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Understanding reprogramming of pancreatic islet cells by transcriptional study and chemical biology

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Doctor of Philosophy

Submitted by

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Declaration

Published results and unpublished data submitted for peer-review journals were described in this thesis. The data in this thesis was produced by experimental work at CeMM - Research Center for Molecular Medicine of the Austrian Academy of Sciences (Vienna, Austria) unless specified below.

The first part of the results for this thesis has been published in *EMBO reports*:

Li, J. et al. Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types. EMBO Rep, doi:e201540946 [pii] embr.201540946 [pii]

JL , MF, TP, SK and CB designed the project. CBa and EB from University of Geneva prepared human islets. JL and MF together with AS from Medical University of Vienna performed the experiments. TP and CBo performed the next generation sequencing. JK did the initial data processing. JL and JK did bioinformatics analysis. JL, SK, CBo, MF, and JK wrote of the manuscript.

The second part of the results for this thesis has been submitted to *Cell* and is currently under review:

Li, J. et al. Artemisinins induce alpha to beta cell transdifferentiation by targeting GABA receptor signaling via gephyrin stabilization

JL and SK designed the project. TF and JHS from Novo Nordisk created the cell lines. MC and PC from University of Nice performed animal experiments. JL, KH, AM, AS, MG, KP, PM, JC, GSF and KB performed the chemical proteomics. JL, MF and JK did the single cell RNA-seq. CS and MD from Children Cancer Research Institute in Vienna performed zebrafish larvae assay. JL together IB and SH from Technical University of Vienna did patch clamp assay. JL and CHL did the chemical screening. CBa and EB from University of Geneva prepared human islets. FP

performed the initial RNA-seq analysis. JL and JK performed the bioinformatics analysis. TP and CBo did the next generation sequencing. The rest of the experiments were done by JL, TC and SS. JL and SK wrote the manuscript.

The third part of the results for this thesis has been submitted to *EMBO J* and is currently under revision:

Li, J. et al. Prevention of beta cell dedifferentiation by PAX4 and approved drugs

JL and SK designed the project. TF and JHS from Novo Nordisk made the cell lines. JL together with CS and MD from Children Cancer Research Institute in Vienna performed the zebrafish larvae assay. FP did the initial RNA-seq data processing. IS and CGR from Karolinska Institutet performed the C.elegans assay. TP and CBo did the next generation sequencing. CBa and EB from University of Geneva prepared the human islets. JL and CHL did the chemical screening. JL and SK wrote the manuscript. The rest of the experiments were done by JL and TC.

Contents

List of abbreviations

ADA: American Diabetes Association

HbA1C: Hemoglobin A1c

OGTT: Oral glucose tolerance test

MODY: Maturity onset diabetes of the young

GWAS: Genome-wide association study

HLA: human leukocyte antigen

RNA: Ribonucleic acid

NOD: Non-obese diabetic

BMI: Body mass index

OECD: Organization of Economic Co-operation and Development

SLC30A8: Solute Carrier Family 30 (Zinc Transporter), Member 8

TNF: Tumor necrosis factors

IFN: Interferon

HNF1A: Hepatocyte nuclear factor 1 homeobox A

HNF4A: Hepatocyte nuclear factor 4 alpha

PAX4: Paired box 4

GCK: Glucokinase

PP cells: Pancreatic polypeptide producing cells

Pdx1: Pancreatic And Duodenal Homeobox 1

Neurog3: Neurogenin 3

Mafa: V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog A

Arx: Aristaless Related Homeobox

Mafb: V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog B

Hhex: Hematopoietically Expressed Homeobox

INS: Insulin

GLP-1: Glucagon-like peptide-1

GLP-1R: Glucagon-like peptide-1 receptor

FDA: U.S. Food and Drug Administration

GCG: Glucagon

TRPM4: Transient receptor potential cation channel, subfamily M, member 4

TRPM5: Transient Receptor Potential Cation Channel, Subfamily M, Member 5

GLP1R: Glucagon-like peptide-1 receptor

AMPK: 5' adenosine monophosphate-activated protein kinase

iPS cells: Induced pluripotent stem cells

SGLT2: Sodium/glucose cotransporter 2

NFAT: Nuclear factor of activated T-cells

FoxO1: Forkhead box protein O1

KRAS: Kirsten rat sarcoma viral oncogene homolog

EGF: Epidermal growth factor

CNF: Ciliary neurotrophic factor

RNA-seq: RNA sequencing

RT-qPCR: Reverse transcription-quantitative polymerase chain reaction

Chip-seq: Chromatin immunoprecipitation sequencing

DOX: Doxycycline

GABA: gamma-Aminobutyric acid

UCSC: University of California, Santa Cruz

REST: RE1-Silencing Transcription Factor

LSD1: lysine-specific demethylase 1

GC: Group-Specific Component

Kir6.2: Potassium Channel, Inwardly Rectifying Subfamily J, Member 11

Mnx1: Motor Neuron And Pancreas Homeobox 1

C.elegans: Caenorhabditis elegans

SLC7A2: Solute Carrier Family 7 (Cationic Amino Acid Transporter, Y+ System), Member 2

Abstract

Islet of Langerhans is the center of glucose homeostasis. Damages on islets are the major cause of diabetes. In Type I diabetes, islets are attacked by an auto immune response; in Type II diabetes, islets are hurt by metabolic stress. These damages lead to apoptosis and dedifferentiation of islets, particularly the insulin producing cells (beta cells). In the end diabetic patients suffer from insulin deficiency. To generate new beta cells, either by inducing proliferation of beta cells or reprogramming other cell types into insulin producing cells, is a promising way to overcome the lack of beta cells. The major aims of my PhD study are to identify chemicals which can induce transdifferentiation from alpha cells to beta cells and to understand the mechanism of transdifferentiation with chemical biology approaches. As the preparation work of phenotype based screening, I performed single cell RNA-seq assay on human islet to establish reference transcriptomes for all the cell types in human islet. Beside the achievement of an extra clean transcriptional data set, with this project we confirmed the cell-type specific expression of transcription factor and observed the unique features of human islets compared to mouse islets. With this starting point cell line models were established for chemical screening. On one hand, a beta-to-alpha transdifferentiation cell line model was built with the inducible misexpression of an alpha cell master transcription factor, ARX, in a mouse beta cell line. A reverse chemical screening was done on this cell line and artemisinins, a group of anti-malaria drugs, were selected as the top hits. Artemisinins' transdifferentiation effects were validated in multiple animal models and human islets. Gephyrin and GABA receptor signaling was discovered by chemical proteomics to be the critical factors of artemisinins-induced alpha to beta transdifferentiation. On the other hand, a beta cell dedifferentiation model was built by chemical inhibition of FoxO proteins as a cell line model more relevant for type 2 diabetes. Further transcriptional analysis on

this model highlighted the importance of lysosomal acidification on beta cell dedifferentiation. Chemical screening was applied on this cell model and loperamide was discovered to rescue beta cell dedifferentiation. Mechanistic study on loperamide revealed loperamide increases mobilization of intracellular calcium and enhances FoxO1.The effects of loperamide on FoxO1 were validated by multiple model organisms. In summary, with the work in my PhD study I provided a reference transcriptome of human islets for the community, established two cell line models which are suitable for high-through put screening, discovered two drugs (artemisinins and loperamide) and three signaling pathways (GABA signaling, lysosomal acidification and calcium mobilization) which can exert dramatic effects on islet reprogramming. Beside of helping us understand islet biology better, these results suggest new therapies for diabetes treatment and have the potentials to be translated into clinical applications.

Zusammenfassung

Die Langerhans Inseln der Bauchspeicheldrüse regulieren die Blutzucker homöostase. Störungen sind kausal für Krankheitsbild des Diabetes mellitus: Während dem Typ 1 Diabetes eine Autoimmunantwort zugrunde liegt, wird Typ 2 Diabetes durch metabolischen Stress verursacht. Diese Schädigungen führen zum Zelltod und Dedifferenzierung der Langerhans Inseln ä- im speziellen der Insulin produzierenden Beta-Zellen - und in weiterer Folge zur Insulindefizienz beimPatienten. Die Generation neuer Beta-Zellen - entweder durch Proliferation oder durch ein Reprogrammierung anderer Zelltypen zu Insulin-produzierenden Beta-Zellen- ist ein vielversprechender Weg den Verlust der Beta-Zellen zu kompensieren.

Das Ziel meiner Dissertation ist es, Moleküle zu identifizieren, welche eine Transdifferenzierung von Alpha-Zellen in Beta-Zellen verursachen und den molekularen Mechanismus dieser Transdifferenzierung aufzudecken. Um ein erstes Referenztranskriptom aller vorhandenen Zelltypen in reifen, humanen Langerhans Inseln zu generieren, sequenzierten wir (die RNA) einzelne(r) Zellen und konnten zelltyp-spezifische Expression einzelner Transkriptionsfaktoren und weitere Besonderheiten humaner Langerhans Inseln im Vergleich zur Maus aufzeigen.

Weiters habe ich einein Beta-zu-Alpha-Transdifferenzierungszelllinienmodell mittels induzierter Missexpression des Alpha-Zell spezifischen Transkriptionsfaktors ARX in einer murinen Beta-Zelllinie generiert. In einem chemischen Screen wurden Artemesinine, eine Gruppe Anti-Malaria Medikamente, identifiziert, welche eine Transdifferenzierung verursachen, welche anhand mehrerer Tiermodelle und menschlicher Langerhans Inseln validiert werden konnte. Mittels proteomischer Analysen konnten wir Gephyrin und den GABA Rezeptor Signaltransduktionsweg als kritische Faktoren der Artemesinin-induzierten Alpha-zu-Beta-Zell Transdifferenzierung aufzeigen.

Um ein Typ 2 Diabetes relevantes Zellmodell zu generieren, wurde die Beta-Zell Dedifferenzierung durch pharmakologische Inhibierung der FoxO Proteine durchgeführt. Mittels Genexpressionsanalysen konnten wir zeigen, dass eine lysosomale Azidifizierung bei der Beta-Zell Dedifferenzierung eine wesentliche Rolle spielt. Loperamid, welches ebenso durch einen chemischen Screen identifiziert wurde, konnte die Beta-Zell Dedifferenzierung wiederherstellen. Durch mechanistische Studien konnten wir zeigen, dass Loperamid die intrazelluläres Kalzium erhöht und ebenso FoxO1 Proteine. Diese Effekte von Loperamid auf FoxO1 und die Langerhans Inseln wurden durch mehrere Modelorganismen validiert. Zusammengefasst, (1) erstellten wir ein Referenztranskriptom einzeler Zelltypen humaner Langerhans Inseln, (2) etablierten wir zwei Zelllinienmodell, welche für Screens geeignet sind. (3) Wir entdeckten zwei Molekülgruppen (Artemisinine und Loperamide) und (4) drei Signaltransduktionswege ((i) GABA Rezeptor Signaltransduktionsweg, (ii) lysosomale Azidifizierung und (iii) intrazelluläre Kalzium Mobilisierung), welche für die Reprogrammierung kausal sind. Die vorgelegten Ergebnisse zeigen neue, molekulare Mechansimen auf, welche es erlauben werden neuartige Therapien zur Behandlung von Diabetes mellitus in die Klinik zu bringen.

Introduction

Diabetes: a metabolic disease

Diabetes is a metabolic disorder characterized by dysfunction of glucose homeostasis. According to the latest guideline from ADA [1], its diagnosis needs to fulfill one of the following four criteria:

1. HbA1C* > or = 6.5%
2. Fasting plasma glucose concentration > or =7.0 mM
3. Plasma glucose concentration > or = 11.1 mM 2 hours after OGTT
4. Random plasma glucose concentration> or = 11.1 mM

* *HbA1C is a form of hemoglobin. It stands for the prolonged average plasma glucose concentration. To measure it is a better assay than to measure plasma glucose concentration.*

Despite of the relatively straightforward diagnostic criteria, diabetes is a rather complicated disease. Like cancer or cardiovascular disease, the causative events of diabetes are mixture of genetic mutations and environmental factors. Based on the causes diabetic patients are divided by Type I (auto-immune attacks to insulin-producing cell) and Type II (decreased insulin sensitivity of hepatocytes, adipocytes and muscular cells). Beside these two types, diabetes can also be caused by inheritable deficiency or dysfunction of insulin producing cells, normally called MODY.

