from day 3-9. After the 6-days treatment period, medium was removed and 100 μl reaction mix was added to each well and incubated for 3 h at 37°C. Then 100 μl developer solution was added into each well for 30 min at 37°C. Fluorescence signals were detected by a FLUOstar® Omega Plate Reader, with excitation and emission filters of 355 nm and 460 nm. All the measures were performed in duplicates or triplicate.

2.3.15 RNA extraction

Total RNA was extracted from NPICCs by using ReliaPrep™ RNA Cell Miniprep System kit according to the manufacturer's instructions. NPICCs were harvested in a sterile centrifuge tube by centrifugation at 300×g for 5 minutes, followed by washing with ice-cold 1X PBS. Cell pellet was lysed by adding 100 μl BL + TG buffer to each tube and pipetted 7–10 times to shear the DNA. Cell pellet was dispersed and mixed with 100 μl 100% isopropanol by vortexing for 5 seconds. Then cell lysate was transferred to a minicolumn in a collection tube and centrifuged at 14,000 g for 30 seconds at room temperature. After that, the minicolumn was washed with 500 μl RNA wash solution and centrifuged at 14,000 g for 30 seconds. For reducing genomic DNA contamination, 30 μl freshly prepared

DNase I incubation mix was added to the minicolumn membrane and incubate for 30 minutes at room temperature. After this incubation, the minicolumn was washed once with 200 µl of column wash solution and once with 500 µl of RNA wash solution followed by centrifugation at 14,000 g for 30 seconds. The minicolumn was transferred into a new collection tube and washed with 300 µl of RNA wash solution by centrifugation at high speed for 2 minutes. Finally, RNA was collected into a 1.5 ml tube by adding 20 µl nuclease-free water to the minicolumn and centrifugation at 14,000 g for 1 minute. The yield of total RNA obtained was determined spectrophotometrically at 260 nm, where 1 absorbance unit (A260) equals 40 µg of single-stranded RNA/ml. The purity also was estimated by spectrophotometry from the relative absorbances at 280 nm with the Micro-Volume Spectrophotometer.

2.3.16 cDNA Synthesis

cDNA synthesis was performed with GoScript™ reverse transcription mix, oligo(dT) kit. 10 µl of GoScript™ reverse transcription mix was prepared for each cDNA reaction by combining components described in the **Table 7**.

Table 7. Reagents composition for reverse transcription of RNA

Nuclease-Free Water	4 µl
GoScript™ Reaction Buffer, Oligo(dT)	4 µl
GoScript™ Enzyme Mix	2 µl
RNA	Up to 10 µl
Total volume	10 µl

Reverse transcription reaction was performed according to the following conditions:

Table 8. The reaction program for RT-PCR

25°C	5 min
42°C	1 h
70°C	15 min
4°C	Hold

2.3.17 Assessment of GPR43 and GPR41 mRNA expression by RT-PCR

Expression of GPR43 and GPR41 mRNA in NPICCs was assessed by gel electrophoresis of amplified cDNA. RT-PCR was run on a T100 Thermal Cycler with the following program: 95°C for 3 min, 45 cycles of 15 s at 95°C and 20 s at 60°C, 1 min at 72°C, and finally 5 min at 72°C. The PCR products were evaluated by 1.5% agarose gel electrophoresis in Tris-borate-EDTA buffer stained with ethidium bromide. Images were taken by Gel Doc Imaging System. Amplification without reverse transcriptase (−) RT served as control.

Table 9. Thermal cycling condition for GPR43 and GPR41 RT-PCR

95°C	3 min	
95°C	15 s	45 x cycles
60°C	20 s	
72°C	1 min	
72°C	5 min	
4°C	Hold	

2.3.18 Real-time quantitative PCR (qRT-PCR)

To quantify mRNA expression of specific gene products, quantitative real-time PCR was established. Amplification efficiencies of the primers for the housekeeping gene and the respective target genes were evaluated using ten-fold serial dilution series of control cDNA (range from 1:10 to 1:10000). Primers, cDNA

and SsoFast™ EvaGreen® Supermix was added to 96-well plates according to **Table 10** and reactions were run in duplicates on MaxPro-Max3000P Real-time PCR system. The amplification conditions for qRT-PCR shown in **Table 11** were as follows: 95°C for 10 min, 40 cycles of 10 s at 95°C and 20 s at 60°C, followed by a melting curve stage of 95 °C for 60 s and 55 °C for 20 s to check primer dimer formation and contamination.

For calculation of the primer amplification efficiencies, the intercross of the respective amplification curves with the threshold line were determined and plotted against the corresponding logarithmized dilution range. Primer efficiencies were calculated from the slope of the resulting linear regression curves according to the following equation [127].

$$\mathbf{E\ (\%) = (10^{-1/slope} - 1) \times 100\%}$$

The amplification plots of each primer used in the experiments and the associated standard curves used to calculate efficiency, slope and intercept are shown in **Figure 5**. The qPCR efficiencies of the primers are stated in **Table 12**.

Table 10. Reagent's composition for qRT-PCR

EvaGreen® Supermix	6 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Nuclease-Free Water	3 µl
Dilute cDNA (1:5)	2 µl
Total volume	12 µl

Table 11 The reaction program for qRT-PC

95°C	10 min	
95°C	10 s	40 x Cycle
60°C	20 s	
95°C	1 min	60 x Cycle
55°C	20 s	

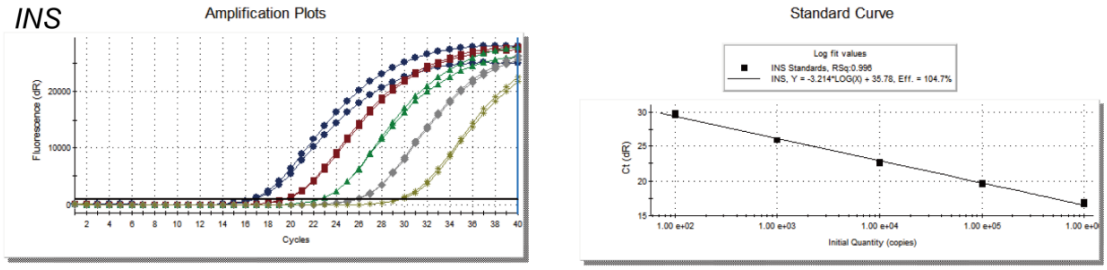
Table 12 Parameters of standard curves for qPCR

	R^2	**Slope**	**Efficiency (%)**	**Intercept**
Insulin	0.998	-3.165	107	32.51
Glucagon	1.000	-3.378	97.7	31.87
PDX1	0.999	-3.405	96.6	38.99
MafA	1.000	-3.356	98.6	36.76
GAPDH	0.998	-3.405	96.6	32.40

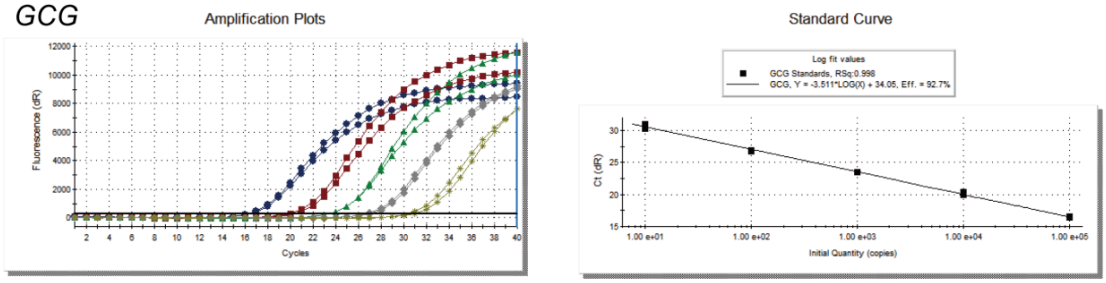
The slope between -3.1 and -3.6 are considered acceptable (90% and 110% efficient, respectively), while a slope of -3.32 is indicative of 100% efficiency.

Transcript abundances of *INS*, *GCG*, *PDX1*, *NGN3*, *Nkx6.1* and *ALDOB* were normalized against the expression level of the *GAPDH* gene via threshold cycle (Ct) value based on the comparative $2^{-\Delta\Delta Ct}$ method. Probes without reverse transcription and buffer without cDNA were used as controls. Results were expressed as a fold change as compared to RNA from NPICCs cultured under basal conditions.

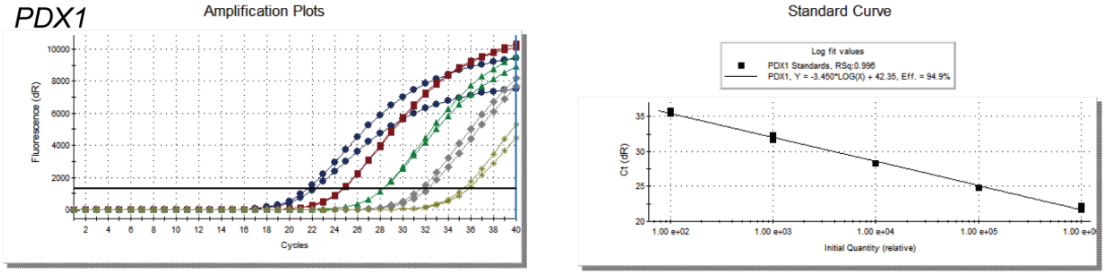
A


INS
Amplification Plots
Fluorescence (dR)
Cycles
Standard Curve
Log fit values
INS Standards, RSq:0.996
INS, Y = -3.214*LOG(X) + 35.78, Eff. = 104.7%
Ct (dR)
Initial Quantity (copies)

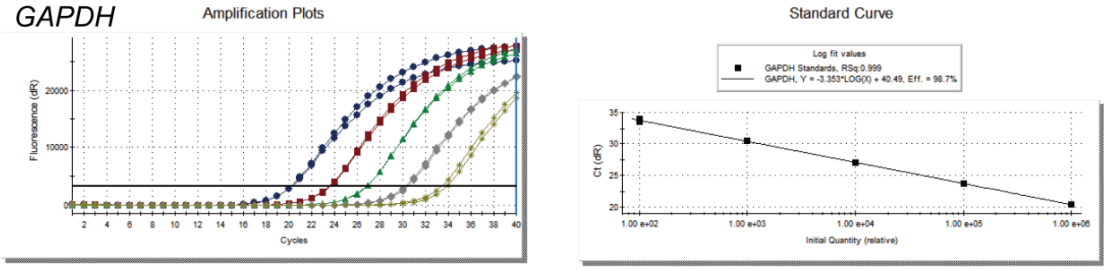
B


GCG
Amplification Plots
Fluorescence (dR)
Cycles
Standard Curve
Log fit values
GCG Standards, RSq:0.998
GCG, Y = -3.511*LOG(X) + 34.05, Eff. = 92.7%
Ct (dR)
Initial Quantity (copies)