Type I diabetes is widely believed as an auto-immune disease. The detection of auto-antibody against beta cells and insulin deficiency soon after birth is its diagnostic criteria. Actually, between these two stages there is a lag which is usually called as “honeymoon” when the children can still live without insulin therapy[2] (**Fig. 1**). The disease process of Type I diabetes is not fully understood yet. Although it related to a lot of genetic components, recent GWAS data suggests

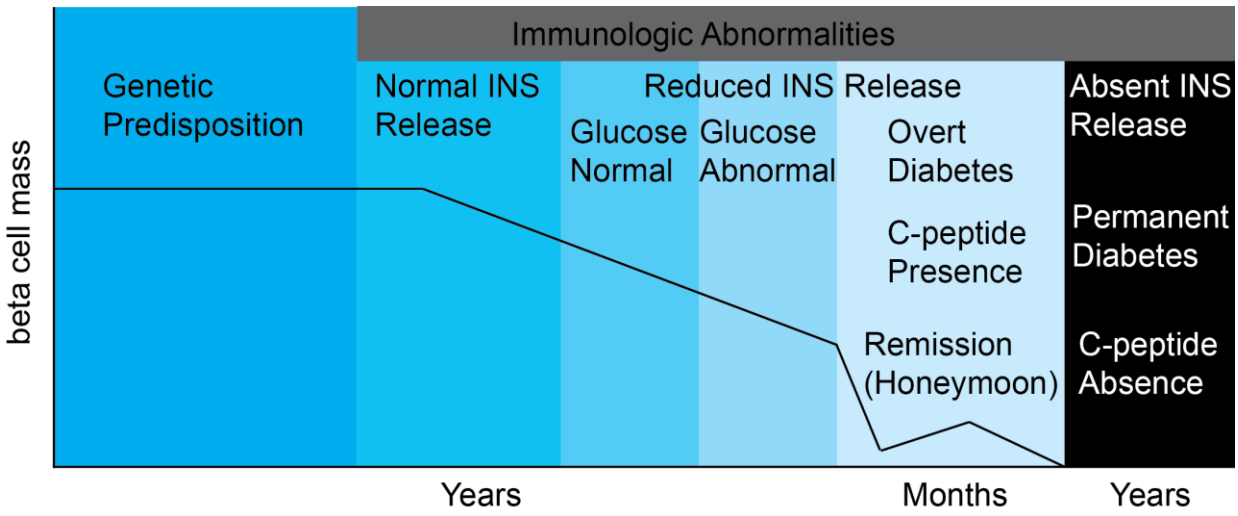


Figure 1. Development of Type I diabetes. Adopted from “Type 1 diabetes: etiology, immunology, and therapeutic strategies”.

the mutations in the patients are rather diverse[3]. Only one gene locus called HLA's odd ratio is high and the other genes carrying mutations in Type I diabetic patients have low odd ratios (**Fig. 2**). The mechanism of Type I diabetes is becoming more complicated because of the studies on identical twins who carry the same mutation but do not always have the same disease process[4,5]. Roughly in 50% of monozygotic twins only one of the two children developed diabetes. Based on these observations several groups develops a hypothesis that Type I diabetes would have an infectious component, most likely virus infection, in the childhood to "awake" the immune system and initiate the auto-immune response. This hypothesis is supported by a few evidences including: 1) Nordic countries which are very cold and have long winters have higher rates of Type I diabetes comparing to the rest of the world [6] (**Fig. 3**) 2) The rate of Type I diabetes is correlated with the Vitamin D deficiency affecting innate immune response[7-9] 3) The presence of virus proteins or RNA have been detected in the islets of Type I diabetic patients[10-12]. The study of Type I diabetes is limited by the absence of mouse models which could perfectly mimic the disease process. The only existing mouse model of Type I diabetes is the NOD model, which indeed develops auto-antibodies against insulin producing cells resulting in insulin deficiency[13-15]. The encouraging aspect of this model is Vitamin D does protect the mouse from diabetes, which highlights the importance of innate immunity to Type I diabetes[16,17]. But the NOD model misses two important features of human Type I diabetes: 1) It does not carry the same mutations which were identified in GWAS data 2) The "honeymoon" period does not exist in the progress of diabetes in NOD mice. Due to all the technical difficulties, the pathogenic details of Type I diabetes is still unveiled.

Type II diabetes, like Type I diabetes, is characterized by the disorder of glucose homeostasis. Type II diabetes is induced by the combination of genetic mutations and environmental factors.

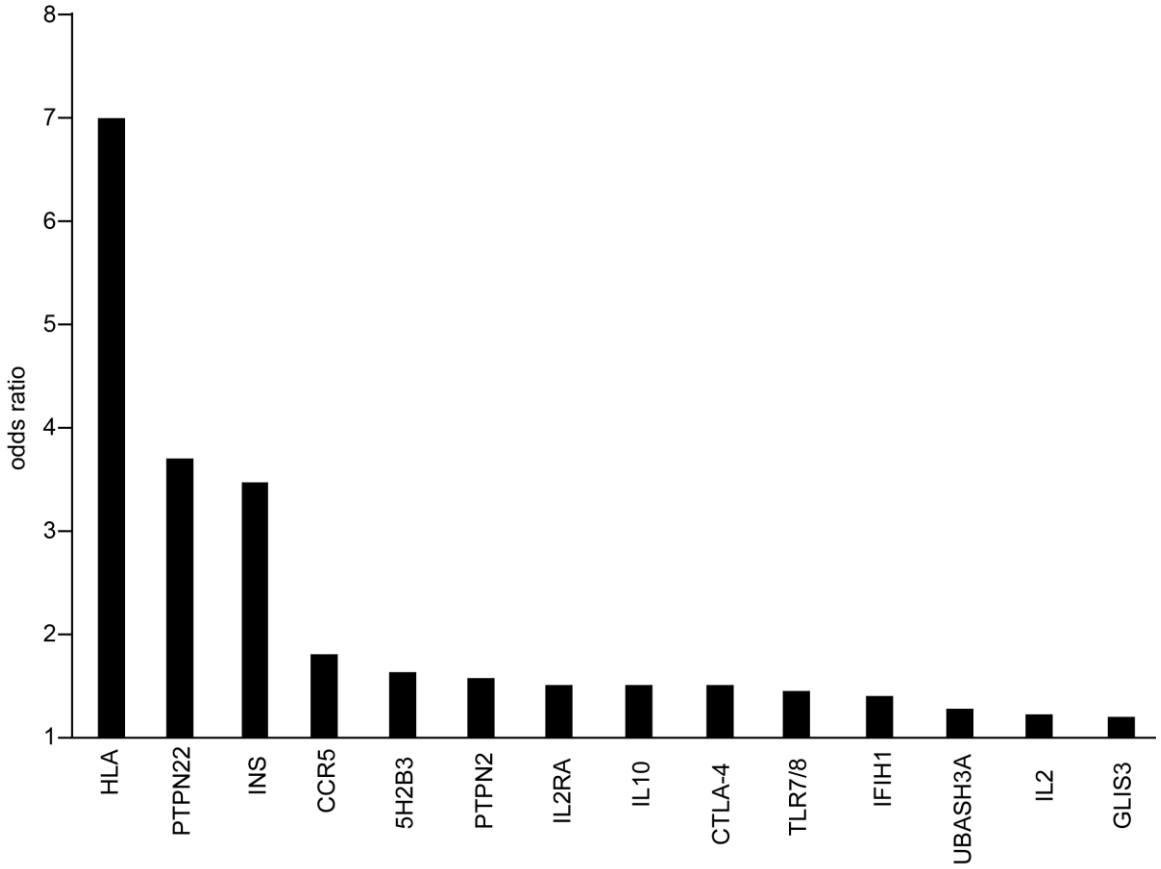


Figure 2. Summary of mutated genes involved in Type I diabetes. The data is got from "Genetics of type 1 diabetes".

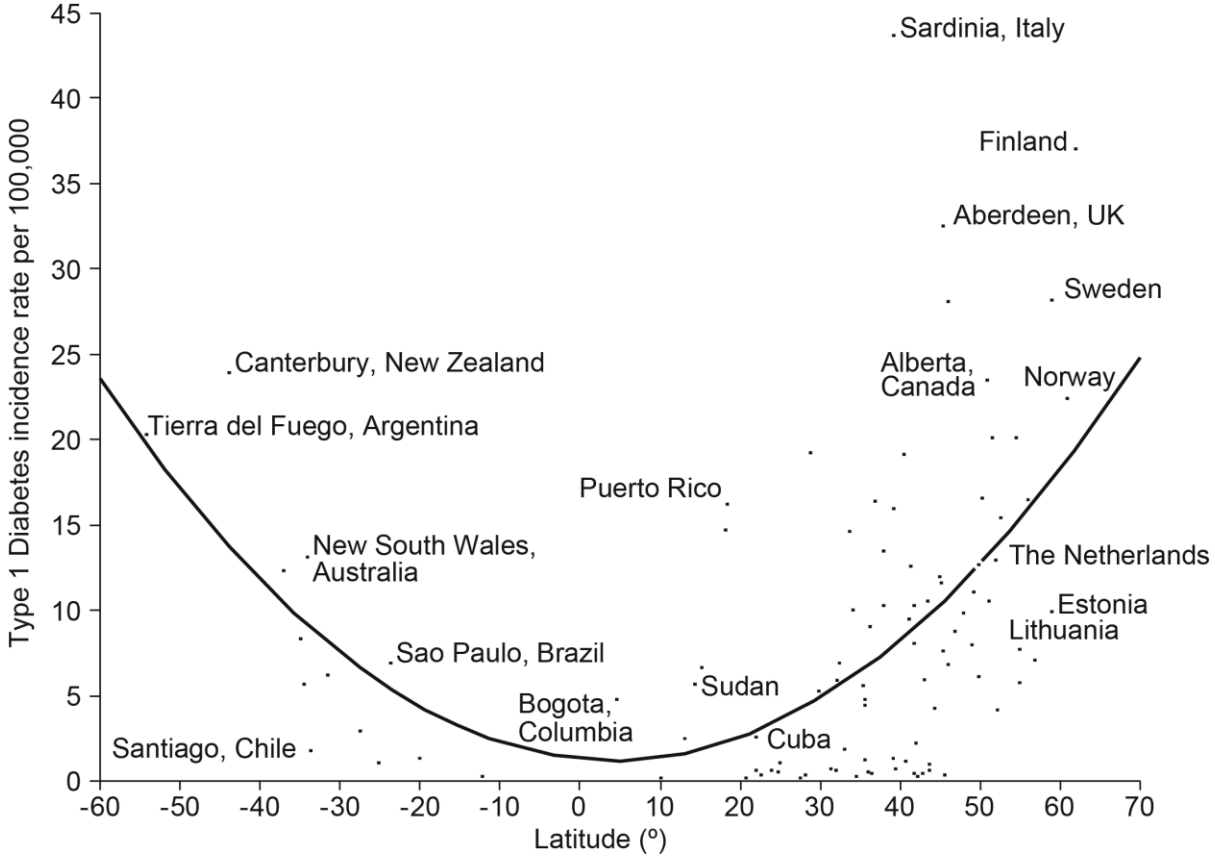


Figure 3. Correlation between latitude and Type 1 diabetes incidence rate. Adopted from “The association between ultraviolet B irradiance,vitamin D status and incidence rates of type 1 diabetes in 51 regions worldwide”.

High BMI (>35), physical inactivity, diabetic history in the first-class relatives and high-risk ethical background are all the risk factors of Type II diabetes[1]. It is the main healthy problems in the western countries (**Fig. 4**). In Europe, 37.0 % to 56.7 % of women and 51.0 % to 69.3 % of men were overweight in 2008 (data obtained from http://ec.europa.eu/eurostat/statistics-explained/index.php/Main_Page). Meanwhile, more than 9% Europeans are diagnosed as diabetic. In total 89 billion Euros are spent on diabetic patients at year 2011 (data obtained from a report of OECD: "the diabetes epidemic and its impact on Europe"). Changes of life style, including restriction of calorie intake and increase of physical exercise are the very first prescriptions physician suggests to Type II diabetic patients at the first diagnosis. However, recent studies pointed out that genetic mutation can also count for a significant part of diabetic incidence. For example, mutations of *SLC30A8* were identified by GWAS to exert protective effects on Type II diabetes patients[18,19] while variants of *SLC16A11* are related to increasing risk of Type II diabetes[20]. With the advance of sequencing technology and bioinformatics more and more diabetes-related genetic mutations are expected to be identified.

Despite the high rate of Type II diabetes, its mechanisms are not fully understood. In general scientists working on Type II diabetes agree that it is an inflammation-related disease. Obesity might induce the accumulation of macrophages and T cells in adipocyte tissue and leads to increase local concentration of TNF-alpha, IFN-gamma and other inflammatory factors[21-27]. The local accumulation of these factors leads to the impairment of insulin sensitivity in adipocytes[28,29]. Researchers have successfully mimicked Type II diabetes in rodent models, such as ob/ob mouse[30] and Zucker rat[31]. Both animal models are based on genetic mutations in the leptin receptor and the mutations lead to excessive hunger and lipid metabolic disorder[32,33]. Therefore these rodent models are severely obese and consistently resistant to insulin in high-fat diet. These models have been widely used in diabetic studies to understand the pathogenesis and

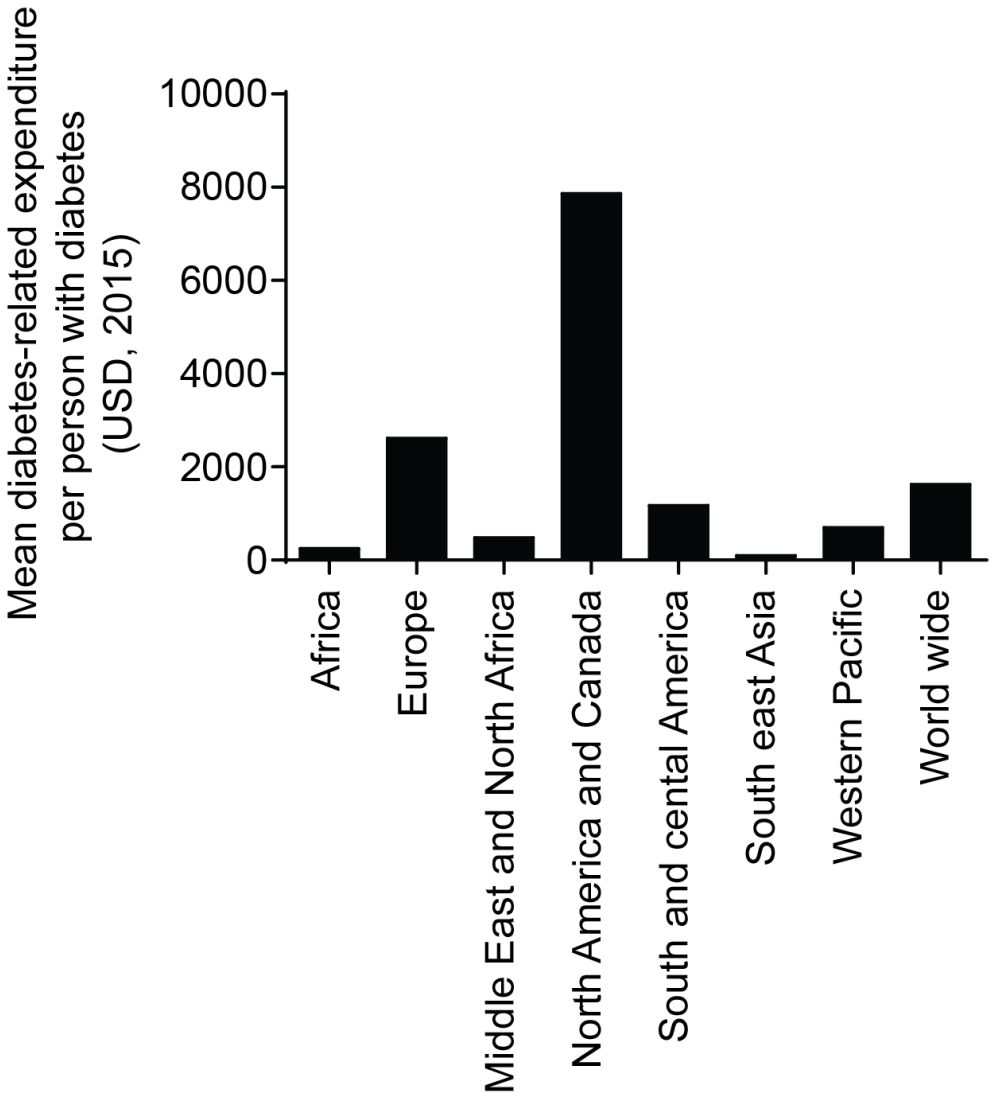


Figure 4. Expenditure of diabetes in different regions. The data is got from “International Diabetes Federation Global Atlas, 2013”.

look for new therapies.

Different from Type I or Type II diabetes, MODY is a well defined disease with clear causes. It is a monogenic disease caused by autosomal dominant mutations in certain genes. The patients are characterized as normal insulin sensitivity but impairment of insulin production or secretion. Some of the genes related with MODY have been studied in genetically modulated mouse models with loss or gain of function, including *HNF1A*[34], *HNF4A*[35], *PAX4*[36], *GCK*[37] (**Fig. 5**). Diabetes is a chronic disease which typically does not cause death immediately. Diabetic patients have good prognosis if their plasma glucose concentration is controlled well. Failure of proper plasma glucose control results in complications including microvascular disease, CVD and renal disease. Therefore, improvement of glucose homeostasis is the major aim of diabetes therapies.

Islets: the center of glucose homeostasis

The pancreatic islet of Langerhans is an endocrine organ which regulates glucose homeostasis by secreting many types of hormones. It is composed of alpha cells, beta cells, PP cells, delta cells and ghrelin cells. Alpha cells secret glucagon which decreases uptake of glucose by hepatocytes, adipocytes and muscular cells and increases plasma glucose concentration, whereas beta cells secret insulin which performs opposite functions to glucagon. The secretions of glucagon or insulin require the processing of the prohormones into the right form of peptides. In details, after the full-length proinsulin/proglucagon is translated from mRNA, certain enzymes and chaperones (e.g. Scg5) are needed to cut the full-length peptides into shorter, or so called mature peptides. Specifically, Pcsk1 is recruited to produce mature insulin and Pcsk2 is recruited to produce mature glucagon. Only mature peptides can be secreted and exert functions. Beside alpha and beta cells, delta cells have been studied in genetically modulated mouse models. Deletion of delta cells induces somatostatin deficiency and impairment of insulin secretion, which hints the

Gene	Prevalence amongst those with MODY
HNF1A	30%-50%
GCK	30%-50%
HNF4A	5%
HNF4B	5%
INS	<1%
PDX1	<1%
NEUROD1	<1% (fewer than five families reported)
CEL	<1% (fewer than five families reported)
PAX4	<1% (fewer than five families reported)

Figure 5. A summary of MODY genes. The table is got from“Clinical features and treatment of maturity onset diabetes of the young (MODY)”.

importance of somatostatin in glucose homeostasis[38]. The roles of PP cells and ghrelin cells in glucose homeostasis are still unclear.

The developmental process of islets is very complicated. It is precisely controlled by multiple transcription factors. The whole pancreas, including the exocrine tissues and endocrine tissues, are all derived from endoderm[39,40]. The transcription factor Pdx1 decides the endoderm cells to differentiate into pancreatic progenitors[41,42]. From this stage, only cells expressing of Neurog3, another transcription factor, can differentiate into pancreatic endocrine progenitors[43,44]. The last critical step of differentiation from pancreatic endocrine progenitors into mature endocrine cell types is regulated by multiple transcription factors. Pax4, Pdx1 and Mafa lead the endocrine progenitors into beta cells[45-47] while Arx and Mafb differentiates endocrine progenitors into alpha cells[48]. The differentiation of delta cells is controlled by Hhex[38] and the transcription factors controlling PP cells or ghrelin cells destiny are still unknown.

For Type I diabetic patients, the auto-antibodies emerges soon after birth. After the emergence of auto-antibodies, patients lose beta cells and develop insulin deficiency within short period. Because human beta cells stop proliferation shortly after birth[49-52] the auto-immune damages on the beta cells are irreversible. Consequently, beta cell is the only cell type which could produce insulin and there is no other cell type which can exert a similar function as beta cells.

For Type II diabetic patients, in the early stage resistance to insulin of hepatocytes, adipocytes and muscular cells is the major phenotypic change. Their islets may still maintain normal function in the beginning but they are actually under consistent inflammatory challenges. There might be the induction of beta cell proliferation upon inflammatory challenges[53-55]. But with the progress of diabetes, islets lose their function due to apoptosis and/or dedifferentiation of beta cells[56-58].

MODY patients carry autosomal dominant mutations of genes which are essential for the development or the function of beta cells, including *INS*, *PDX1*, *PAX4*, et al. For example, *PDX1* is the master regulatory transcription factor of beta cells which controls the transcription of *INS*. Loss-of-function mutation of *PDX1* leads to dysfunction of beta cells in the islets. Importantly, these mutations do not induce a complete loss of beta cells but rather cause a deterministic process of diabetes in juvenile or early adult age.

In brief, insulin deficiency due to loss of functional beta cells in the islet is one of the major problems in all the types of diabetes. Clearly, providing new beta cells/protecting beta cells from damages/improving the function of existing beta cells become the aims of diabetic therapies.

Existing therapies for diabetes

The therapies for diabetes are very diverse. Beside different forms of insulin and GLP-1 agonists, oral agents to increase insulin secretion, insulin sensitivity or decrease uptake/reabsorption of glucose have also been approved by FDA. Beside these medicines, islet transplantation and bypass surgery are making encouraging improvements and increasing their impacts on diabetes.

Insulin

The invention of insulin is one of the most important events in biomedical research. So far insulin therapy is the most effective way to control glucose homeostasis. Almost after one century since the first application of insulin on diabetic patients[59,60], scientists are still improving it to overcome the disadvantages.

One common side effect of insulin therapy is hypoglycemia which is very dangerous for patients. The sad fact of insulin treatment-induced hypoglycemia is it usually happens at night when the patients are sleeping[61-63]. To monitor the plasma glucose concentration and inject insulin according to the change of plasma glucose concentration, a lot of efforts have been made to

invent small insulin pumps which can be implanted into patients' body. The aims of these pumps are to monitor the plasma glucose concentration and to provide insulin in the real-time fashion. It has been shown that patients equipped with insulin pumps have better prognosis and lower rate of hypoglycemia[64-66]. However, to make a perfect insulin pump which can maintain its function in the long term in patient's body and release insulin as accurately as endogenous beta cells is still a significant challenge.

In parallel with engineers, chemists and biologists have been working hard to create different forms of insulin which could be used for different purpose. There are rapid-acting insulin (start to work after 15min/peak time 1h/last for 2-4h), regular insulin (start to work after 30min/peak time 2-3h/last for 3-6h), intermediate-acting insulin (start to work after 2-4h/peak time 4-12h/last for 12-18h) and long-acting insulin (last for >24h). In addition to them, FDA in 2014 approved the first inhaled insulin powder (Afrezza) which is rapid-acting insulin for inhalation before each meal. It could be used together with long-acting insulin for diabetic patients.

GLP-1/GLP-1R agonist

GLP-1/GLP-1R agonist is the most effective anti-diabetic drug family except insulin. GLP-1 is a neuropeptide majorly produced by intestinal L cells as a gut hormone[67,68]. It shares the same mRNA (preglucagon) and prohormone (proglucagon) as GCG, while it requires PCSK1 but not PCSK2 to convert the proglucagon into GLP-1[69-72]. GLP-1 treatment benefits multiple organs, including islets, microvascular, heart/marcrovascular, liver and adipocytes in diabetic patients[73-76]. Particularly GLP-1 treatment induces beta cell proliferation, enhances beta cells function and protects beta cells from damages of diabetes[77-82]. The mechanism of GLP-1's protective effects on diabetes is not clear yet. A recent study suggests the activation of GLP-1 receptor triggers TRPM4 and TRPM5, which leads the increase of sodium influx and insulin secretion[83]. Since the

endogenous production of GLP-1 is not enough to exert protective effects against diabetes, patients need GLP-1 recombinant protein supplements. Alternatively a few GLP-1R agonists (exenatide and liraglutide, et al) could be used instead of GLP-1[84,85].

Oral agents

This is a big family of drugs which have very diverse effects. They target on different stages of glucose homeostasis, such as inhibiting glucose absorption in intestine (acarbose)[86], preventing reuptake of glucose from kidney (gliflozins)[87,88], increasing insulin secretion of beta cells (sulfonylurea and glipizide)[89,90] and improving insulin sensitivity in multiple organs (metformin)[91]. Acarbose works by inhibition of glycoside hydrolases, the major enzyme responsible for digestion of carbohydrates. Gliflozins prevents the renal reabsorption of glucose by antagonizing SGLT2, a sodium/glucose transporter expressing in kidney. However, gliflozins induces hyperglucagonemia in some patients with Type II diabetes by increasing glucagon secretion in alpha cells[92]. This side effect actually attenuates the therapeutic effects of gliflozins. Sulfonylurea can force the secretion of insulin by binding to ATP-sensitive potassium channel and inducing depolarization of beta cell membrane. Metformin is used as the initial treatment for Type II diabetic patients according to the latest ADA guideline. It is widely believed that metformin works through activation of AMPK and increasing glucose uptake to lower the plasma glucose concentration[93,94]. Beside metformin, the other oral agents should only be used in combination with insulin or GLP-1 when metformin alone in the maximal tolerant dose cannot properly control the glucose homeostasis.

Islet transplantation

So far the only way to cue Type I diabetic patients, meaning to achieve insulin therapy independence, is islet transplantation. It is recommended to transplant kidneys in the same time

as the islet transplantation because these patients usually develop renal complications due to diabetes. This therapy has been proved to be effective and patients could be cured shortly after the surgery[95]. However, beside all the common limitations of all transplantation therapies, the islet transplantation is rendered less effective by the facts that the transplanted islets quickly loose function and the patients need to have the second round transplantation[96,97]. The long term successful rate has been improved by the major revisions on the islet isolation protocol. According to the latest report, 44% of patients receiving islets transplantation stay insulin independence within three years[98]. A special problem of islet transplantation is it takes two to three donors' islets to make a successful transplantation on one patient. Another special problem of islet transplantation is unlike kidney transplantation, physicians can only get islets from donors who have passed away. The shortages of organs together with the technical difficulties severely limit the wide use of islet transplantation.

Bariatric surgery

Bariatric surgery, particularly the gastric bypass surgery (Roux en-Y) is a very promising therapy for Type II diabetes. It refers to surgery on stomach to divide it into two pouches, a small one and a big one. Then the intestine is linked to the small pouch to bypass the majority of stomach. Regardless of the high cost of this therapy, patients with proper post-surgery maintenance dramatically loose body weight and increase sensitivity to insulin[99,100]. Side effects include hypoglycemia and its mechanism is still controversial. A few factors, including abnormal insulin secretion and clearance[101,102] or decrease of renal gluconeogenesis[103] may contribute to the hypoglycemia. This therapy is currently used in patients whose BMI is more than 35.

Innovative therapies for diabetes

Despite of the rapid progress of diabetic therapies, none of them fit perfectly to what patients need. An ideal diabetic therapy should meet the following criteria:

1. Physiological control of insulin release.
2. Minimal risk of hypoglycemia.
3. Minimal rejection from the patients.
4. No neoplasm in other organs.
5. No repetitive surgical operations.

According to these criteria several innovative approaches have been developed for diabetic therapies. Here I would like to discuss the approaches concerning improvement of islet function.