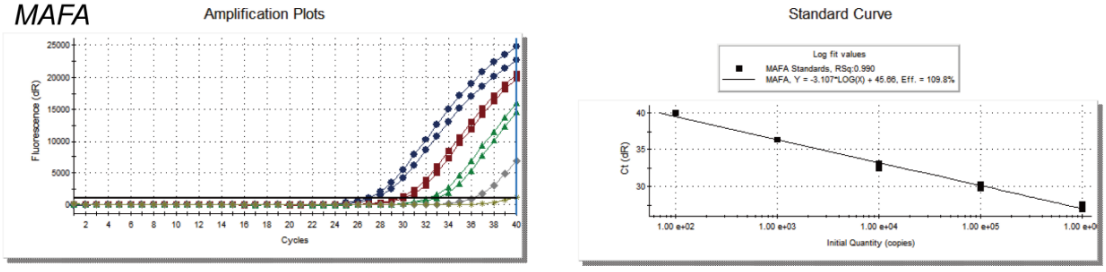
C


PDX1
Amplification Plots
Fluorescence (dR)
Cycles
Standard Curve
Log fit values
PDX1 Standards, RSq:0.996
PDX1, Y = -3.450*LOG(X) + 42.35, Eff. = 94.9%
Ct (dR)
Initial Quantity (relative)

D


GAPDH
Amplification Plots
Fluorescence (dR)
Cycles
Standard Curve
Log fit values
GAPDH Standards, RSq:0.999
GAPDH, Y = -3.353*LOG(X) + 40.49, Eff. = 98.7%
Ct (dR)
Initial Quantity (relative)

E


MAFA
Amplification Plots
Fluorescence (dR)
Cycles
Standard Curve
Log fit values
MAFA Standards, RSq:0.990
MAFA, Y = -3.107*LOG(X) + 45.66, Eff. = 109.8%
Ct (dR)
Initial Quantity (copies)

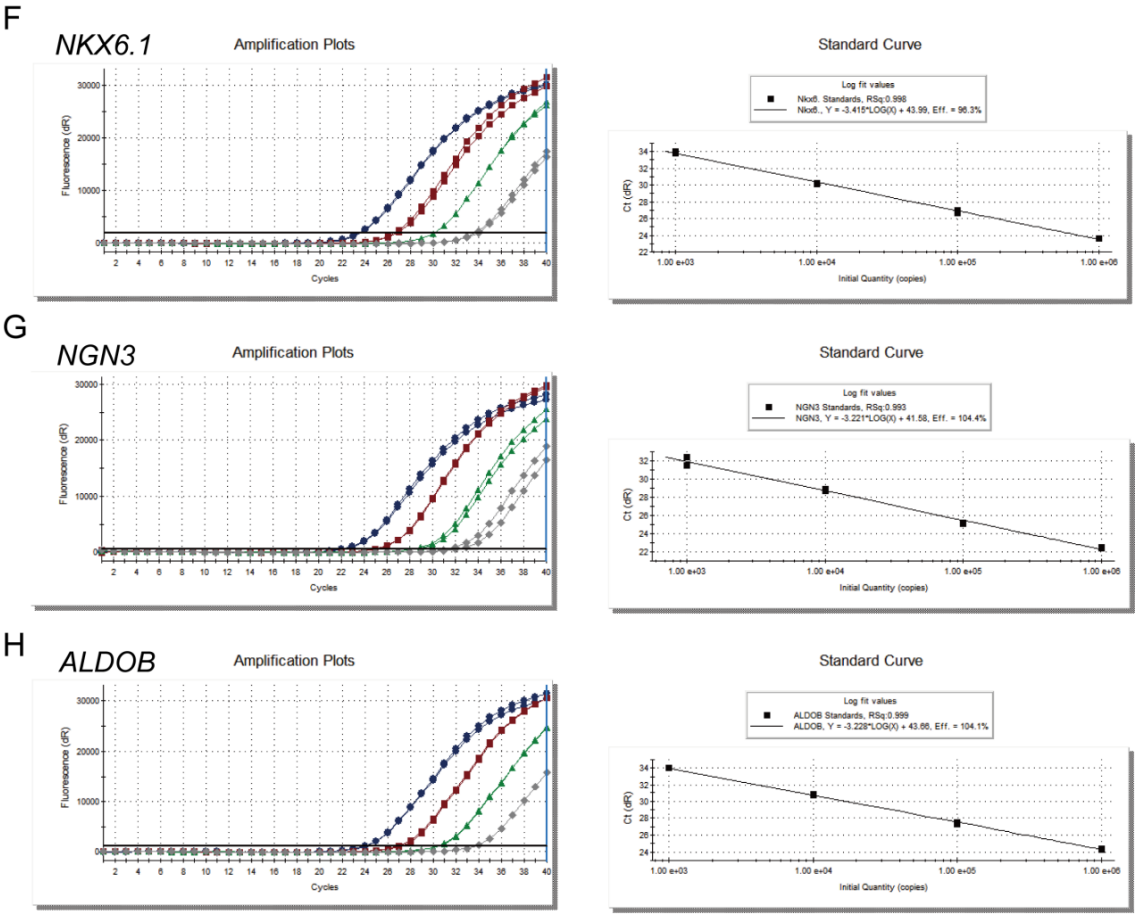


Figure 5. qPCR amplification of the control cDNA dilution series. A-E: Amplification plots (left panel) diluted cDNA measured in duplicate and the calibration curve (right panel) with equation and R^2 of the linear regression of *INS* (**A**), *GCG* (**B**), *PDX1* (**C**), *GAPDH* (**D**), *MafA* (**E**), *Nkx6.1* (**F**), *NGN3* (**G**) and *ALDOB* (**H**).

2.3.19 Immunofluorescence staining of paraffin sections of NPICCs

2.3.19.1 Preparation of paraffin sections of NPICCs

Histogel was heated in a water bath at 65°C for 1 hour until it melts to a liquid state. Meanwhile, islet clusters were collected by centrifugation at 1000 rpm for 2 min and washed twice with cold PBS. Then 3 ml of 4% formalin was added to fix the cells at room temperature for 15 minutes, after which the cells were washed twice with PBS and centrifuged to collect the cell pellets. 50 ul of pre-warmed Histogel was added to resuspend the islet clusters at a centrifuge tube, and pipette for several times to adequately and thoroughly mix cells and HistoGel

together. Then HistoGel was solidified by cooling to near 4°C. HistoGel pellet containing the specimen was removed and placed inside a HistoScreen Tissue Cassette. Cassettes were fixed in 4% paraformaldehyde for 24 h to prepare for the preparation of paraffin sections.

The fixed NPICCs were placed in alcohol with different concentration gradients for dehydration and put into xylene solution for transparency before dipping into paraffin, the details of whole procedure were listed in **Table 13**. When the Histo-Gel was transparent, it was embedded into melted paraffin. The embedded NPICCs paraffin blocks were cut into 4-μm slices and slices were baked in the incubator at 37°C~45°C for subsequent staining experiments.

Table 13. Procedure of dehydration for paraffin embedding.

Step	Reagent	Time (min)	Temperature
1	70% ethanol	45	25°C
2	80% ethanol	45	25°C
3	95% ethanol	45	25°C
4	100% ethanol	60	25°C
5	100% ethanol	60	25°C
6	100% ethanol	60	25°C
7	Xylene	60	25°C
8	Xylene	60	25°C
9	Paraffin	60	60°C
10	Paraffin	60	60°C
11	Paraffin	60	60°C

2.3.19.2 Immunofluorescence staining of NPICCs sections

For deparaffinization, slices were immersed in xylene (I) for 10 minutes → xylene (II) for 10 minutes → 100% ethanol (I and II) for 5 minutes each → 96% ethanol

(I and II) for 5 minutes each → 80% ethanol for 5 minutes → 70% ethanol for 5 minutes. The slices were removed and immediately rinsed with tap water and then with deionized water to remove the ethanol. Before the blocking step, slices were heated in a steamer oven in sodium citrate buffer (pH 6.0) for 20 minutes for antigen retrieval. Then slices were cooled to room temperature and washed 3 times with PBS buffer for 5 minutes. To reduce non-specific hydrophobic interactions between the primary antibodies and the specimen, the sections were blocked with 5% serum blocking reagent for 45 minutes. Blocking reagent was removed and slides were incubated for one hour at room temperature or in a wet box at 4°C overnight with the respective primary antibody diluted to the desired concentration in TBS buffer (pH 7.4). Then slides were washed again in TBS buffer (pH 7.4) for 10 minutes × 3 and incubated with the corresponding fluorescent secondary antibody containing 5% porcine serum for 45 min at room temperature, avoid light. Slides were washed with TBS buffer for 10 min × 3. The antibodies used in the experiment and their dilution ratios are shown in **Table 14**. DAPI was added to the sections for cell nuclei staining. Images were visualized and taken by Leica DM2500 microscopy.

Table 14. Double immunostaining protocols

Step	Insulin + Glucagon	Insulin + PDX1
1	Deparaffinization	Deparaffinization
2	N/A	Antigen repair with sodium-citrate
3	N/A	0.1% Triton X-100, 10 min
4	5% Donkey + 5% Goat Serum blocking	5% Donkey + 5% Goat Serum blocking
5	guinea pig anti-insulin, 1: 300, 1 hour, 25°C	guinea pig anti-insulin, 1: 300, 1 hour, 25°C
6	rabbit anti-glucagon, 1: 300, 1 hour, 25°C	rabbit anti-PDX1, 1: 500, overnight, 4°C
7	Donkey Anti-Guinea Pig, Alexa Fluor 594, 1;1000, 45 min, 25°C	Goat anti-Guinea Pig, FITC, 1;1000, 45 min, 25°C
8	Goat anti-Rabbit, Alexa Fluor 488, 1;1000, 45 min, 25°C	Goat anti-Rabbit Alexa Fluor Plus 555, 1;1000, 45 min, 25°C
9	DAPI	DAPI

Antibodies details are given in **sections 2.2.4.1 and 2.2.4.2**

2.3.20 Statistical analysis

Data are expressed as mean ± standard deviation (SD) for normal distribution or data are expressed as median for non-normal distribution. To determine the normality of the distribution, we applied the Shapiro–Wilk's test. We used two-tailed Student's t-tests for two groups and one-way ANOVA with Tukey post-test for multiple comparisons on normally distributed data. For non-normally distributed data, we used Mann–Whitney U-tests for two groups and Kruskal–Walli's test with Dunn's test for multiple comparisons, respectively. p value < 0.05 was considered significant. Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad, San Diego, USA)

3. Results

3.1 Comparison of NPICC cultivation media

Many different media are currently used for the culture of neonatal porcine islets, and this, together with various supplementary factors, has led to a great diversity of media being used. However, there is no conclusive evidence regarding which medium is more appropriate for NPICCs culture. Therefore, we compared the effect of the medium used in our group with four other media that were described for the culture of NPICCs in other publications. After a 5-day incubation period post-isolation NPICCs were harvested to detect the expression of *Insulin*, *PDX1*, *Glucagon* and *MafA* genes. NPICCs cultured in basal medium had significantly higher *Insulin* and *PDX1* mRNA levels compared to cells in Ham's F10 medium supplemented with IBMX and the RPMI 1640 medium supplemented with BSA. NPICCs cultivated in Ham's F10 medium with HSA or RPMI 1640 with IBMX had comparable gene expression levels of *Insulin*, *PDX1* and *MafA*. The above results indicated that the combination of RMPI 1640 medium plus HSA and exenidin-4 is effective for *in vitro* culture of NPICCs and other media based on Ham's F10 medium or supplementation with IBMX or BSA are not superior with respect to the yield of pancreatic endocrine cells.