Inducing/Enhancing beta cell proliferation

Beta cells stop proliferating soon after the completion of differentiation process. Scientists can hardly detect proliferating beta cells in adult human islets. But there is the presence of proliferating beta cells in diabetic mouse, which hints the possibility to induce or enhance beta cell proliferation. Dramatic efforts have been made looking for reagents which have effects on beta cell proliferation. Betatrophin, a secreted peptide which is supposed to specifically increase beta cell proliferation, was a very promising drug candidate for both Type I and Type II diabetes[104]. But it meets problems in re-producing the results in either genetic modified mouse model[105,106] or islets from human donors[107]. One highlight coming out recently about chemical-induced beta cell proliferation is targeting NFAT signaling. Two chemicals identified by two groups independently, harmine and GNF4877 are both enhancers of NFAT and they both increase beta cell proliferation[108,109]. Another highlight is a secreted protein Serpinb1[110]. It was discovered from a similar model as betatrophin to specifically increase beta cell proliferation.

Comparing to other anti-diabetic therapies the beta cell proliferation-related therapy is still in its beginning stage and does have a promising prospect.

De novo production of beta cells from iPS cells

With the advantages of iPS cells[111], theoretically doctors can take any cells from patients, reprogram them into a specific cell type and transplant them back to the same patients. Obviously in this way all the transplantation treatments are not limited by the donors. Furthermore, the host-rejection is no longer a problem because the cells are from the same patient. But differentiate iPS cells into functional beta cell is an extremely difficult work. Only very recently this is achieved by two groups independently[112,113]. It seems like the iPS cell-based therapy is a promising way for diabetes treatment but it needs a lot of efforts on the pre-clinical studies before translating it into clinical application.

Reprogramming non-pancreatic cells to insulin-producing cells

The initial effort was made by Ferber and his colleagues to reprogram hepatocytes into insulin-producing cells by misexpression of Pdx1 in mouse liver. This result is validated in human fetal liver by the similar strategy with around 60% reprogramming efficiency[114]. With the progress in this field Pdx1 was substituted with the misexpression of three genes in one construct (Pdx1-Neurog3-Mafa) to gain higher reprogramming efficiency[115,116]. But due to the complicated three-gene-misxpression system it is very difficult to translate this strategy into clinical application. However, these are very important proof-of-concept experiments showing the possibility of reprogramming non-pancreatic adult mature cell types into insulin-producing cells.

An alternative approach was achieved by Talchai and his colleagues in 2012 when they deleted FoxO1, a transcription factor conditionally in Neurog3 expressing intestine cells and these cells turned into insulin-producing cells[117]. The effect of deleting FoxO1 in intestine cells in human

tissues was confirmed by Bouchi and his colleagues from the same group[118]. The obvious benefit of FoxO1 deletion is that it only disrupts one gene. Another benefit is the chemical inhibitor of FoxO1 does exist and has protective effects on diabetic mice[119]. However, since FoxO1 is an extremely important transcription factor and has diverse effects on different tissues the patients need an organ-specific delivery system for FoxO1 inhibitor.

Reprogramming pancreatic exocrine cells to insulin producing cells

The pancreatic exocrine cells including pancreatic duct and acinar cells, are developmentally close to endocrine cells (**Fig. 6**). Zhou and his colleagues showed that misexpression of a combination with three transcription factor, Pdx1-Neurog3-Mafa, is enough to induce reprogramming of acinar cells to beta cells[120,121]. Meanwhile pancreatic duct cells could also be reprogrammed to insulin-producing cells by misexpression of Neurog3, MafA and Pdx1 in human primary pancreatic duct cells[122,123]. Similar to the non-pancreatic cells to insulin-producing cells reprogramming, the three-transcription-factors induced reprogramming by retrovirus delivery or conditional misexpression is very difficult to be translated to clinical operation.

Some groups chose to study the reprogramming of exocrine cells to insulin-producing cells in a different way. The mouse primary exocrine cells could be converted to insulin producing cells (up to 5%) with the combinational treatment of EGF and nicotinamide in suspending cell culture[124]. This method is revised and improved by Baeyens and his colleagues by combination treatment with EGF and CNF. This combination reprograms the acinar cells to insulin-producing cells *in vivo* and fully rescues the insulin deficiency phenotype in a mouse model[125]. Comparing to the genetically modified mouse model the EGF and CNF combination treatment is one step

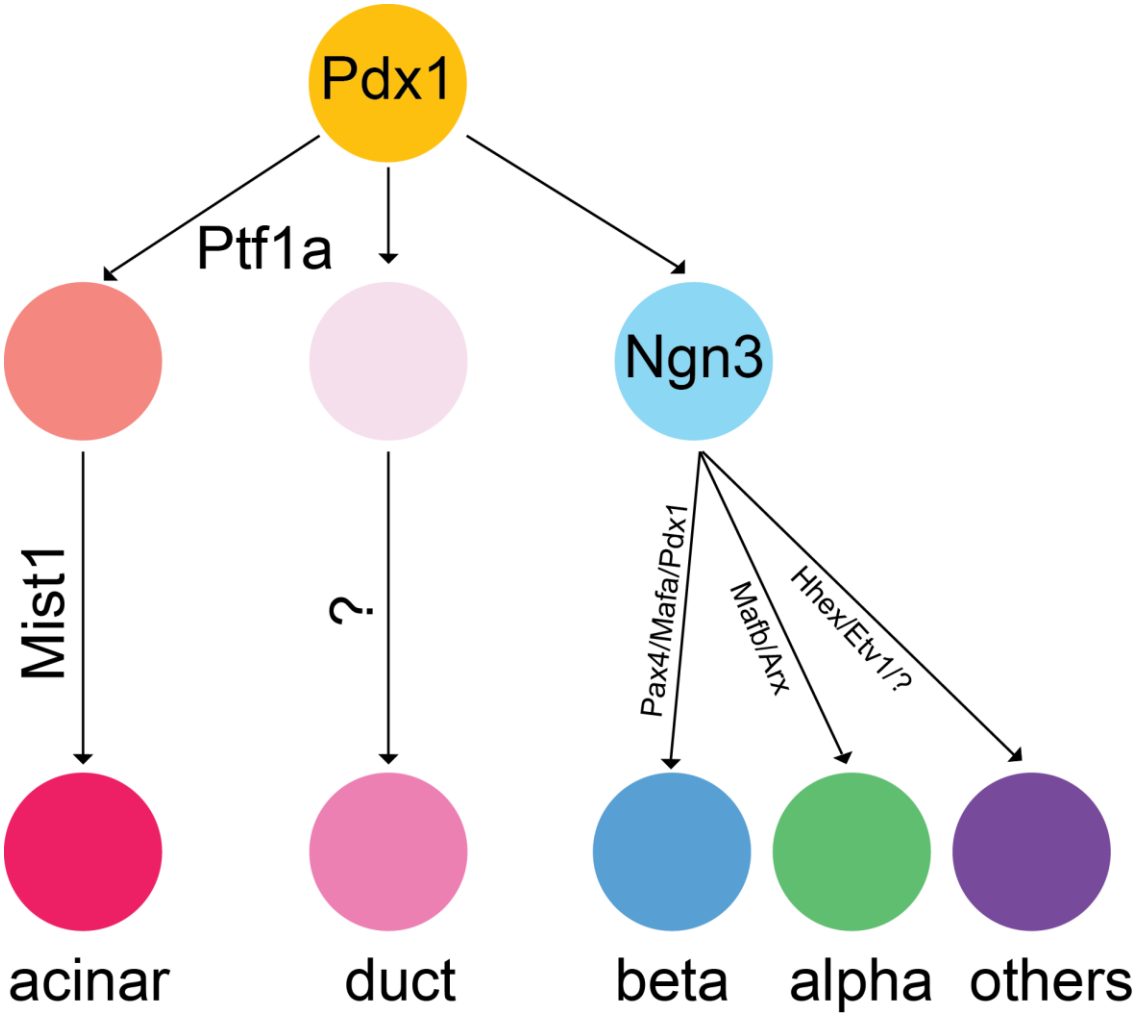


Figure 6. Scheme of pancreatic developmental process.

nearer to clinical application. However, both EGF and CNF have certain risks to induce neoplasm in multiple organs and it is difficult to initiate clinical trials with this strategy.

Reprogramming pancreatic endocrine cells to insulin producing cells

Pancreatic alpha cells and delta cells are very different from beta cells in their epigenome and transcriptome but very close developmentally. They share several transcription factors like Isl1 and Neurod1 which are important for the endocrine specification from pancreatic progenitors and are still expressed in mature cells. Thorel, Chera and their colleagues observed transdifferentiation from alpha cells or delta cells to beta cells under extreme loss of beta cells in moue islets[126,127]. Although this transdifferentiation is not enough to rescue insulin deficiency in adult mice, it provides a proof-of-concept that alpha cells and delta cells can be reprogrammed to beta cells.

Unlike the reprogramming of exocrine cells to insulin producing cells, the deletion or misexpression of single transcription factor in alpha cells is enough to induce reprogramming towards beta cells. Collombat and his colleagues performed extensive works on two genes, Pax4 and Arx. Their work convincingly showed that misexpression of Pax4 or deletion of Arx specifically in mature alpha cells is enough to induce transdifferentiation from alpha to beta cells. Mice with streptozotocin-induced beta cell loss and insulin deficiency could be fully rescued by misexpression of Pax4 or deletion of Arx. The alpha cell pool is replenished by duct cells to maintain the proper number of glucagon producing cells. A further study suggests the simultaneously deletion of Pax4 and Arx can still induce alpha to beta transdifferentiation, highlighting the importance of Arx in the transdifferentiation context[48,128-131]. Arx belongs to the homeobox family of transcription factors which is consider as “undruggable” because of the lack of binding pocket. Pharmacological exploitation requires new ideas on developing Arx inhibitors to mimic the deletion of Arx.

Identifying reprogramming agents as the next generation anti-diabetic drugs

Since insulin was used on diabetic patients for the first time, islet biology has made enormous progress in understanding the detailed biochemical events in this field. Transgenic animal models have been made to dissect the developmental process of islet formation and the effects of a lot of transcription factors and ion channels on pancreatic endocrine cell differentiation and function have been clarified. However, scientists are missing three important tools to discover new drugs for diabetes:

1. Transcriptional profiles of all the cell types in the islet of Langerhans.

With the development of next-generation sequencing technology, more and more transcriptional profiles have been produced in different organs by RNA-sequencing. Efforts have been made in understanding the transcriptional change of islets in diabetic mice or patients by sequencing RNA from the whole islets[132,133]. Undoubtedly this is a dramatic improvement comparing to RT-qPCR and microarray assay on whole islets because RNA-seq has a much better coverage and resolution. This knowledge is very important for islet biology research, particularly for the reprogramming studies. However, due to the fact that islets contain a mixture of a few extremely different cell types, the specific changes in one cell types might be covered in the bulk islet RNA samples. This brings difficulties to study diabetes-induced transcriptional change in specific cell types. It also brings difficulties to study the genome-wide effects of reprogramming chemicals or genes in specific cell types.

Assays have been developed to purify fractions of cells from islets to investigate cell type specific transcriptional profiles. For example, mouse models with linage specific reporters (eg. insulin minimal promoter driven mcherry) have been established and RNA-seq analysis was performed on reporter-positive purified cells[134]. The benefit of this assay is to provide a relatively

clean data set for a specific cell type. Unfortunately, the promoters with specificity for one cell type may have low misexpression in other cell types. Such kind of "leaky expression" introduces certain contaminations to the data set. Another shortcoming of the linage specific reporter based assay is it obviously could not be employed on human islets.

Except the linage specific reporter models antibodies against cell-type specific surface proteins were also applied to purify cells from islets. This strategy was majorly used on human islets. Grompe's group has identified a pair of famous antibodies, HPa2 and HPi2, to purify alpha and pan-endocrine cells specifically from human islets[135]. With these two antibodies, Grompe and Kaestner's group have produced a lot of data sets, including both RNA-seq and Chip-seq data to investigate the difference of human alpha and beta cells in physiological and diabetic conditions[136-138]. Although the authors claimed they achieved a very high purity (>95%) with these antibodies, the raw expression data does not fit to this high purity. Beside these assays, Eizirik's group developed a method to sort out alpha cell and beta cells based on the size of the different cell types[139]. Regardless of the purity of sorted cells, this purifying strategy was only reported on rat islets.

In summary, there are quite a few islet cell type specific transcriptional profiles available. All of them are either for alpha cells or beta cells. Transcriptional profiles for delta and pp cells are missing. All of these data sets are based on some kind of purification strategy and none of them are clean enough to be used as a reference for future studies.

2. Cell line models which can recapitulate the similar reprogramming events as animal models.

All the reprogramming events observed in islets, including transdifferentiation of alpha cells to beta cells and dedifferentiation of beta cells to endocrine progenitor cells, were mainly done in mouse models. The advantages of mouse models are obvious, as they achieve a maximal

physiological relevance. However, it is almost impossible to perform any phenotype-based compound screenings on mouse models. Furthermore, because the complex cell-cell interaction among different cell types in islets, it is difficult to prove if it is an autonomous reprogramming or it is affected by other cell types. For drug discovery it is important to have a screening model in cell culture condition which can quickly and economically identify compounds affecting the reprogramming events. After identification of a few hits from the screening models the validation could be done on mouse models to test the physiological relevance.

Such kind of work has been done by Kubicek and his colleagues with alpha-TC1 cell, which is mouse alpha cell line. In this cell line the small molecular BRD7389 was identified from a chemical library to increase insulin expression in alpha-TC1 cells as well as human islets. This is a proof-of-concept story suggesting that mouse cell lines could be used as a screening model for diabetic drug discovery. However, based on our experience achieving the robust and full reprogramming of this alpha cell line to beta cells is very difficult. Indeed, so far we have not find a genetic or chemical method to fully reprogram it into beta cells.

On the other hand, there is no cell line model which can recapitulate the dedifferentiation phenotype of beta cells with FoxO1 inhibition. The genetic deletion of FoxO1 in a mouse beta cell line Min6 cell does not present any dedifferentiation phenotypes[140]. One explanation is, because the growth of Min6 cells depends on the insulin promoter driven large T expression the absence of dedifferentiation phenotype in this model may due to the loss of cells whose insulin promoter are silenced by FoxO1 deletion. The other explanation is the loss of FoxO1alone is not enough to induce dedifferentiation in beta cells and it may require triple knock-out of all FoxO members[141]. Zhu and his colleagues reported to induce beta cell dysfunction by application of glycated-serum on Min6 cell. The authors incubated the serum with glucose at 37°C for 3 weeks to generate glycated-serum[142]. In my mind this method is not robust enough for high-through put

screening. Recent publication suggests triple knock-out of FoxO1/3/4 can induce beta cell dedifferentiation without metabolic stress[141]. However, it is very time and money-consuming to perform a primary islets-based high-through put screening.

A screening cell line model for islet reprogramming research should meet a few criteria:

a) The factor(s) inducing reprogramming is/are validated in animal models
b) Robust reprogramming with minimal manipulations
c) Suitable for high-through put screening

3. Proteins as drug targets for reprogramming

Despite of all the knowledge we gained in the past decades on alpha cell to beta cell transdifferentiation and on beta cell dedifferentiation, the underlying mechanisms are poorly understood. It is interesting to identity proteins interacting with Pax4 or Arx in order to find "druggable" proteins for pharmacological development. Unfortunately there is no literature reporting a successful pull-down of either Arx or Pax4 in islets so far. Without knowing how Arx and Pax4 induce alpha to beta transdifferentiation we are in lack of drug targets to develop reprogramming agents.

We meet similar problems on the beta cell dedifferentiation induced FoxO1 inhibition. Like Arx or Pax4, the interacting proteins of FoxO1 in beta cells have never been reported. Encouragingly, the mechanism behind it has been partially addressed by Kim-Muller and his colleagues in 2014. They believed the inflexibility of beta cell energy metabolism is the major causes of dedifferentiation[141]. It is a big progress but still quite far from discovering a druggable protein target.

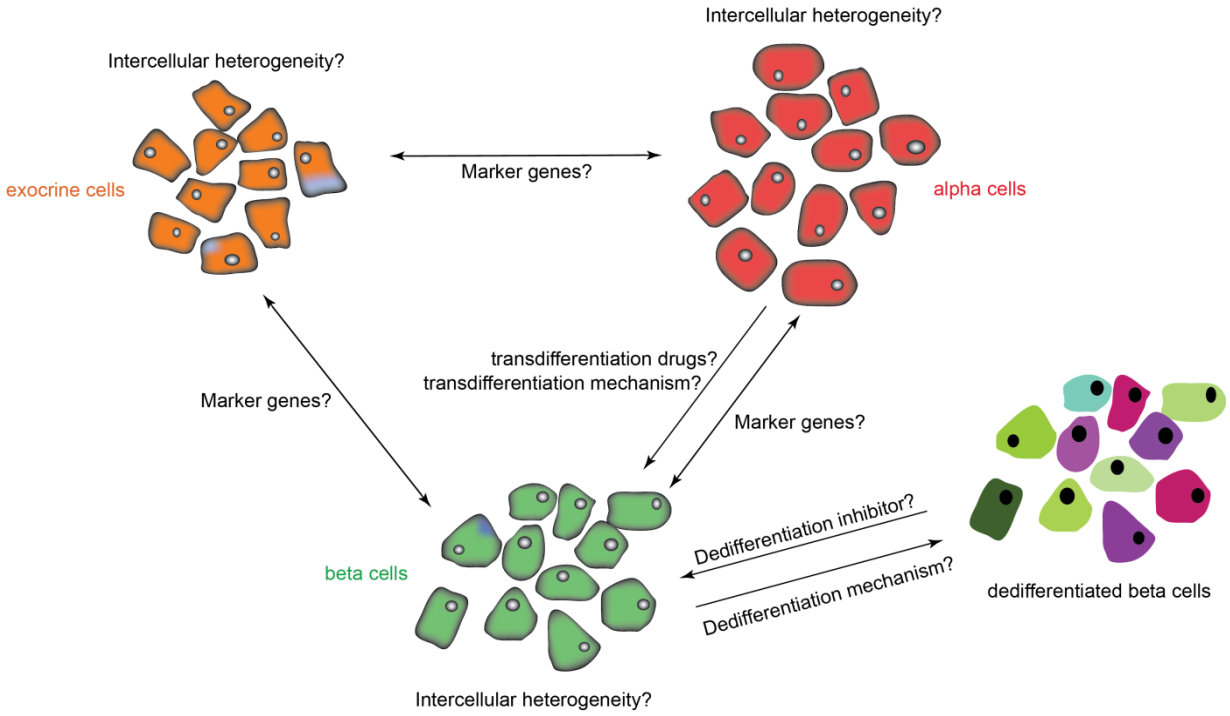
Aims

1. As the first part of my PhD study, together with my colleague I performed single cell RNA-seq to establish reference transcriptomes for the major mature cells types in human islets. This is the first cell type specific transcriptional data set on human islets achieved in single cell resolution. This data set was used in the second part of my thesis to understand the reprogramming effects of chemicals. In the same study I also identified novel transcription factors for different cell types, compared human islets transcriptional profiles to mouse islets and predicted the major transcription factors defining exocrine and endocrine cells. This study is published in *EMBO reports* now[143].

2. As the second part of my PhD study, I performed a high-content chemical screening with a representative compound library of approved drugs on a newly established model cell line. In this cell line the beta-to-alpha transdifferentiation can be initiated by inducible misexpression of ARX. The screening was done on this cell line to look for chemicals counteracting the effects of ARX misexpression. A group of approved drugs, artemisinins were identified to induce alpha to beta transdifferentiation and were validated in multiple animal models. With the chemical proteomics assay artemisinins were observed to interact with gephyrin, a cofactor of GABA receptor subunits. We further proved the enhancement of GABA receptor signaling is essential for artemisinins induced alpha-to-beta transdifferentiation. With current data I provided a model cell line for alpha cell to beta cell reprogramming, a group of chemicals to induce insulin-producing cells from pancreatic alpha cells and an important signaling pathway for transdifferentiation. This study is submitted to *Cell* now.

3. As the third part of my PhD study, I established a cell line model to study beta cell dedifferentiation. With chemical inhibition of FoxOs on Min6 cell I managed to mimic the *in*

vivo dedifferentiation phenotypes in the cell culture system within two days treatment. With this model I identified the increase of lysosomal acidification as the key factor of beta cell dedifferentiation by analyzing transcriptional profiles. Further study found an approved drug loperamide could inhibit glucagon transcription in the dedifferentiated model beta cell line from a high-content chemical screening. Loperamide also suppressed glucagon transcription in mouse alpha cell line and human islets. This effect is mediated by the mobilization of intracellular calcium ions and increase of FoxO1 expression. With this project I made a cell line model for beta cell dedifferentiation, found a few drugs which can affect the dedifferentiation and two signaling pathways which are important for beta cell identity. This work has been submitted to *EMBO J* and is currently under revision. The details of these three parts are presented in the following pages.



Summary of Aims for this thesis.

Results

Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types

In order to establish high-quality transcription profiles for all different cell types in human pancreatic islets, we used a single cell RNA-seq approach. Different from previous approaches, single cell RNA-seq does not require any pre-enrichment or purification and can achieve transcriptional information at single cell resolution. This provides an extra clean and robust data set for islet biology. Together with my colleagues I used this data set to study three important questions for pancreatic islets:

1. Identification of novel cell type specific transcription factors.
2. Investigation on the driver genes differ exocrine cells from endocrine cells.
3. Species specificity of islet transcription profiles between human and mouse.

The findings of this project are published in *EMBO report*[143].

Beside achievement of the reference transcriptome, the single cell RNA-seq assay is also a powerful assay to study cell type specific response to drug treatments. This assay is used in the second project to study the differential effects of arteminins on alpha and beta cells in human islets.

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Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types

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Abstract

Pancreatic islets of Langerhans contain several specialized endocrine cell types, which are commonly identified by the expression of single marker genes. However, the established marker genes cannot capture the complete spectrum of cellular heterogeneity in human pancreatic islets, and existing bulk transcriptome datasets provide averages across several cell populations. To dissect the cellular composition of the human pancreatic islet and to establish transcriptomes for all major cell types, we performed single-cell RNA sequencing on 70 cells sorted from human primary tissue. We used this dataset to validate previously described marker genes at the single-cell level and to identify specifically expressed transcription factors for all islet cell subtypes. All data are available for browsing and download, thus establishing a useful resource of single-cell expression profiles for endocrine cells in human pancreatic islets.

Keywords alpha cells; beta cells; diabetes; marker genes; single-cell RNA-seq
Subject Category Systems & Computational Biology
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Introduction

Located within the pancreas, the islets of Langerhans are composed of endocrine cells expressing glucagon (alpha cells), insulin (beta cells), somatostatin (delta cells), pancreatic polypeptide (PP cells), and ghrelin (epsilon cells). Furthermore, they are heavily vascularized and innervated, and in contact with the surrounding acinar and ductal cells of the exocrine pancreas. Pancreatic islets function as highly specialized micro-organs that monitor and maintain blood glucose homeostasis. While damage to beta cells causes diabetes, the other pancreatic cell types may also contribute to pathogenesis in ways that are not well understood. Recent studies showed that both alpha [1] and delta cells [2] have the potential to replenish beta cell mass in animal models.

Development of diabetes correlates with global changes in the transcriptome of pancreatic islets [3]. These gene expression changes could reflect alterations in the cell subtype composition of the islet and/or changes in the transcriptomes of beta cells or other individual cell types. Analyzing islet cell-specific gene expression changes has the potential to shed light on the etiology of diabetes. Recently, alpha and beta cell purification protocols from human [4–6] and mouse islets [7,8] have yielded initial maps of cell type-specific transcriptomes. The available transcriptome datasets further comprise primary mouse and human alpha cells, beta cells, and delta cells, a number of rodent alpha and beta cell lines, and one human beta cell line [4,9–12]. Despite the rapid progress in this field, a comprehensive transcriptome database for individual human islet cell types is still missing, and no transcriptome data are currently available for PP cells.

Recent advances in next-generation sequencing and library preparation enabled for the first time the transcriptome characterization of single cells from primary tissue. For example, this approach was successfully used to establish transcriptome profiles and dissect cell type heterogeneity for primary tissue obtained from the lung [13], the spleen, and the brain [14,15].

Here, we used single-cell RNA-seq to establish a comprehensive transcriptome database for the cell types that are present in primary human pancreatic islets. Principal component analysis in combination with visualization as biplots identified alpha cells, beta cells, delta cells, PP cells, acinar cells, and pancreatic duct cells directly from the single-cell transcriptome profiles. We illustrate the utility of this resource by discovering novel cell type-specific marker genes, and we identified human-specific expression patterns in alpha and beta cells. All data are readily available for user-friendly online browsing and download to foster research on pancreatic islet biology and diabetes-related mechanisms in human.

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Results and Discussion

Single-cell transcriptomes recapitulate pancreatic endocrine cell types

Primary human pancreatic islets of Langerhans were disassociated into single cells, and these cells were sorted into individual wells of a 96-well plate by FACS [16]. The Smart-Seq2 protocol [17] was then applied to obtain single-cell transcriptomes. Following the generation and amplification of cDNA, we determined the levels of beta-actin expression by qRT–PCR and selected all cell-containing wells for library preparation and next-generation sequencing (Fig 1A). Seventy cells were sequenced in total, of which 64 cells passed quality control (see Materials and Methods) and were included in the analysis (Fig EV1A and B, and Dataset EV1). We obtained an average of 12.7 million high-quality reads per single cell, of which 62.9% aligned to the human reference genome. RNA expression levels were calculated using the BitSeq software which uses RPKM normalization and corrects for non-uniform read distribution along the transcripts (e.g., 3-prime bias) [18]. Data quality was validated by assessing the relation between expression level and transcript length in native RNA (Fig EV1C) as well as ERCC spike-in controls (Fig EV1D). While transcript length and expression level were not correlated in the ERCC spike-in controls, we detected a negative correlation ($r = -0.405$) in the native RNA which was in the range of what had been previously reported as biologically significant finding [19]. However, a potential bias due to transcript length normalization cannot be completely excluded; therefore, comparing expression levels of different transcripts/genes should be performed with caution. To define global similarities among the single cells and the marker genes that drive these similarities, we performed principal component analysis (PCA) on the transcriptome dataset and displayed the results as biplots. PCA on the full dataset separates a group of 18 cells based on high *glucagon (GCG)* and *transthyretin (TTR)* expression and a group of 9 cells expressing *pancreatic polypeptide (PPY)* from a heterogeneous group of 37 cells (Fig 1B). In a second PCA on the 37 yet undefined cells, we identified a group of 12 cells with high *insulin (INS)* expression, a group of 11 cells characterized by *PRSS2*, *CTRB2*, *REG3A*, *REG1A*, and *REG1B* and a group of two *somatostatin (SST)*-expressing cells. In a third PCA on the remaining 12 undefined cells, a group of 8 cells was characterized by *keratin18 (KRT18)* and *keratin8 (KRT8)*. Based on the expression profiles of the identified marker genes, we were able to uniquely assign 60 out of 64 single-cell transcriptomes to the alpha, beta, delta, PP, acinar, or ductal cell type (Fig 1C).