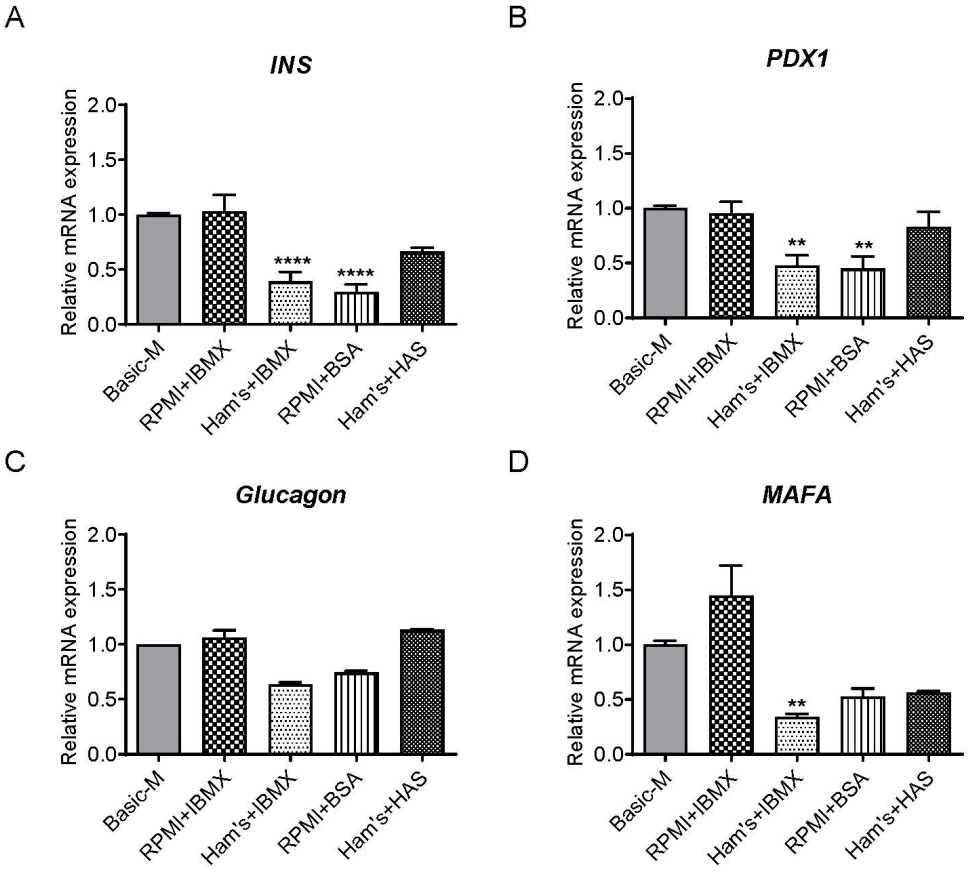


Figure 6. Comparison of different NPICC cultivation media. NPICCs were cultured in five different media (Basic-M; RPMI + IBMX; Ham's + IBMX; RPMI + BSA; Ham's + HSA) for 5 days. Relative gene expression of *insulin* (*INS*) (**A**), *pancreatic and duodenal homeobox 1* (*PDX1*) (**B**), *glucagon* (*GCG*) (**C**) and *MAF bZIP transcription factor A* (*MafA*) (**D**) assessed by RT-qPCR. Data are presented as mean ± SD of three to six independent experiments. $^{**}p < 0.01$ and $^{****}p < 0.0001$ vs. NPICCs cultivated in basic medium.

3.2 The effect of SCFAs on islet yield and islet viability

To observe the effect of SCFAs on islet yield, islets were counted at the third day after isolation and IEQ was calculated. Islets were divided into four groups (basic medium, basic medium supplemented with 1000 µM acetate, propionate and butyrate) and the IEQ was calculated again after further three days. At baseline (day 3 after isolation) the NPICC count, determined as IEQ per g of pancreas (IEQ/g), was 15,880 ± 3315 (n = 9). On day 6, the islet yield was standardized based on the basal medium group, which was 94.5 ± 7.6% for the acetate group, 87.7 ±

6.3% for the propionate group, and 94.8 ± 4.8% for the butyrate group (**Table 15**). The result suggested that treatment with SCFAs did not negatively affect NPICC yield compared to cells cultured in basal medium. In addition, the live/dead assay revealed that treatment with 1000 µM acetate (89.2 ± 2.5%), 1000 µM propionate (90.8 ± 3.0%) and 1000 µM butyrate (91.7 ± 1.7%) had no effect on cell viability as compared to basal conditions (93.1± 2.2%).

Table 15. Islet yield per gram of pancreas after 3 days of culture in either basic media or basic medium with 1000 µM SCFAs.

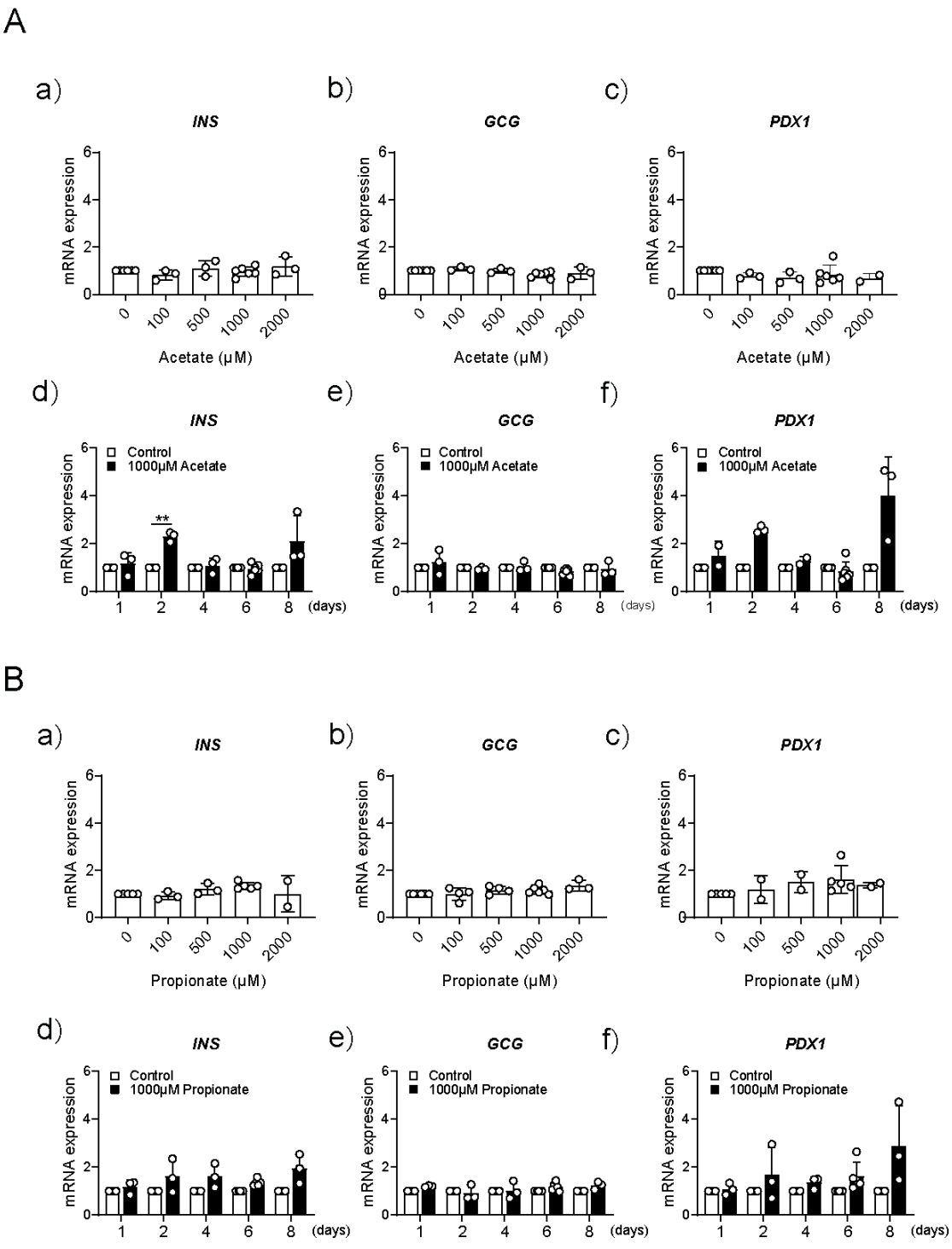
IEQ / g (Normalized to NPICCs cultured in basic medium)	**Day 6**
Acetate	94.5 ± 7.6%
Propionate	87.7 ± 6.3%
Butyrate	94.8 ± 4.8%

IEQ/g: Islet equivalent per gram of pancreatic tissue. Data are presented as mean ± SD.

3.3 The role of SCFAs on beta cell maturation

To investigate the potential effect of SCFAs on beta cells maturation, we cultured NPICCs in basic culture medium with 0, 10, 100, 500, 1000, 2000 µM acetate, butyrate, and propionate for different times (1, 2, 4, 6 and 8 days). **Figure 7A** illustrated that 1000 µM acetate had a minor influence on *insulin* gene expression. Only after 2 days of treatment, there was a significant increase in *insulin*, and a slight rise of *PDX1* gene expression. Treatment with propionate had no significant effect on *insulin*, *glucagon*, or *PDX1* expression. At 2, 4, and 6 days after treatment with propionate at 1000 µM concentration, there was only a slight tendency of increase in *insulin* and *PDX1* gene expression (**Figure 7B**). As shown in **Figure 7C**, butyrate results in a strong time- and dose-dependent upregulation of *insulin* gene expression. Specially, treatment with 1000 µM butyrate for 6 days also led to a significant upregulation of *glucagon* and *PDX1* compared to the untreated group (**Fig 7. C b, c and e, f**). These results demonstrate that butyrate

strongly increases *insulin* gene expression in a dose- and time-dependent manner. Therefore, we selected butyrate in a concentration of 1000 µM and a culture time of 6 days as the optimal conditions for subsequent experiments.