As an additional validation of our cell type classification, we visualized the global transcriptional similarity of individual pancreatic cells by multidimensional scaling (MDS), where each single-cell transcriptome was colored by the cell type derived from PCA (Fig 1D). When mapped upon the MDS plot, the known cell type-specific marker genes *INS*, *GCG*, *PPY*, *SST*, *REG1A*, and *KRT8* show the expected expression patterns, with different amounts of variability within the subgroups (Fig 1E). The validity of our single-cell RNA-seq dataset was further confirmed in direct comparison to an external dataset consisting of bulk RNA-seq data for whole islet, beta, and acinar cells [20]. Using MDS, we show high transcriptional similarity between the corresponding cell types of both datasets (Fig EV1E). The expression information of individual cells and merged expression values for each cell type is available in Dataset EV2.

To rule out technical reasons as a major source of gene expression variability, we identified presumably pure alpha and beta cells among the assessed single cells (Fig EV2A). Their transcription profiles were used to simulate transcriptomes with defined percentages of alpha and beta cell contribution (Fig EV2B). Individual alpha and beta cells were then compared to these virtual transcriptomes to estimate upper limits for potential cross-contamination (Fig EV2C–E). All beta cell transcriptomes were found to be free from any alpha cell contribution, whereas beta cell profiles could explain a small proportion ($< 3\%$) of the variance observed in 8 of the 18 alpha cells studied. However, given that these alpha cells further show higher unexplained variance, it is likely that they are characterized by high inherent variability rather than cross-contamination from beta cells. We conclude that the differences between alpha and beta cell heterogeneity are in line with biological rather than technical effects which supports the hypothesis that alpha cells might be more plastic than beta cells [4].

The heterogeneity within the different cell types was further explored by separate PCAs for each cell type (Appendix Fig S1). Particularly for endocrine cells, heterogeneity was mainly driven by expression differences of marker genes as identified in the initial cell type classification by PCA, suggesting that these cell types are characterized by a spectrum of marker gene expression levels. While this analysis provides evidence for transcriptional heterogeneity, more cells are needed to thoroughly characterize subgroups within the different cell types.

A transcriptome resource to reveal marker genes of human pancreatic cell types

To maximize the utility of our dataset for the identification of cell type-specific expression patterns, we generated a resource of genome browser tracks of all individual cells as well as cumulative tracks for the cell type clusters identified by PCA (http://islet-transcriptome.computational-epigenetics.org/). One interesting use of

Figure 1. Single-cell transcriptomes recapitulate the major pancreatic cell types.

A Workflow for obtaining and analyzing single-cell RNA-seq data from human pancreatic islets.
B Iterative PCA/biplot-based approach for the identification of cell types and cell type-defining transcripts from single-cell RNA-seq data.
C Expression (scaled RPKM values) of cell type-defining genes as identified in (B) across all single cells. Transcripts and single cells are grouped by cell type as identified in (B).
D Display of transcriptional similarity between all single cells by MDS. The coloring scheme is based on the cell types as identified in (B).
E Relative expression (scaled RPKM value) of canonical marker genes for the 6 identified pancreatic islet cell populations represented by bubble size and projected onto the MDS profile.

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Jin Li et al Single-cell transcriptomes of human pancreatic islets **EMBO reports**

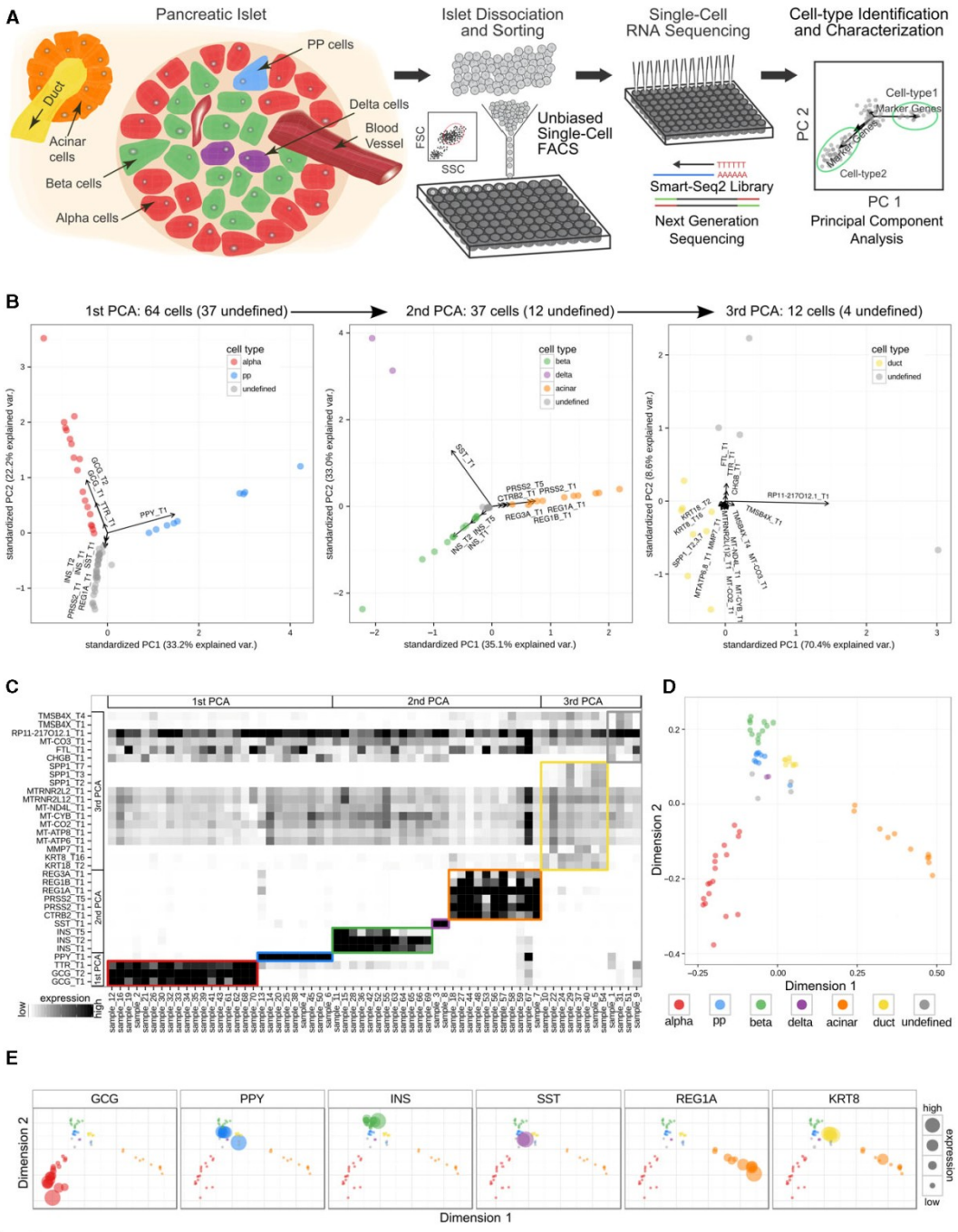

Figure 1.

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this resource is the analysis of master regulatory transcription factors, which are fundamental for the development and the maintenance of different pancreatic cell types based on animal models and human genetics. The genome browser tracks illustrate the beta cell-specific expression of *PDX1*, a master regulatory transcription factor directly controlling insulin expression. In contrast, the alpha lineage-defining factor *ARX* is expressed in both alpha and some PP cells (Fig 2A and Appendix Fig S2). Other transcription factors that are important for pancreas development have different degrees of cell type-specific expression in mature human islets, including pan-endocrine (*PAX6*), beta cell-specific (*PAX4*), and duct/delta (*HHEX*) patterns (Fig 2B). While *MAFA* is transcribed in beta cells specifically, we observed robust *MAFB* expression in alpha, beta, and delta cells. Half of the beta cells studied expressed *MAFA* and *MAFB* concomitantly. In addition to these previously described factors, we also observed cell type-specific expression for transcriptional regulators, which have not yet been extensively characterized in the endocrine pancreas. For example, *MORF4L1* shows a similar pan-endocrine pattern to the canonical islet cell marker *NEUROD1* (Fig 2C). A subset of alpha cells express *IRX2* (Fig 2D), some beta cells show high expression of *polycomb ring finger oncogene (BMI1)* (Fig 2E), and PP cells can be characterized by the transcription factors *ETV1* and *MEIS1* (Fig 2F).

We further performed pairwise correlation analysis on transcript level to identify genes, of which the expression profiles correlate highly ($r > 0.9$) with those of the endocrine marker genes *INS*, *GCG*, *SST*, and *PPY* (Fig EV3). While several highly correlated genes could be identified for *INS* and *SST* (e.g., zinc transporter *SLC39A4* and Notch pathway component *DLK1* for *INS* and transcription factors *NKX6-3*, *ZNF430* for *SST*), the expression profiles of *GCG* and *PPY* did not show high correlation with any other genes.

To extend our analysis beyond transcription factors and known marker genes, we performed pairwise comparisons of cell type-specific transcriptomes by gene set enrichment analysis (Dataset EV3). Interestingly, we observed strong enrichment of a gene set containing the REST-binding motif in all endocrine cell types compared to acinar and ductal cells (Fig 3A). Most genes that contain the REST motif in their promoters are expressed in alpha, beta, delta, and PP cells, whereas they are repressed in ductal and acinar cells (Fig 3B). The transcriptional repressor REST targets the REST-binding motif. In line with the target gene expression pattern, *REST* is specifically expressed in ductal and acinar cells (Fig 3C and Appendix Fig S3).

Finally, based on pairwise differential expression analysis between the pancreatic cell types, genes with highly specific expression patterns were identified (Fig EV4 and Appendix Fig S4, Datasets EV4 and EV5). We then used these data to assess islet cell type-specific expression in two areas of high relevance for diabetes research–diabetes risk genes and mouse–human species differences.

Genomewide association studies (GWAS) have identified genomic loci conferring increased risk for the development of diabetes. We examined whether any of the diabetes-related genes predicted by GWAS were specifically expressed in one of the pancreatic islet cell types and genes differentially expressed between the endocrine and exocrine cell types (Fig EV5A). For both type 1 and type 2 diabetes, we identified GWAS genes with beta cell- and endocrine-specific expression. Other genes show broader expression patterns, emphasizing the complexity of functional annotation of diabetes GWAS results. Furthermore, key MODY (Mature Onset of Diabetes in Young) [21] genes *PDX1, PAX4, INS, HNF1A, GCK* are predominantly specific to beta cells (Fig EV5B).

To investigate species-specific differences of alpha and beta cell transcriptomes, we assessed the degree to which the previously identified differentially expressed mouse genes [7,9] are also differentially expressed in human islets and vice versa (Appendix Fig S5). We found that the human alpha cell-specific gene *group-specific component (vitamin D binding protein) GC* and the human beta cell-specific gene *DLK1* (Fig 3D) displayed opposite expression patterns as to what had been reported in mouse islet cells. To confirm the cell type-specific expression of *DLK1* and *GC*, we performed immunofluorescence staining on both human and mouse pancreatic tissue sections. In human islets, *DLK1* was specifically expressed only in insulin-positive cells (Fig 3E), whereas this protein was observed in glucagon-positive cells in mouse tissue (Fig 3F). Similarly, *GC* expression showed alpha cell specificity in human tissues (Fig 3G), whereas it was co-expressed with insulin in mouse tissues (Fig 3H). These results suggest that two of the most differentially expressed cell type-specific marker genes for human alpha and beta cells have opposite expression patterns in mouse islets.

Pancreatic islets comprise different cell types with characteristic transcriptomes, which confounds transcriptome studies that focus on whole pancreatic islets in physiological and pathological conditions. Lineage-labeled transgenic mice have made it possible to obtain transcriptomes for highly pure alpha and beta cell populations in mouse. For human islets, however, cell type-specific enrichment strategies depend on the availability of specific antibodies. Efforts have been made to measure the transcription of individual genes in single human islet cells by qRT–PCR [22], but our dataset is the first to provide genomewide transcriptional information of human islets at single-cell resolution. Using single-cell data, we also for the first time defined the transcriptomes of human delta cells and PP cells, thereby providing reference transcriptomes for all major endocrine cell types in human pancreatic islets.

We illustrated the practical utility of our resource and dataset by three case studies. First, after confirming the cell type specificity of the major transcription factors involved in pancreatic endocrine lineage determination, we identified transcripts encoding transcription factors expressed in islet cells. These include the pan-endocrine marker *MORF4L1*, alpha cell-specific *IRX2*, beta cell-specific *BMI1*, and PP cell-specific *MEIS1* and *ETV1*. These data can provide the basis for future functional studies in the roles of these transcription factors in the pancreas and in diabetes.