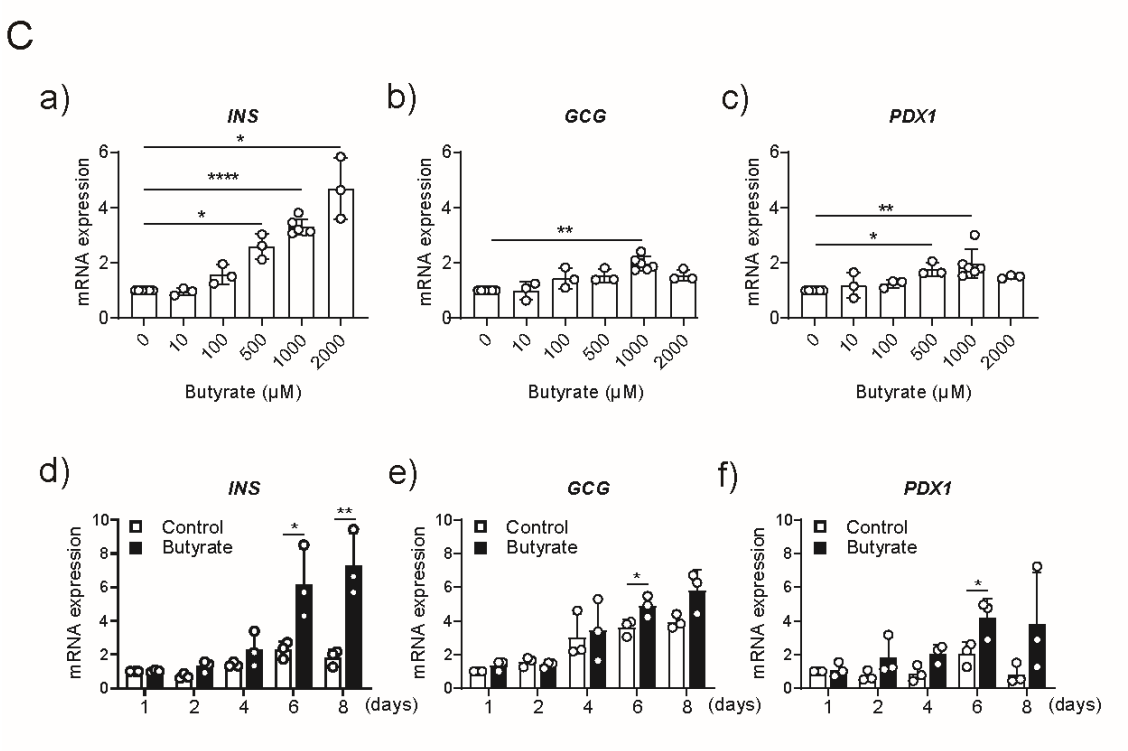


Figure 7. The effect of SCFAs on beta cell maturation of NPICCs. Acetate and propionate have minor and not consistent effects on expression of proendocrine genes, while butyrate significantly promotes beta cell maturation in a concentration- and time-dependent manner. **A**: Relative gene expression of *insulin* (*INS*) (a, d), *glucagon* (*GCG*) (b, e), *pancreatic and duodenal homeobox 1* (*PDX1*) (c, f) after treatment with 10–2000 µM acetate for 6 days (a, b, c) or 1000 µM acetate for one to eight days (d, e, f), assessed by RT-qPCR. **B**: Relative gene expression of *insulin* (*INS*) (a, d), *glucagon* (*GCG*) (b, e), *pancreatic and duodenal homeobox 1* (*PDX1*) (c, f). NPICCs were treated with 10–2000 µM propionate for six days (a, b, c) or 1000 µM propionate for one to eight days (d, e, f). **C**: Relative gene expression of *insulin* (*INS*) (a, d), *glucagon* (*GCG*) (b, e), *pancreatic and duodenal homeobox 1* (*PDX1*) (c, f). NPICCs were treated with 10–2000 µM butyrate for six days (a, b, c) or 1000 µM butyrate for one to eight days (d, e, f). Cells incubated without SCFAs were used as controls. Data are presented as mean ± SD of three to six independent experiments. *p < 0.05, **p < 0.01, and ****p < 0.0001 vs. control groups.

3.4 Butyrate enhances insulin protein expression

To further explore the effect of butyrate on islet maturation, insulin, glucagon, somatostatin, PDX1 and Nkx6.1 protein expression were analyzed by FACS on day 3 post-isolation (before butyrate treatment) and after exposure of NPICCs to

1000 µM butyrate for 6 days. As shown in **Figure 8**, butyrate treatment significantly increased the number of insulin-positive cells (43.8 ± 4.7% vs 33.1 ± 3.0%) and median fluorescence intensity of insulin-positive cells (100111.2 ± 26020.4 vs 53718.4 ± 17089.2) ($p < 0.01$).

The numbers of glucagon-positive cells (12.8 ± 4.9% vs 19.0 ± 7.0%), somatostatin-positive cells (10.1 ± 1.0% vs 10.0 ± 2.7%), PDX1-positive cells (39.3 ± 6.1% vs 43.2 ± 9.1%) and Nkx6.1-positive cells (43.7 ± 9.1% vs 44.5 ± 7.6) were not significantly different. However, the median fluorescence intensity (MFI) of PDX1-positive cells was higher than that of the control group (34179.8 ± 2202.5 vs 28511.5 ± 820.1).

In addition, a higher proportion of mature cells, defined by co-staining of insulin+ and Nkx6.1+, were observed in the butyrate treated group as compared to cells cultured in B-IC medium (33.4 ± 8.3% vs 21.0 ± 8.0%).

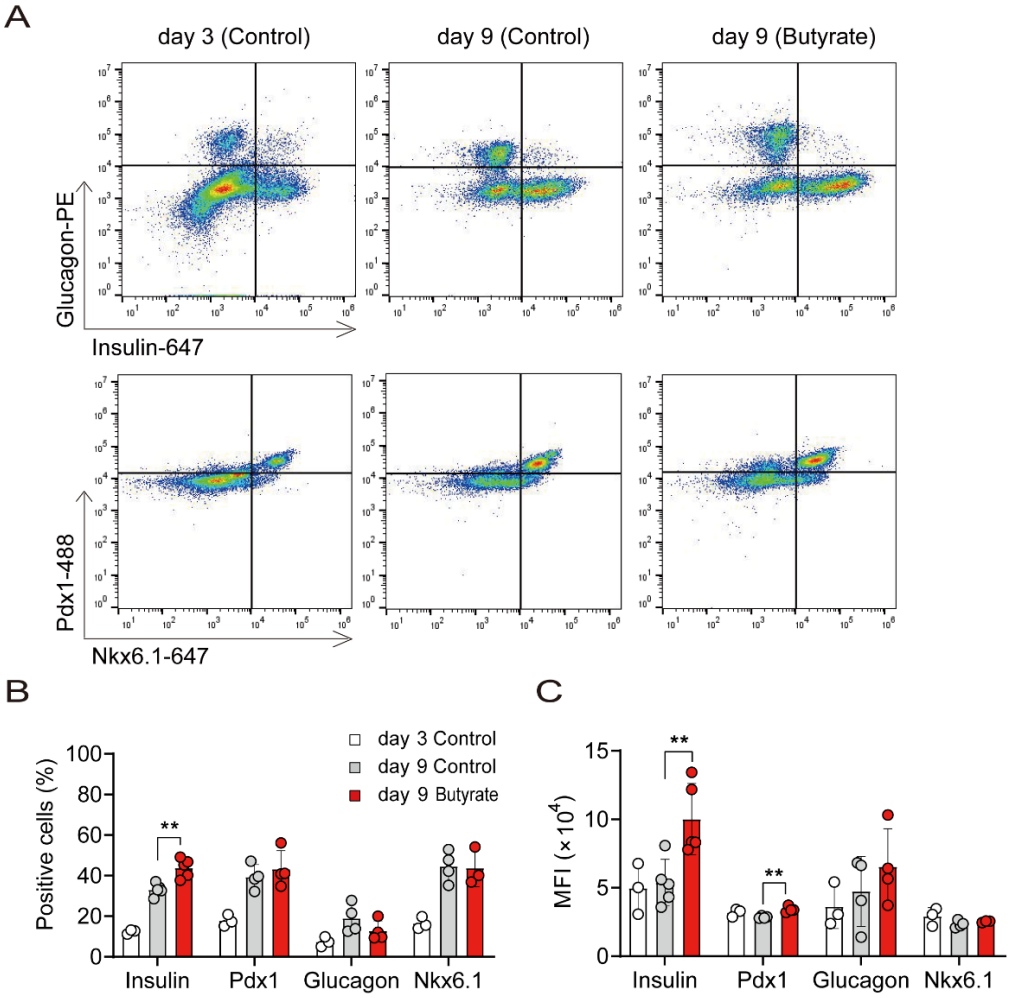


Figure 8. Butyrate increases insulin and glucagon expression. Flow cytometry analysis of insulin (AF647) and glucagon (PE) expression in NPICCs before and after treatment with butyrate (1000 µM) for 6 days. **B, C:** Butyrate increased the number of insulin positive cells and the median fluorescence intensity (MFI) of insulin-stained cells. Data are presented as mean ± S.D. (**B**) or median ± S.D. (**C**) of 3-5 independent NPICCs preparations. **p < 0.01 vs. control group.

To further confirm these results immunofluorescence staining was performed using NPICCs treated with or without butyrate. As shown in **Figure 9**, a significant higher percentage of in insulin-positive cells was observed (**Figure 9 A-B**) ($p < 0.01$).

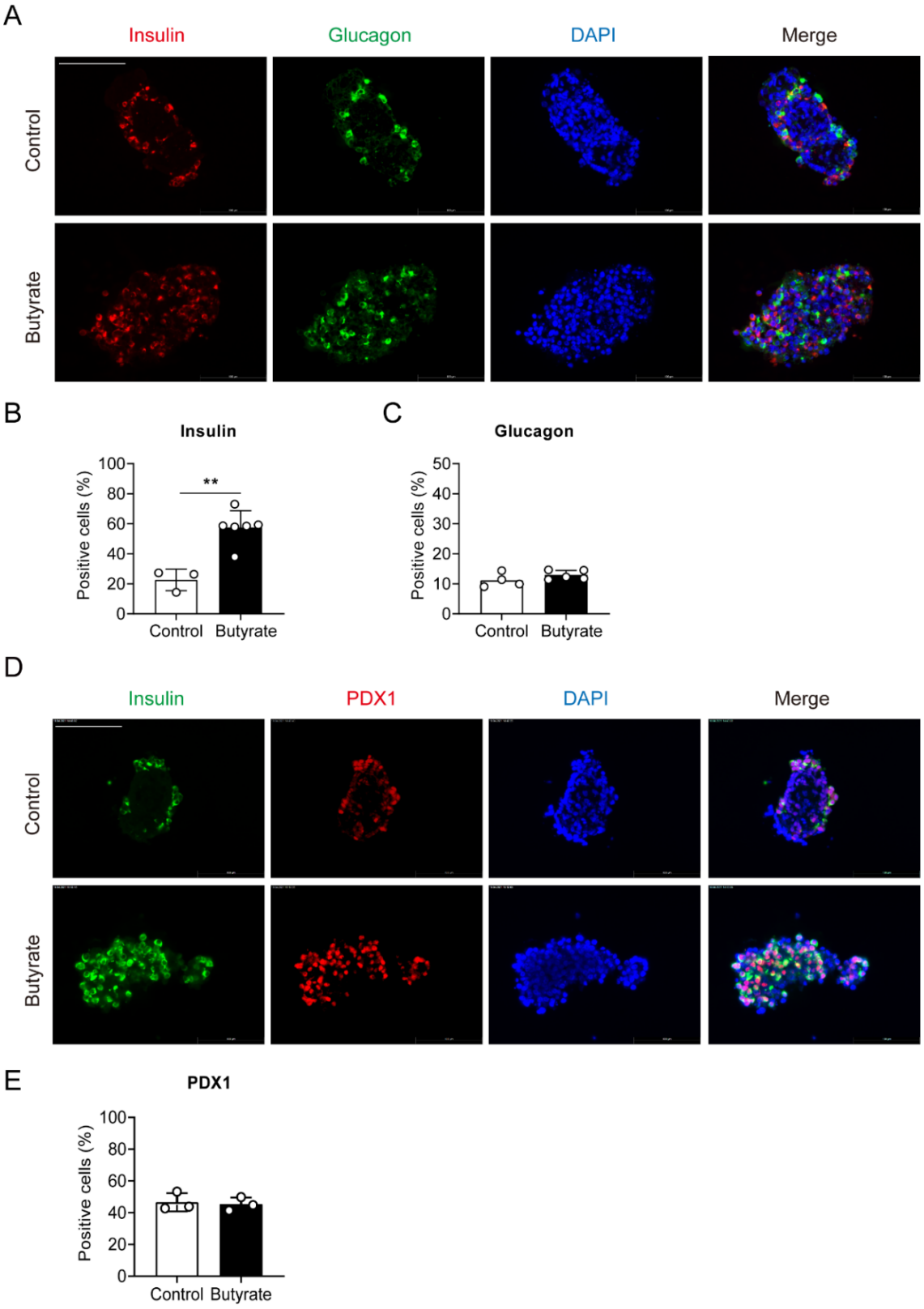


Figure 9. Butyrate increases insulin protein expression in NPICCs. A: Immunofluorescence staining for insulin (red) and glucagon (green) with / without 1000 µM butyrate treatment for 6

days; nuclei are stained with DAPI (blue); scale bar 100 µm. **B-C**: Statistical analysis of percentage of insulin (**B**) or glucagon positive cells detected by immunofluorescence staining (**C**). **D**: Immunofluorescence analysis for insulin (green) and PDX1 (red) with / without 1000 µM butyrate treatment for 6 days; nuclei are stained with DAPI (blue); scale bar 100 µm. **E:** Percentage of PDX1 positive cells in NPICCs cultured in basic medium (open bar) and butyrate-containing medium (black bar). Data are presented as mean ± S.D. (**B**, **C**, **E**) of 3-5 independent NPICCs preparations. **p < 0.01 vs. control group.

3.5 Butyrate has the potential to promote pancreatic beta cell differentiation

Along with insulin expression and the expression of pancreatic progenitor marker PDX1, markers of endocrine progenitors (NGN3), functionally immature beta cells (Aldolase B) and mature beta cells (Nkx6.1 and MafA) were investigated. Treatment with butyrate resulted in a decrease of critical markers of progenitor/immature cells (*NGN3* and *ALDOB*). *PDX1*, which is required to maintain beta cell function, was not affected. *Nkx6.1* gene expression was significantly up regulated in islets after butyrate stimulation further confirming the hypothesis that butyrate favors the differentiation of NPICCs to a beta cell phenotype. *MafA* expression, which indicates mature beta cell identity, was not significantly altered suggesting that butyrate treatment is not sufficient to induce a complete differentiation to mature beta cells.

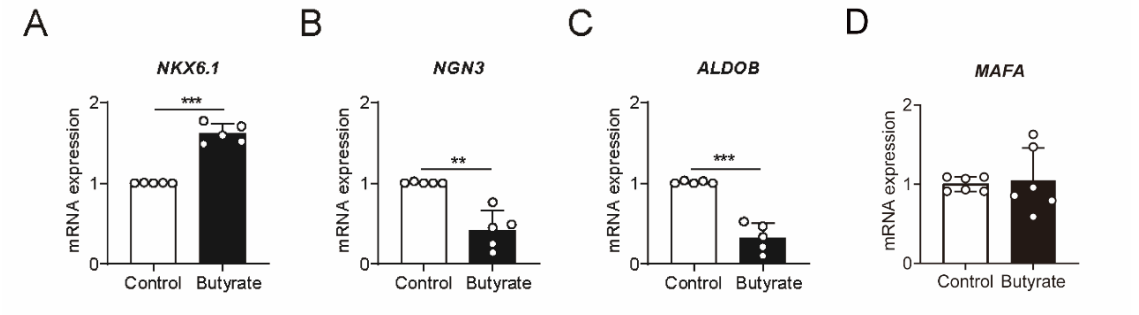


Figure 10. Butyrate increases beta cell differentiation marker expression in NPICCs. A-D: The mRNA expression of NK6 homeobox 1 (*Nkx6.1*) (**A**), neurogenin 3 (*NGN3*) (**B**), aldolase fructose-bisphosphate B (*ALDOB*) (**C**) and MAF bZIP transcription factor A (MafA) (**D**) in NPICCs

after induction of 1000 μM butyrate for 6 days. White bars mean NPICCs maturated in basic medium, and black bars represent Butyrate-containing medium. The data were presented as mean ± S.D. of 3-5 independent NPICCs preparations. **$p < 0.01$ and ***$p < 0.001$ vs. control group.

3.6 Butyrate had no effect on islet cell proliferation

Islet mass is influenced by the proliferation, hypertrophy or apoptosis of existing islet cells and the differentiation of endocrine progenitor cells. We used the WST-1 assay to investigate whether SFACs affect islet proliferation. After incubation with 10-2000 μM acetate, propionate and butyrate, 100 IEQ islets were seeded in triplicates in 96-well plates. The absorbance was measured three hours after the addition of WST-1 reagent. The results showed that treatment with either of the SCFAs did not promote islet cell proliferation (**Figure 11 A-C**).

We additionally investigated islet cell proliferation during butyrate treatment by an Edu assay. Analysis by immunohistochemistry revealed no differences in Edu-positive cells in the presence or absence of butyrate (**Figure 11 D-E**).

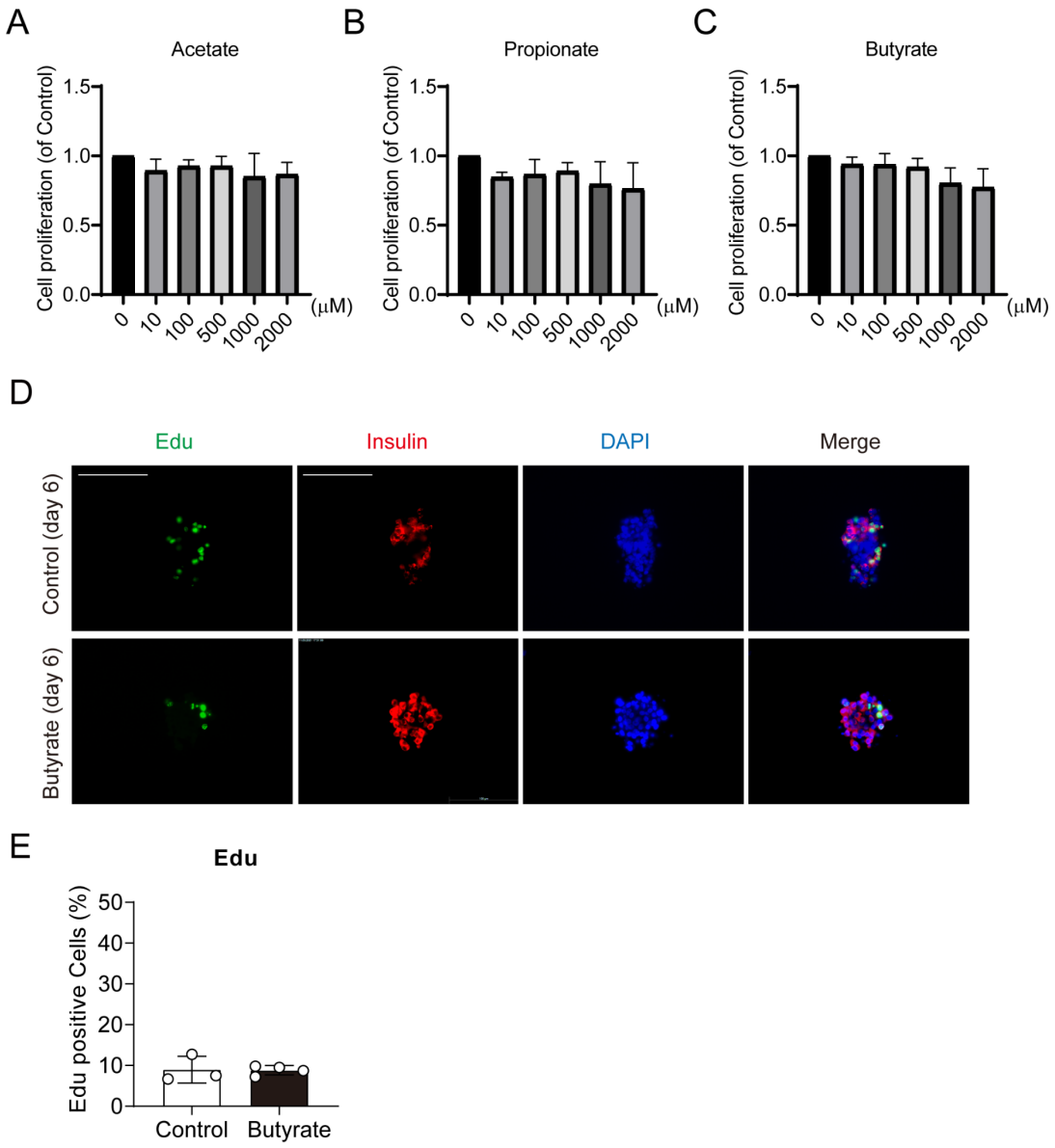


Figure 11. SCFA treatment do not affect beta cell proliferation. A-C: WST-1 assay was performed for cell proliferation of NPICCs after incubation with 0-2000 µM acetate (**A**), propionate (**B**) and butyrate (**C**) for 6 days. **D-E:** The percentage of proliferating cells is not affected by butyrate treatment. (**D**) Immunofluorescence analysis for Edu (green) and insulin (red) after treated with 1000 µM butyrate for 6 days; scale bar 100 µm. (**E**) Quantification of Edu-positive cells as a percentage of the total cell number, after treated with 1000 µM butyrate for 6 days. Data are presented as mean ± SD of three independent experiments.

3.7 The effect of butyrate on islet function and insulin content

Static glucose stimulated insulin release assay was used to evaluate islet function. NPICCs treated with / without 1000 μM butyrate for 6 days were hand-picked and stimulated with low glucose (2.8 mM) or high glucose (28.0 mM). The amount of secreted insulin in each glucose media was normalized to the islets number. Total insulin content (51.7 vs 29.6 μU/ng DNA) and GSIS was only moderately increased from 1.7 to 2.1 when compared to untreated islets (**Figure. 12 A**), indicating that NPICCs still have not acquired physiological glucose response.

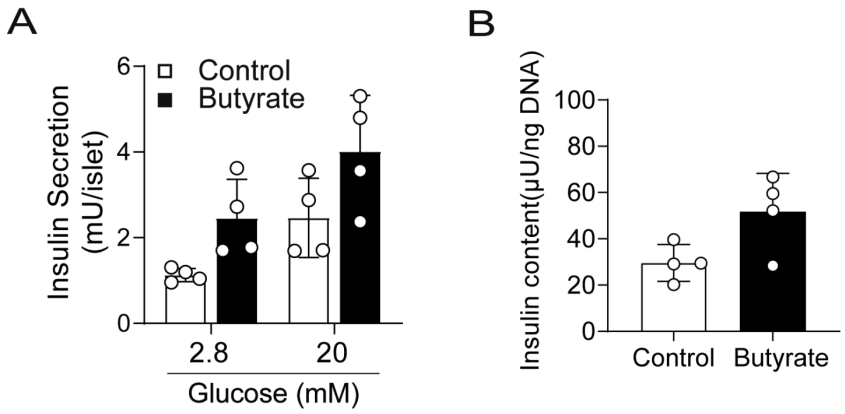


Figure 12. Butyrate marginally improved NPICCs glucose stimulated insulin secretion capacity. **A:** Glucose stimulated insulin secretion is expressed as insulin concentration normalized to the number of islets. **B:** Total insulin content was normalized to DNA content. NPICCs maturated in basic medium (white bars) or butyrate-containing medium (black bars). The data were presented as mean ± S.D. of 3-5 independent NPICCs preparations.