In a second example, we analyzed cell type-specific enrichment of previously characterized gene sets. The specific expression of REST-motif-containing genes in the endocrine cell types led us to identify the specific expression of the transcriptional repressor *REST* in the exocrine pancreas. REST recruits a large complex of chromatin regulators, including many factors that allow pharmacological modulation like histone deacetylases and the histone demethylase LSD1. REST repression in non-endocrine cells activates the promoters of important beta cell transcription factors, including PAX4 and PDX1 and is a key step in reprogramming to insulin-producing cells [23–26]. Future studies will show whether REST is critical in restricting ductal differentiation potential and may be a target for inducing beta cell neogenesis from duct cells.

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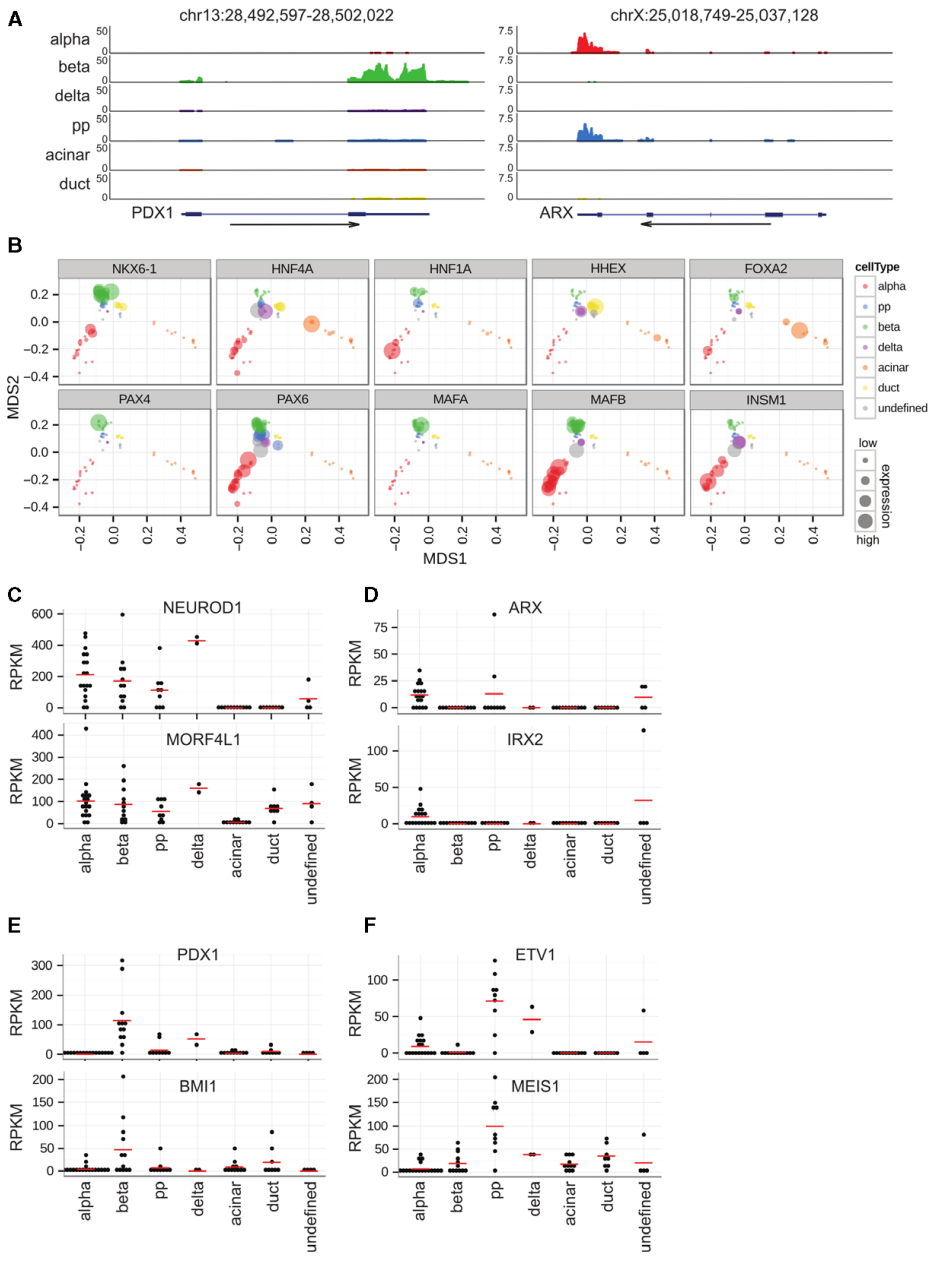

Figure 2. Expression of cell type-specific transcription factors at single-cell resolution.

A Merged UCSC Genome Browser tracks for the *PDX1* and *ARX* loci. The respective tracks for all single cells are presented in Appendix Fig S2.

B Relative expression (scaled RPKM value) of important transcription factors represented by bubble size and projected onto the MDS profile.

C–F Cell type-specific expression of pan-endocrine (C), alpha cell (D), beta cell (E), and PP cell (F) transcription factors (red bar: mean expression). The statistical significance of the differential gene expression is presented in Appendix Fig S6.

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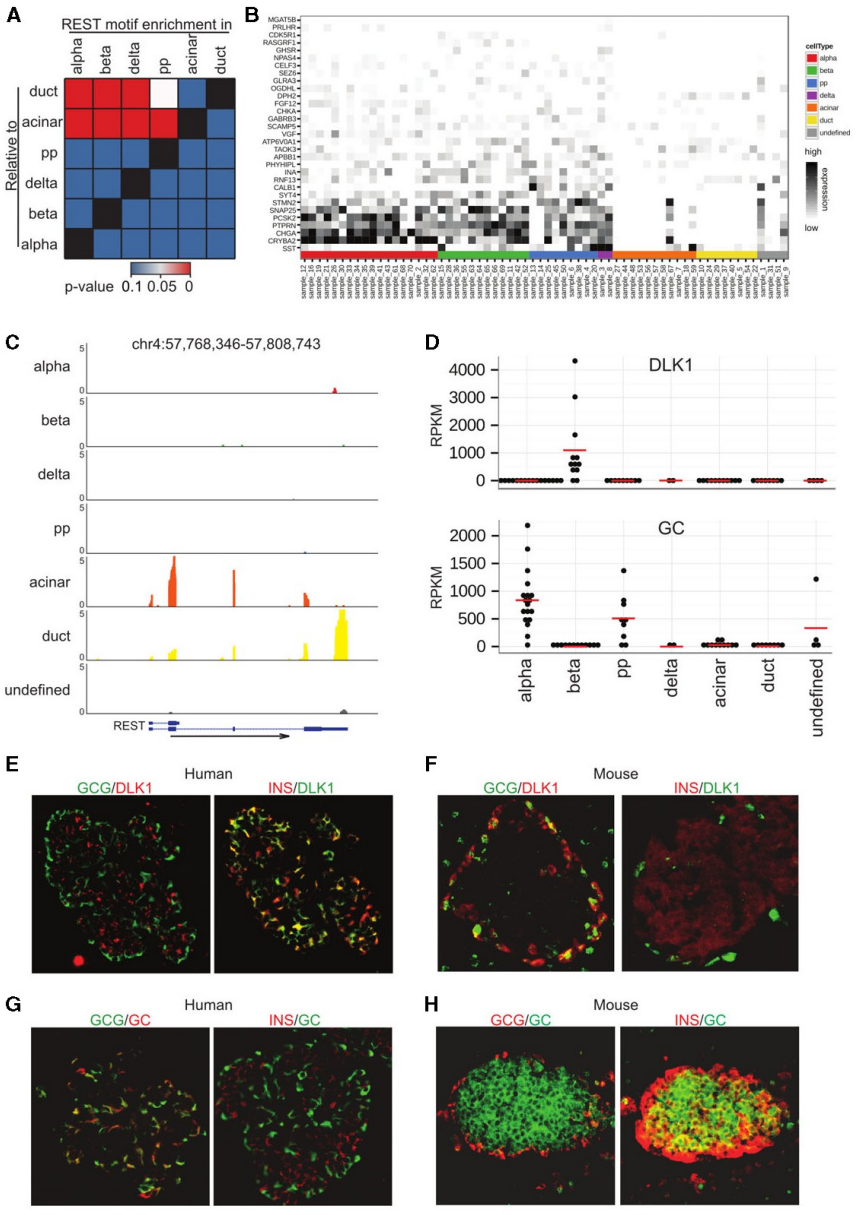

Figure 3. Single-cell transcriptomes reveal unique features of human islets.

A Heatmap displaying the *P*-values obtained by pairwise Gene Set Enrichment Analysis (GSEA) for the REST-binding motif.

B Relative expression (scaled RPKM value) of genes contained in the REST-binding motif gene set.

C Merged UCSC Genome Browser tracks for *REST*. The respective tracks for all single cells are presented in Appendix Fig S3.

D Expression of DLK1 and GC in human islet cell types (red bar: mean expression). The statistical significance of the differential gene expression is presented in Appendix Fig S6.

E–H Co-staining of *DLK1* (E, F) or *GC* (G, H) with insulin and with glucagon in representative human (E, G) and mouse (F, H) islets.

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Finally, in a third example, we focused on differences between mouse and human islets. Previous studies have noticed such differences regarding the overall architecture and specific physiological properties [7,27]. Our human islet single-cell transcriptomes confirm that the expression of hormones and canonical transcription factors is conserved between human and mouse. However, two genes—*GC* and *DLK1*—that are among the most characteristic for human alpha and beta cells, respectively, are expressed in opposite patterns in the mouse. Both *DLK1* and *GC* are relevant to diabetes [5,28], and further research is necessary to dissect their roles in both human and mouse islet biology.

These examples highlight the utility of the current single-cell transcriptome database for islet biology. In addition, we expect future growth of our resource with the addition of single-cell expression data from diabetic donors and from islets treated with drugs and metabolites *ex vivo*, contributing to the utility of the presented resource for studies on all aspects of human islet biology. In summary, our study establishes a transcriptional dataset for all the cell types in human pancreatic islets with single-cell resolution and defines distinctly human features in the patterns of alpha and beta cell-expressed genes.

Materials and Methods

Reagents

Antibodies used in this project are directed against insulin (Sigma I8510), glucagon (Abcam ab92517), DLK1 (R&D MAB1144-100), and GC (Abcam ab81307). The sequences of primers for actin have been published recently [29]. All the fluorescently labeled secondary antibodies were purchased from Life Technologies Corporation. The reagents used for the Smart-seq2 protocol for cDNA synthesis, amplification, and sequencing library preparation have been published recently [17].

Cell culture

Human islets were provided through the JDRF award 31-2012-783 (ECIT: Islet for Research program). They were from a 37-year-old male donor whose BMI was 22. Islets were cultured in CMRL medium (Life Technologies) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Islets were collected following overnight culture after receiving them. To disassociate islets into single cells, islets were incubated in Accutase (Life Technologies) in 37°C for 20 min, neutralized by CMRL medium. Purification of single cells was performed by flow cytometry cell sorting on a Moflo AstriosEQ (Beckman Coulter, Miami) as previously described in [16].

Immunofluorescence

The human pancreatic histology slides were ordered from Abcam (ab4611). The mouse pancreatic histology slides from 129SV mice were gifts from Patrick Collombat. The staining followed a published protocol [30]. Briefly, the paraffin was removed from the tissues. Afterwards, rehydration and antigen retrieval was performed. The tissues were blocked by 3% BSA for half an hour and incubated overnight at 4°C with primary antibodies in 1:1,000 dilutions. After washing with PBST, tissues were incubated with secondary antibodies and Hoechst 33342 for half an hour. Finally, the slides were mounted and sealed with nail polish and images were taken with Leica CRT6000.

Single-cell RNA-seq sample and sequencing library preparation

cDNA synthesis and enrichment were performed following the Smart-seq2 protocol as described Picelli *et al* [17]. ERCC spike-in RNA (Ambion) was added to the lysis buffer in a dilution of 1:1,000,000. Library preparation was conducted on 1 ng of cDNA using the Nextera XT library preparation kit (Illumina) as described Picelli *et al* [17]. Sequencing was performed by the Biomedical Sequencing Facility at CeMM using the 50 bp single-read setup on the Illumina HiSeq 2000/2500 platform.

qRT–PCR

After the cDNA was synthesized and amplified from single cells, quantitative PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) on a LightCcycler 480 qPCR instrument (Roche).

Single-cell RNA-seq data processing

The raw sequencing data were processed using a custom bioinformatics pipeline which consists of the following main steps: (i) trimming of contaminating sequencing adapter sequences, (ii) alignment of the trimmed reads to the human transcriptome as well as genome, (iii) calculation of expression estimates for each transcript, differential expression analysis and visualization as genome browser tracks.

Trimming of adapter sequences was performed with trimmomatic (v 0.32). Only reads with a minimum length of 25 bp after adapter trimming were included in the downstream analysis. Alignment of the trimmed reads to the human transcriptome (hg19 GRCh37 ftp://ftp.ensembl.org/pub/release-74/fasta/homo_sapiens/cdna/Homo_sapiens.GRCh37.74.cdna.all.fa.gz) was performed with bowtie1 (v 1.1) [31] recording up to 100 different mapping positions for each read which takes into account that one read might originate from any of the different transcripts of one gene. Alignment to the human genome (hg19/GRCh37) was performed using Tophat (v 2.0.13) [32]. These genomic alignments were purely performed for the purpose of visualization in genome browser tracks. Conversion of the alignment files to the files needed to display the data as genome browser tracks (bigWig) was performed with RSeQC (v 2.3.9) bam2wig.py followed by UCSC tools' wigToBigWig. Calculation of normalized transcript-wise expression estimates (rpkm values) as well as differential expression analysis was performed based on the transcriptome alignments using the R (v 3.1.2) package BitSeq (v 1.10.0) [18]. In order to correct for potential biases in the read distribution, the BitSeq function getExpression() was run with the "uniform" option disabled.