3.8 Butyrate up-regulated insulin expression is independent of binding to G-protein coupled receptors

It is known that butyrate can bind to free fatty acid receptor FFA3 (GPR41) and FFA2 (GPR43) to exert physiological effects in vivo. We examined the expression of these two genes in porcine islets and demonstrated that GPR41 and GPR43 were expressed in NPICCs (**Figure 13 A**).

To further explore the underlying mechanism of butyrate-induced insulin gene and protein expression, we analyzed the involvement of GPCRs. Treatment with

butyrate and specific blockers of GPR41 (β-hydroxybutyrate) or GPR43 (GLPG0974) did not significantly reduce the upregulation of insulin expression (**Figure 13 B**). The islets were then treated with AR420626, a selective agonist of FFAR3. Results in **Figure 13 C** showed AR420626 also did not alter gene expression of *insulin.* These data suggest that the effect of butyrate on NPICCs is independent of fatty acids receptor activation.

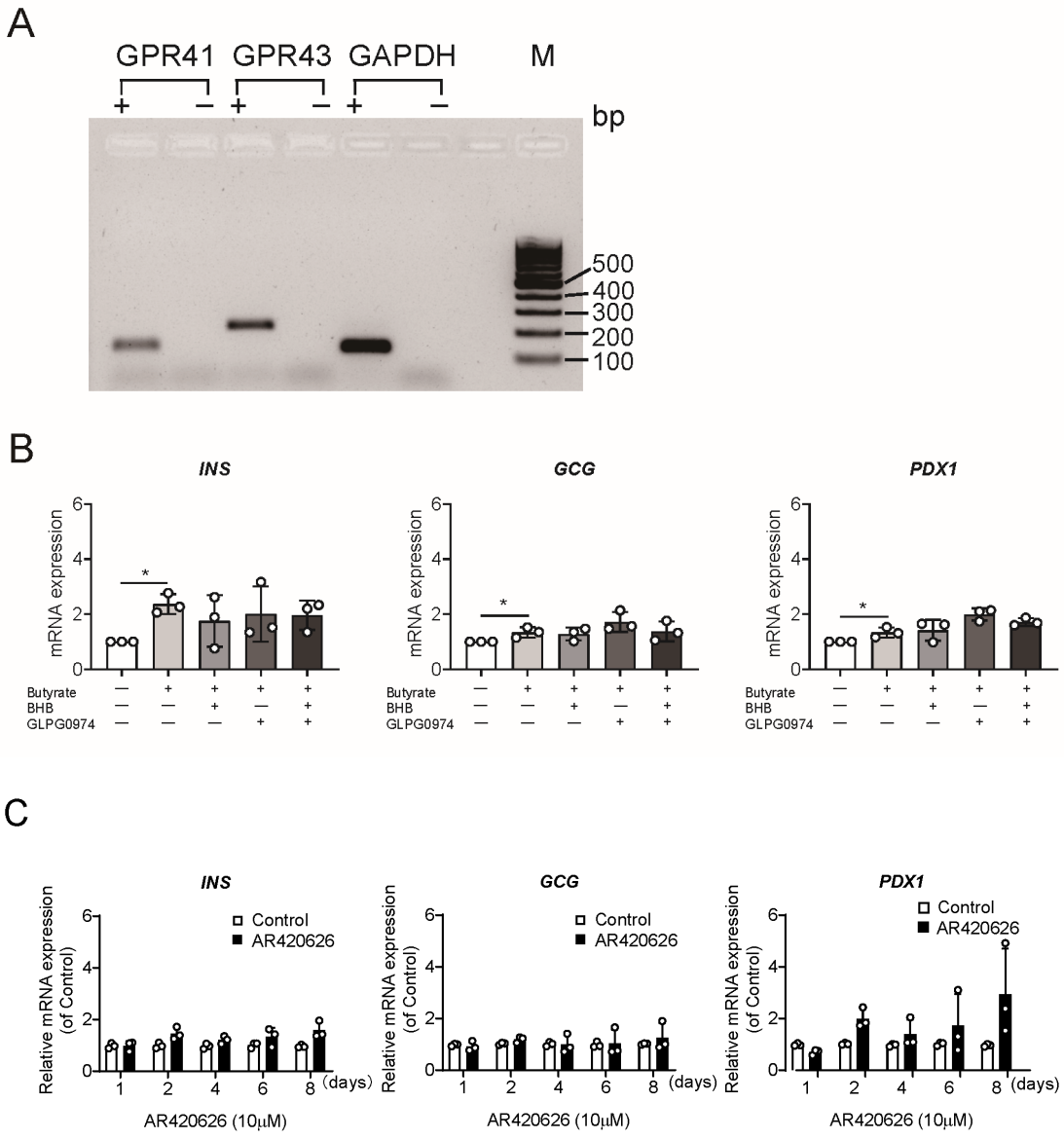


Figure 13. Antagonists of short-chain fatty acid receptors and GPR41 agonist did not significantly affect gene expression in NPICCs. **A:** The expression of GPR41 and GPR 43 was assessed by PCR and Agarose gel electrophoresis showing PCR amplification product (+) of *GPR41* (lane1, 2), *GPR43* (lane3, 4), *GAPDH* (lane5, 6). Reverse transcriptase-minus (−) samples were included as controls. **B:** NPICCs were cultured in basic medium or in medium containing butyrate (1000 μM, 6 days), butyrate + β-hydroxybutyrate (butyrate 1000 μM + BHB 5 mM),

or butyrate (1000 µM) + GLPG0974 (200 nM), as indicated. The mRNA expression level of *INS*, *GCG* and *PDX1* were examined by qRT-PCR and value were expressed as ploidy changes and calculated by dividing the expression level of the stimulated group by the basic medium control. **C:** The expression of *INS*, *PDX1* and *GCG* after treatment of 10 µM AR420626 from different time points were assessed by qRT-PCR. The data were presented as mean ± S.D. of 3 independent experiments. *p < 0.05 vs. basic medium control group.

3.9 Butyrate triggers insulin transcription via inhibition of HDAC

Since butyrate is considered to be an HDAC inhibitor (HDACi), we first tested the ability of butyrate to inhibit HDAC activity using a cell-free HDAC activity kit. We then tested HDAC activity in butyrate treated NPICC using a fluorescent in vitro enzyme assay with the non-specific HDAC inhibitor TSA as a positive control. The results showed that 500 µM, 1000 µM and 2000 µM butyrate significantly reduced HDAC enzyme activity in the cell-free assay in a dose-dependent manner (**Figure 14 A**) and that butyrate inhibited class I HDAC activity in NPICCs (**Figure 14 B**).

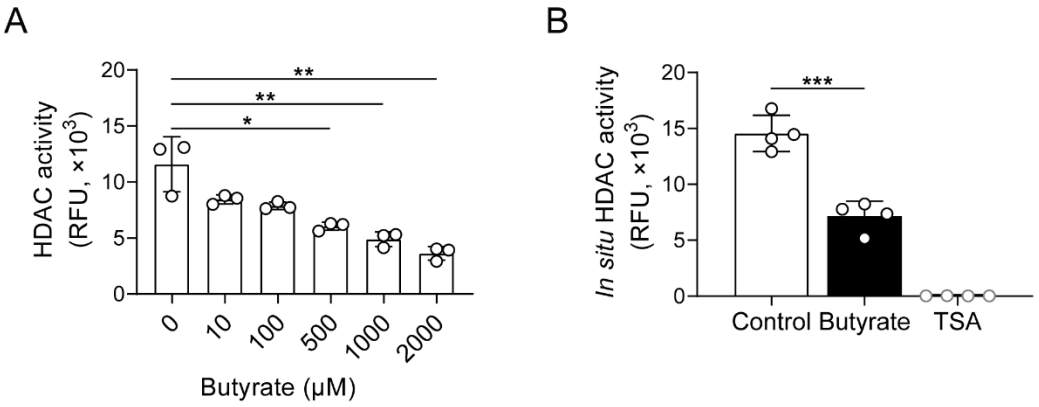


Figure 14. Butyrate inhibits HDAC activity in NPICCs. A: HDAC classic I activity in nuclear extraction incubate with butyrate in different concentrations. **B:** HDAC activity in NPICCs in the presence of butyrate or trichostatin A (as a positive control). The data were presented as mean ± S.D. of 3-5 independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001 vs. basic medium control group.

To further elucidate which HDAC enzymes may induce proendocrine effects, we tested several additional specific HDACis, including the class I inhibitors MS275 and mocetinostat, class IIa inhibitors MC1568 and TMP269, and the unspecific HDAC inhibitor trichostatin A. Stimulation of NPICC with mocetinostat resulted in a highly significant upregulation of *insulin* gene expression, which was similar to the effect of butyrate. Trichostatin A and MS275 moderately increased insulin expression levels whereas MC1568 and TMP269 had no effect (**Figure 15 C**), providing evidence that class I HDAC inhibition enhances beta cell differentiation in NPICCs. These findings suggest that butyrate may induce the maturation of neonatal pig islets toward the beta cell phenotype through its HDAC inhibitory activity.

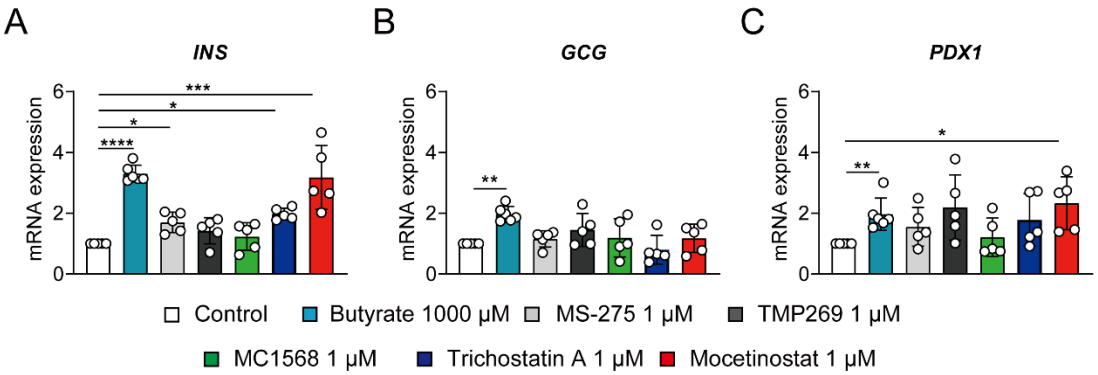


Figure 15. Butyrate improves beta cells maturation by inhibiting class I HDAC activity. A-C: The mRNA expression of *INS* (**A**)*, GCG* (**B**) and *PDX1* (**C**) in NPICCs with basic medium or in medium containing butyrate or different HDAC inhibitors: MS275 (1 µM, selective class I inhibitor), TMP269 (1 mM, selective class II inhibitor), MC1568 (1 µM, selective class II inhibitor), mocetinostat (1 µM, selective class I inhibitor) and trichostatin A (1 µM, TSA, unspecific inhibitor) for 6 days, as indicated. The mRNA expression level was expressed as a fold change of control group. The data were presented as mean ± S.D. of 3-5 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.001$ vs. basic medium control group.

The above results show that treatment with HDAC class I inhibitors increased *insulin* gene expression in NPICCs. To further investigate which HDAC class I isoforms is involved in the regulation of beta cell differentiation, we treated NPICCs with Romidepsin (FK228, 10 nM), a potent inhibitor of HDAC1 and

HDAC2, the selective HDAC2 inhibitor Santacruzamate A (1 μM) and the selective HDAC3 inhibitor RGFP966 (1 μM) for 6 days. Then the expression of *insulin*, *PDX1* and *glucagon* genes in NPICCs was examined. FK228 significantly increased *INS* mRNA expression by 3.5-fold as compared to controls. The HDAC3 inhibitor RGFP966 significantly up-regulated the expression of *insulin* by 1,5-fold and *glucagon* by 1.5-fold, respectively. Santacruzamate A did not change *insulin* gene expression. None of these inhibitors had a significant effect on *PDX1* gene expression.

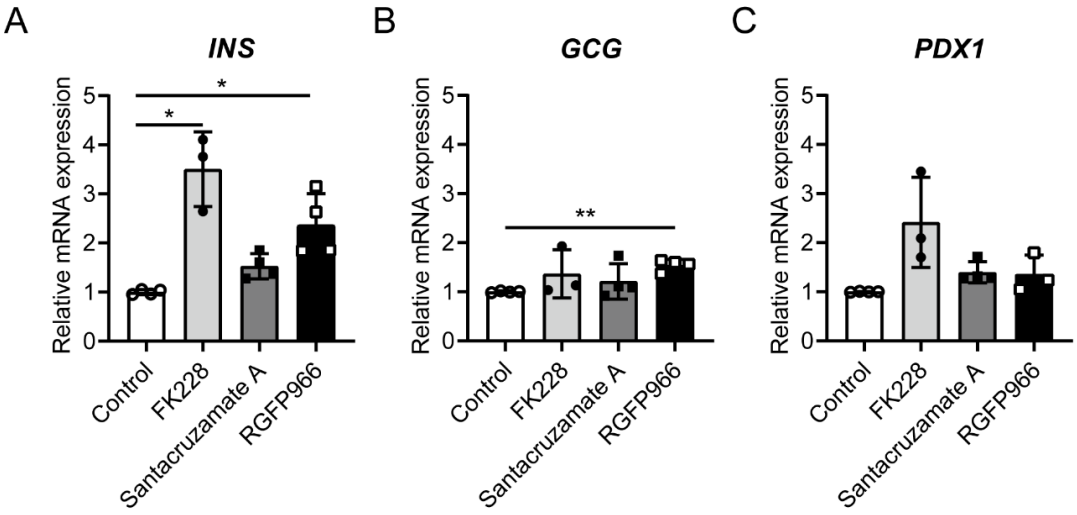


Figure 16. Simultaneous inhibition of HDAC1 and 2 and selective inhibition of HDAC3 activity promotes beta cells maturation. **A-C**: mRNA expression of *INS* (**A**), *GCG* (**B**) and *PDX1* (**C**) in after treatment with HDAC class I inhibitors: FK228 (10 nM, selective HDAC1 and 2 inhibitor), Santacruzamate A (1 μM, selective HDAC2 inhibitor), RGFP996 (1 μM, HDAC3 inhibitor) for 6 days. The mRNA expression levels were expressed as fold change from the control group. Data are presented as mean ± standard deviation. 3-4 independent experiments. *p < 0.05, **p < 0.01 vs. basic medium control group.

4. Discussion

Cellular replacement is considered as the only therapy, which has the capacity to physiologically restore glucose metabolism and cure T1DM. The shortage of organ donors and side effects of the required systemic immunosuppression limit its success. Transplantation of porcine islets has the potential to overcome the organ shortage as shown by the results of porcine islet transplantation in diabetic monkeys, which achieved long-lasting islet survival with near-physiological control of blood glucose [43, 128]. The challenges for the translation in the clinic are to reduce immunogenicity of the islets by genetic modifications and the development of islets isolation protocols to provide high numbers of high quality porcine islets.

4.1 NPICCs culture and treatment with SCFAs

Due to the better standardization and reproducibility of the islet isolation, the cost-effectiveness and the pre- and posttransplant cell robustness, there is consensus among most researcher that neonatal or very young pigs may be the better organ donors as compared to adult pigs [129-131]. The limitations of neonatal islet-like clusters (NPICCs) are their relative immaturity upon isolation and the lower islet and beta cells number leading to a delayed reversal of hyperglycemia after transplantation and the need to use more donor animals for the treatment of one recipient, respectively. To overcome these problems several studies investigated different media and various supplements. Results of these studies were inconsistent with respect to the NPICCs yield, viability and *in vitro* beta cell maturation. In the present study, we demonstrate that the addition of butyrate to established culture media strongly increased beta cell mass and induced beta cell maturation in isolated NPICCs suggesting that it may be a valuable novel supplement to generate an improved cell product for transplantation.

One advantage for the use of neonatal porcine pancreas is the simple isolation procedure. After digestion of the pancreas with collagenase, the cell suspension includes single cells and small cell clusters composed of exocrine, ductal and endocrine cells. During the first *in vitro* cultivation days pancreatic exocrine cells

spontaneously die whereas endocrine and endocrine progenitor cells form cell clusters and further differentiate into endocrine cells. The resulting islet beta cell mass depends on the balance of cell proliferation, differentiation and loss due to apoptosis. If the culture time is short, the beta cells cannot mature, and if the culture time is too long, a large number of islet cells are lost.

The current porcine islet cell culture protocols are mainly based on serum-free cultivation in Ham's F10, DMEM or RPMI 1640 media supplemented with nicotinamide and BSA or HSA together with IBMX or exendin-4. Since few studies directly compared different media, the present study investigated the influence of the source of albumin and the addition of IBMX versus exendin-4 in media based on Ham's F10 and RPMI 1640 with 10 mM nicotinamide. Nicotinamide was added to all media because many studies consistently reported on pro-endocrine *in vitro* effects in isolated NPICCs, fetal pancreatic cells, human pluripotent stem cells as wells as adult rodent and human islets [85, 132-134].

In the present study, a trend towards a higher *insulin* gene expression was observed in NPICCs cultured in RPMI 1640 medium compared to islets cultured in Ham's F10 medium when other components were the same. This is in line with a study comparing the effects of different islet cultures such as TCM 199, RPMI 1640, CMRL 1066, MEM, and Ham's F10 on the quality and functional maturity of islets. Islet cells cultured in Ham's F10 had the highest insulin content, while that the RPMI 1640 cultured islet cells had the highest rate of insulin synthesis [133]. We observed that HSA was superior to BSA and exendin-4 was equal to IBMX. Exendin-4 is a long-acting analogue of GLP-1. In a previous study exendin-4 was described to improve islet yield and enhance insulin secretion of NPICCs after glucose stimulation [135, 136]. Although HSA and exendin-4 are more expensive as compared to BSA and IBMX, use of media supplemented with HSA and exendin-4 would allow GMP-conform islet isolation with respect to the transfer of xenotransplantation to clinical application. Our findings confirm that the combination of RPMI 1640 plus nicotinamide, exendin-4 and HSA is very appropriate for the culture of NPICCs and can be used as basic culture medium for NPICCs.

With the currently used basic medium only 20-25% of cells in NPICCs obtained at day 5 after isolation are insulin positive. Therefore, a screening for novel supplements was performed to optimize *in vitro* maturation and differentiation of NPICCs. Numerous studies demonstrated that SCFAs affect metabolism at different levels including direct binding to Langerhans´ islet cells. We here investigated changes of *insulin*, *glucagon* and *PDX-1* gene expression after exposure of NPICCs to 10-2000 µM acetate, propionate and butyrate for 1-8 days. Hormonal markers insulin, glucagon and somatostatin are widely used to specifically define pancreatic beta, alpha and delta cells, respectively. The transcription factor Pdx-1 is one of the earliest cell markers in pancreas development. It is expressed in beta cell progenitor cells, mature beta cells and plays a critical role for the development and function of pancreatic beta cells [137, 138]. There was a strong time- and concentration-dependent up-regulation of *insulin* gene expression in NPICCs treated with butyrate. Treatment with acetate and propionate has significant lower and only marginal influence on gene expression indicating a specific effect of butyrate. Peak expression of insulin, glucagon and Pdx-1 was observed at a concentration of 1000 µM butyrate and an incubation time between 6-8 days. Butyrate at a concentration of 1000 µM is higher than the physiological serum concentration, but is clearly in the non-toxic range. SCFAs are produced in the colon as end-products of dietary fiber fermentation by gut microbes (colon concentration 20-140 mM) [139]. Dependent on the diet a butyrate concentration of 50-140 µM was measured in the portal vein and a concentration between 4-8 µM and 9-25 µM has been described in human and porcine peripheral blood, respectively [140, 141]. The butyrate-induced beta cell maturation was significantly higher than the well-known spontaneous rise in the proportion of alpha and beta cells reported from NPICC cultures between day 3 to day 9 [68, 80, 85, 142]. In agreement with the hypothesis that butyrate may induce endocrine differentiation we also observed an increased expression of the Nkx6.1, which is expressed in beta cell progenitor cells and mature beta cells [143, 144]. Interestingly, butyrate-mediated maturation was accompanied by a down-regulation of NGN3, a transcription factor known to be expressed in endocrine progenitor cells, which possess the capacity to differentiate into four distinct islet endocrine cell types: beta, alpha, delta and PP cells [145]. In addition, gene expression of the enzyme

AldoB, a marker for immature beta cells, which is suppressed in mature and stressed beta cells, was decreased after butyrate treatment [146]. These data are in agreement with previous studies which demonstrated that butyrate increased *glucagon* and *insulin* gene expression in rat insulinoma (RIN) cells and in rat and human islet [147-149].

Flow cytometry using cells from dissociated NPICCs stained for insulin, glucagon, somatostatin, PDX1 and Nkx6.1 confirmed the butyrate mediated increase in the number of beta and alpha cells after several days of *in vitro* culture. NPICCs had roughly 65% hormone-positive islet cells, with about 43% insulin-, 12% glucagon-, and 10% somatostatin-producing cells after butyrate therapy. Among these cells about 33% were matured beta cells defined by co-expression of insulin and Nkx6.1 and only very few cells were double-hormone positive (< 1.5%). A two to threefold increase of insulin expression with 23% insulin positive cells was reported in porcine fetal cell clusters cultured for one week in medium supplemented with 10% human serum with either nicotinamide or butyrate [68]. Lopez-Avalos described a three to fivefold increased insulin content and threefold increased *insulin* gene expression in hydrogel encapsulated NPICCs after 14 days incubation in medium with 10% fetal calf serum plus 10 nM IGF-1, plus/minus nicotinamide and 500 µM butyrate [150]. In our NPICC culture we exclude serum supplement, because serum growth factors are not well defined. Serum contains many adhesion factors including fibronectin that promote cell attachment to the matrix. In previous studies of our laboratory serum supplementation leads to an attachment of NPICCs on the plastic surface of the culture dishes followed by a spreading of attached cells and rapid loss of endocrine and beta cell markers (data not shown). A similarly high number of endocrine cells was reported in a recent study, which shows that a 20-days culture period of NPICCs in medium with sequential addition of dexamethasone and oncostatin M, nicotinamide/exendin-4, and TGF-beta1/thrombin generated 42% insulin and 17% glucagon positive cells [80]. Taken together, these data indicate that butyrate is a strong inducer of beta cell differentiation and maturation in NPICCs, which is important for the stepwise refinement of the optimal *in vitro* culture conditions.

There are two possible explanations for the observed increase in the number of beta cells: replication of pre-existing beta cells and/or differentiation of islet precursor and/or ductal-like cells [142, 151]. Beta cell neogenesis is primarily regulated by beta cell proliferation, transdifferentiation from other endocrine cells and precursor cell differentiation [152, 153]. To determine whether butyrate promotes cell proliferation butyrate treated NPICCs were tested by WST assay and Edu labeling. In both assays no difference in the cell proliferation rate was detected as compared to controls. Therefore, the observed increase in beta cell number is most likely explained by ductal/progenitor cell differentiation. To completely answer this issue, lineage tracing investigations of ductal and progenitor cells are necessary.

4.2 The effect of butyrate on insulin secretion and signaling through G protein-coupled receptors

The strong up-regulation of *insulin* gene and protein expression raises the question how butyrate promote this effect. SCFAs including butyrate act on pancreatic cells by binding to two G protein-coupled receptors (GPCR) GPR43 and GPR41, respectively. These two receptors have different affinities in the binding of different SCFA in the following order: GPR41: propionate > butyrate > acetate, GPR43: acetate = propionate > butyrate. Butyrate binds and activates GPR41 and GPR43 with ligand affinity (EC_{50}) of 42 to 158 μM and 28 to 371 μM, respectively [124].

Because it was unknown whether GPCR are localized in NPICCs, we first verified that GPR41 and GPR43 receptors are expressed in neonatal pig islets by RT-PCR. Next, we used specific inhibitors to block binding of butyrate to both receptors. Beta-hydroxybutyrate, a potent antagonist of GPR41 [154] and GLPG0974, a potent and selective GPR43 antagonist [155] did not significantly alter *insulin* gene expression in butyrate treated cells. Treatment with the GPR41 agonist AR420626 confirmed this result. Thus, our results suggest that the butyrate induced increase of insulin expression may not be predominantly mediated by binding to or signaling through one of these receptors.

Although butyrate strongly promoted beta cell differentiation and maturation, surprisingly, glucose-stimulated insulin secretion was only moderately, statistically not differently increased by approximately 1.5-fold. GRP41 and GRP43 promote different effects on GSIS. Mouse knockout and transgene studies highlighted the importance of GPR signaling on beta cell function. GPR43 KO mice exhibited lower beta cell mass from birth through adulthood [156]. Other studies reported that there is no difference in islet morphology and beta cell mass between WT and GPR43 KO mice [123]. GPR43 activation increased beta cell mass via a direct influence on beta cell proliferation and expression of differentiation genes such as *MafA*, *PDX1* and *NeuroD* [157]. In the GPR41 KO model, increased basal and glucose-induced insulin secretion was detected, while islet size, beta cell area and proliferation were significantly decreased. Conversely, an impaired glucose response in the beta cell-specific GPR41 overexpression model was compensated by increasing beta cell area and proliferation [158].

Two GPR43 receptor agonists, 2-butyric acid (SCA15) and 2-propionic acid (SCA14), stimulated islet cell proliferation in mice [156]. GPR43 signaling is mediated through pertussis toxin (PTX)-sensitive Gαi/o and PTX-insensitive Gαq/11 pathway, whereas GPR41 selectively signals through the Gαi/o pathway [159]. Activation of Gαi/o pathway restricted the proliferation of pancreatic beta cells, particularly during the critical perinatal period and inhibited insulin secretion [160]. In contrast, Gαq/11 signaling activation improved beta cell function, enhanced GSIS and triggered an increase in beta cell mass [161, 162]. Thus, butyrate binding to GPCR can transmit stimulatory and inhibitory signals on insulin secretion. Some studies in isolated human and mouse islets showed a stimulation of insulin secretion and a protective effect of SCFAs on beta cells. Pingitore et al. reported that pretreatment of human and mouse islets with 1 mM propionate or acetate significantly stimulated insulin secretion and reduced cell death caused by cytokines and palmitate [163, 164]. Interestingly, butyrate did not substantially promote GSIS in primary isolated human islets. Insulin production from human islets was reduced when treated with selective GPR43 agonists [165]. Incubation with high concentrations of butyrate at 2.5 to 10.0 mM stimulated insulin secretion in isolated sheep islets [166]. In addition, treatment with GPR43 agonists and GPR43 antagonists showed heterogeneous effects on insulin secretion in human

and rodent islets indicating species differences in GPCR signaling or differences in the composition of beta, alpha and delta cells, which can affect beta cell function by paracrine mechanisms [155]. Data on the role of GRP41 and GRP43 on GSIS in adult and neonatal porcine islets are missing. It only can be speculated that butyrate treatment induced the expression of signature genes characterizing the beta cell lineage (*Nkx6.1* and *INS*), but have not achieved a functional maturity with a physiological GSIS or in parallel attenuated GSIS of NPICCs by signaling through GPR43. The hypothesis of a still incompletely matured beta cell population is supported by the fact that *MafA* gene expression, an important marker of mature beta cells, was not up-regulated by butyrate treatment. Further experimental studies with specific blockers of the Gαi/o and Gαq/11 pathways are required to discover how ligation of butyrate to GPCR modulate regulation of insulin secretion in NPICCs.

4.3 Butyrate inhibit HDAC activity to promote islet maturation

One part of the physiological effects of butyrate is epigenetic modification through inhibition of the activity of class I and II histone deacetylases (HDAC), which regulate gene transcription [167]. Class I and class II HDACs are expressed in developing pancreas and in adult human, mouse and rat islets and play an essential role in cell differentiation and regulation of glucose-dependent insulin secretion. It has been shown that HDACs regulate insulin synthesis, insulin resistance, glucose intolerance and inflammation in Type 1 and Type 2 diabetes [168].

In general, histone acetylation increases gene transcription by opening the chromatin structure, whereas histone deacetylation has in most cases the opposite effect by inhibiting gene transcription [113]. Histone hyperacetylation is associated with increased insulin gene transcription by the recruitment and binding of specific transcription factors such as PDX1 to the insulin promotor. Under low glucose concentration PDX1 directly interacts with HDAC1 and HDAC2 causing hypoacetylation of histone H4 and downregulation of insulin gene expression [169, 170].

In vitro treatment of rat embryonic explants with the HDAC inhibitors TSA and butyrate enhanced beta/delta lineage differentiation and increased beta cell mass

[171]. Several studies on embryonic stem (ES) cells have demonstrated that butyrate enhances ES cell differentiation into islet-like clusters that produce insulin and glucagon [172, 173]. Among class I HDACs, HDAC1 silenced beta cell specific PDX1 expression which is associated with impeding beta cell development [174]. HDAC3 induced beta cell apoptosis by enhancing endoplasmic reticulum (ER) stress [175]. *In vivo* studies have reported that intraperitoneal administration of 500 mg/kg/day butyrate for 21 days to streptozotocin-diabetic mice resulted in decreased beta cell apoptosis and increased beta cell proliferation, which was achieved through altered histone acetylation [176]. In the current study, we confirmed that butyrate inhibits histone deacetylase activity in NPICCs by measuring intra-islet HDAC activity. Using class I and class II selective HDAC inhibitors we provide evidence that inhibition of class I HDACs but not class IIa HDACs is associated with an up-regulation of *insulin* gene expression. Treatment with mocetinostat, an HDAC1, 2, 3 and 11 inhibitor with most potent inhibitory activity against HDAC1 followed by HDAC2 and 20-30-fold lower inhibitory activity against HDAC3 and HDAC11, and FK228 (also named romidepsin), an inhibitor with 10-fold preference towards HDAC1 and HDAC2 versus HDAC4 and about 300-fold preference over HDAC6 [177], significantly induced *insulin* gene expression by about 3.5-fold, but does not significantly promote differentiation of alpha and delta cells in NPICC. Thus, some class I HDACs appear to modify specifically histones in the chromatin of the promoter regions of genes involved in porcine beta cell differentiation and maturation. The cell phenotype was very similar to that observed by butyrate treatment. Based on this observation, it is likely that butyrate mainly exerts its pro-endocrine effect by HDAC inhibition.

These novel findings are in contrast to previous studies in fetal rat pancreatic cells reporting treatment of pancreatic explants with butyrate, TSA and MS275, respectively [171]. During in vitro culture up-regulation of *NGN3* gene expression and a strong increase in alpha cells and pancreatic polypeptide (PP)-expressing cells was described [171]. Treatment with selective class II HDACi MC1568 triggered *Pax4* expression and caused increased beta and delta cell development [178]. Consistent with our results, exposure of fetal rat explants to TSA and butyrate strongly promoted the differentiation of insulin-expressing beta cells [171].

These data imply that the regulation of pro-endocrine transcription factor and *insulin* gene expression may differ significantly between rat and porcine pancreatic cells. The present data clearly demonstrate that some class I HDACs, in particular HDAC1 and HDAC2, may be critically involved in the specification of NPICC progenitors towards pancreatic endocrine cells. This opens the door for the development of novel well defined and robust differentiation protocols using specific HDAC inhibitors.

4.4 Limitations

There are some limitations to our study. The exact molecular mechanisms and the signaling pathways involved in butyrate-mediated enhancement of pancreatic endocrine cell differentiation have not been fully explored. It is unknown whether the short-term treatment with butyrate for some days promote a long-lasting effect on the endocrine differentiation status leading to a terminal differentiation and physiological beta cell function. It would be also very interesting to determine how HDACi exert the regulation of the pancreatic endocrine maturation pathway in future studies. Chromatin immunoprecipitation studies are required to determine which promotor regions are modified by HDAC inhibition and which chromatin modifications favor beta cell maturation in NPICCs. Finally, transplantation studies in diabetic SCID-mice are necessary to assess their functionality after transplantation and evaluate whether butyrate- or HDACi-treated NPICCs reduce the time to develop normoglycemia and improve beta cell function *in vivo*. However, experiments covering these research questions go beyond the scope and the objectives of the present thesis.

4.5 Conclusion

In the present study, we investigated the potential of SCFAs to stimulate NPICC development and the maturation of pancreatic beta cells. This study demonstrated that butyrate is a strong beta cell differentiation and maturation trigger in NPICCs. Further studies are required to unravel which factors are needed to induce a terminal differentiation of beta cells to achieve a physiological insulin se-

cretion. Our study highlights the contribution of class I HDAC inhibition in the development of an endocrine cell fate derived from neonatal porcine islets. Modifications of class I HDAC activity may be the major mechanism whereby butyrate favors beta cell maturation. Direct application of class I HDAC inhibitors was identified as novel tool to improve *in vitro* beta cell specification. These findings hold great promise regarding the development of novel, efficient and stable protocols for *in vitro* generation of beta cells from immature progenitor cells and for the development of beta cell replacement therapies.

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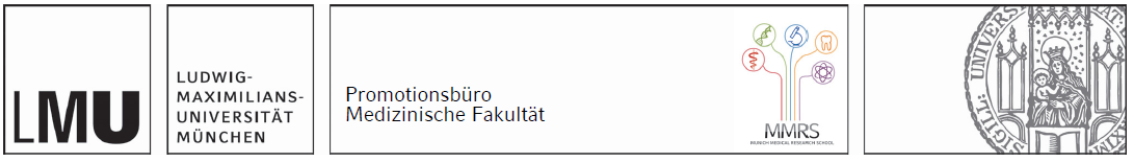
Affidavit



Affidavit

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I hereby declare, that the submitted thesis entitled:

Studies on the effects of short-chain fatty acids on beta cell differentiation and maturation in neonatal porcine islet-like cell clusters

is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

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Yichen Zhang

Signature doctoral candidate

List of publications

Title of the Publication		
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