Quality filtering

The minimal number of reads needed to obtain reliable RPKM values as estimates of gene expression was determined by taking

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advantage of a synthetic RNA mix consisting of 92 RNAs covering a 10^6-fold concentration range (ERCC spike-in controls) that had been carried along through the entire library preparation and sequencing process with each single cell. Starting from ~25 reads per transcript, we observed the expected linear relationship between ERCC transcript abundance and measured RPKM values (Fig EV1B). For the purpose of noise reduction, we defined transcripts covered by less than 25 reads as "not expressed" and set their RPKM values to a minimal value. Furthermore, 6 samples showed less than 500 (arbitrary cutoff) reliably covered transcripts and were excluded from the analysis (Fig EV1A).

Grouping the single cells based on their gene expression profiles

In order to determine groups of cells with similar expression profiles and at the same time identify the primary defining genes for each group, we performed a stepwise principal component analysis (PCA) based on the quality-filtered expression values. PCA was performed using the function prcomp() in R. The results were displayed as a biplots showing samples (cells) as dots and the most highly loaded variables (transcripts) as vectors. Biplots were constructed using a slightly modified version of the R function ggbiplot() (https://github.com/vqv/ggbiplot).

External data

External RNA-seq raw data (next-generation sequencing reads) for bulk samples of human acinar cells, beta cells, and islet cells were obtained from ArrayExpress (E-MTAB-1294: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-1294/samples/) [20]. We used the samples HI10 (islet), HI25 (islet), HI32 (islet), HIE1 (beta cells), HIE2 (beta cells), and acinar tissue donor (acinar cells). External data were processed using the same pipeline as the single-cell data. For the comparison of external and single-cell as well as 500 cell data by multidimensional scaling, batch effect correction was performed using the function ComBat() of the R package sva.

Defining cell type-specific gene expression profiles

Cell type-specific gene expression profiles were defined by performing pairwise differential expression analysis between all previously defined groups of cells. Differential expression analysis was performed using the function estimateDE() of the R package BitSeq. For each cell type in each comparison, the specificity of the expression of each transcript was deduced under consideration of effect size (absolute difference and log2 fold change) as well as statistical significance (probability of positive log ratio, PPLR) of the measured differential expression. Technically, for each comparison, all transcripts were ranked by absolute difference in gene expression, log fold change of gene expression, and probability of positive log ratio and a combined rank for each transcripts was produced by selecting the worst (i.e., highest) of these three ranks as a representative rank. Finally, the representative ranks from all comparisons for each cell type were again combined by selecting the worst rank for each transcript (Appendix Fig S7). Therefore, the lower the combined rank, the more specific the expression of the respective transcript for the assessed cell type. To identify the cell type for which the expression of a given gene is most specific, we compared the assigned combined ranks between all cell types and selected the cell type that showed the lowest combined rank for this gene.

Assessing cross-contamination between cell types

We assessed potential cross-contamination between two cell types using a four-step approach: (i) selection of cell type-specific genes (profile genes), (ii) selection of the purest single cells for each cell type (profile cells), (iii) calculation of pure and increasingly contaminated gene expression profiles *in silico* (mix profiles), and (iv) identification of the mix profiles that best match the expression profile of each single cell.

As profile genes, we selected all genes among the top 500 cell type-specific genes for each of the two cell types that showed an absolute mean expression difference of greater than 0.5 and a relative mean expression difference of at least twofold. This selection resulted in 233 profile genes for alpha cells and 252 profile genes for beta cells.

To identify the purest cells of each cell type, we calculated a weighted mean of scaled expression values (sample-wise, scale 0 to 1; lower percentile: 0.05, upper percentile: 0.95) for both groups of profile genes for each single cell (profile scores). We used a rank-based weighting system in order to give more power to more cell type-specific profile genes. All single cells were then plotted according to their profile scores, and per cell type, the three cells with the most cell type-specific profile scores (highest distance to the diagonal) were selected as profile cells (Fig EV2A).

Pure expression profiles consisting of both groups of profile genes were calculated as the mean expression values of the three profile cells. We then used these two cell type-specific profiles to artificially construct expression profiles that represented different degrees of contamination by computationally mixing the two profiles in different ratios. Specifically, we calculated weighted means of the two pure expression values for each profile gene, with the weight increasing from 0 to 100 in steps of 1 for one of the pure profiles and at the same time decreasing from 100 to 0 for the other pure profile. This resulted in 100 profiles, two pure (cell type specific) and 98 mixed profiles (Fig EV2B).

We then calculated the Pearson correlation of each of the artificial 100 profiles with the actual expression profiles of each of the single cells (Fig EV2C) and selected the highest correlating mix profile for each single cell. These selected mix profiles represent the fraction of variance in profile gene expression that is explained by either of the two cell type-specific profiles as well as the fraction of variance that remains unexplained (Fig EV2D and E).

Gene set enrichment analysis

Binding motif analysis was done with Gene Set Enrichment Analysis (GSEA) [33,34]. For each single cell, the most highly expressed transcript was selected as representative for the respective gene. Finally, gene expression values for each cell type were found by calculating the median across all cells of a particular cell type. These median expression values were used as input for GSEA. Genes that were not found to be expressed in any of the cell types were removed from the input dataset. Pairwise comparisons were done among all six

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assigned cell types except the "undefined" amounting to 30 comparisons in total. The REST-binding motif was significantly enriched ($P < 0.05$, FDR < 25%) in all of the comparisons between endocrine cell types and exocrine cell types.

GWAS analysis

GWAS results relevant for diabetes (search for "diabetes") were downloaded from the GWAS catalog (https://www.ebi.ac.uk/gwas/). We categorized the reported traits into type 1 and type 2 diabetes according to whether "1" or "2" appeared in the trait description. Each gene that was identified as significant in a GWAS (reported gene) was assigned to the cell type for which it was identified as most specific (see "Defining cell type-specific gene expression profiles"). Because in this analysis specificity among the endocrine cells (alpha cells, beta cells, delta cells, PP cells) and among the exocrine cells (acinar cells, duct cells) was not paramount, cell type specificity was determined only in comparison with cell types of the other group. This approach was chosen in order to not dismiss genes as unspecific if they are endocrine or exocrine specific but not necessarily cell type specific. The eight MODY genes were taken from [21].

Data deposition

Sequencing datasets described in this work have been deposited in the Gene Expression Omnibus (GEO) repository under accession number GSE73727.

Expanded View for this article is available online.

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Author contributions
SK, CBo, MF, JL and TP conceived and designed the study; CBa and EB provided human islets; JL, AS and MF performed the experiments; TP and CBo generated next-generation sequencing data; JK processed the raw data; JK and JL performed the bioinformatic analysis; SK, CBo, MF, JL and JK wrote the manuscript with contributions from all co-authors.

Conflict of interest
The authors declare that they have no conflict of interest.

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Expanded View Figures

Figure EV1. Statistical analysis of single-cell RNA-seq data.

A Number of detected transcripts and total aligned reads for each single cell. The red line denotes 500 transcripts, below which samples were excluded from the analysis.

B Scatter plots displaying the correlation between the number of input ERCC RNA molecules and measured RPKM values in four representative single cells. The Pearson correlation (r) is noted in the upper left corner.

C Scatter plots correlating raw read counts (left) and RPKM normalized expression values (right) with transcript length. All adequately covered transcripts (> 25 reads) of all 64 samples were included in this analysis. The observed negative correlation after RPKM normalization lies in the range of what had been reported previously [19].

D Raw counts and RPKM normalized expression values for 10 groups of ERCC spike-in controls. The amount of molecules spiked into the sequencing reaction is constant within one group, whereas the length of the transcripts varies considerably. Within each group, equal expression values across different transcript lengths thereby confirm that RPKM normalization does not systematically penalize longer transcripts in the assessed ERCC transcript length range of ~200 to 2,000 bp. ERCC spike-in controls covered by more than 25 reads are indicated in blue and those with ≤25 reads in red.

E MDS displaying transcriptional similarity between corresponding cell types of a published dataset [20] (prefix: ext) and the current dataset.

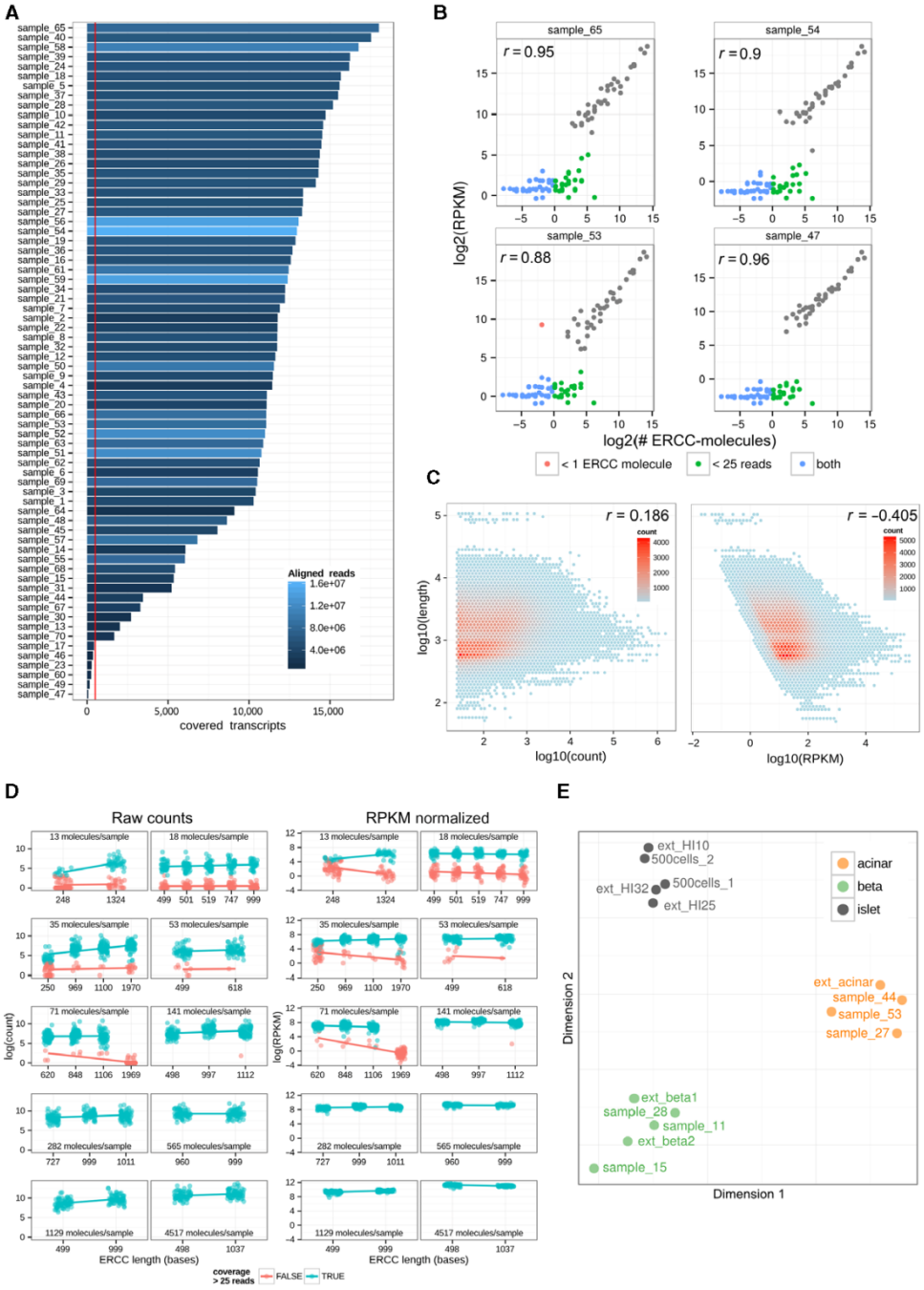

Figure EV1.

Figure EV2. Assessing cross-contamination between alpha and beta cells.

A Scatter plot displaying single alpha and beta cells, 500-cell islet samples, as well as bulk islet and beta cell samples from published datasets according to their weighted mean of scaled expression values in alpha and beta cell-specific profile genes. The three selected profile cells for each cell type are indicated by their sample ID.

B Pure and mixed expression profiles consisting of 233 alpha cell-specific genes and 252 beta cell-specific genes. Alpha and beta cell-specific profiles are calculated based on the expression values of the three selected profile cells only, while profile genes were selected based on all single cells classified as alpha or beta cells, which is why the expression gradients in the mix profiles do not always follow the same direction.

C Profile correlation curves for each individual sample. The maximum of each curve defines the maximum variance that can be explained (*y*-axis) by the corresponding mix profile (*x*-axis) providing a measure for the composition of the respective sample.

D Diagram explaining the transition from profile correlation curves to sample composition estimates. The profile composition that explains most variance is linearly scaled to the maximum variance explained.

E Sample composition estimates for each assessed sample. The differences between the 500-cell islet samples and bulk samples might be explained by technical effects that enrich for alpha cells during islet cultivation, disassociation, and FACS purification.

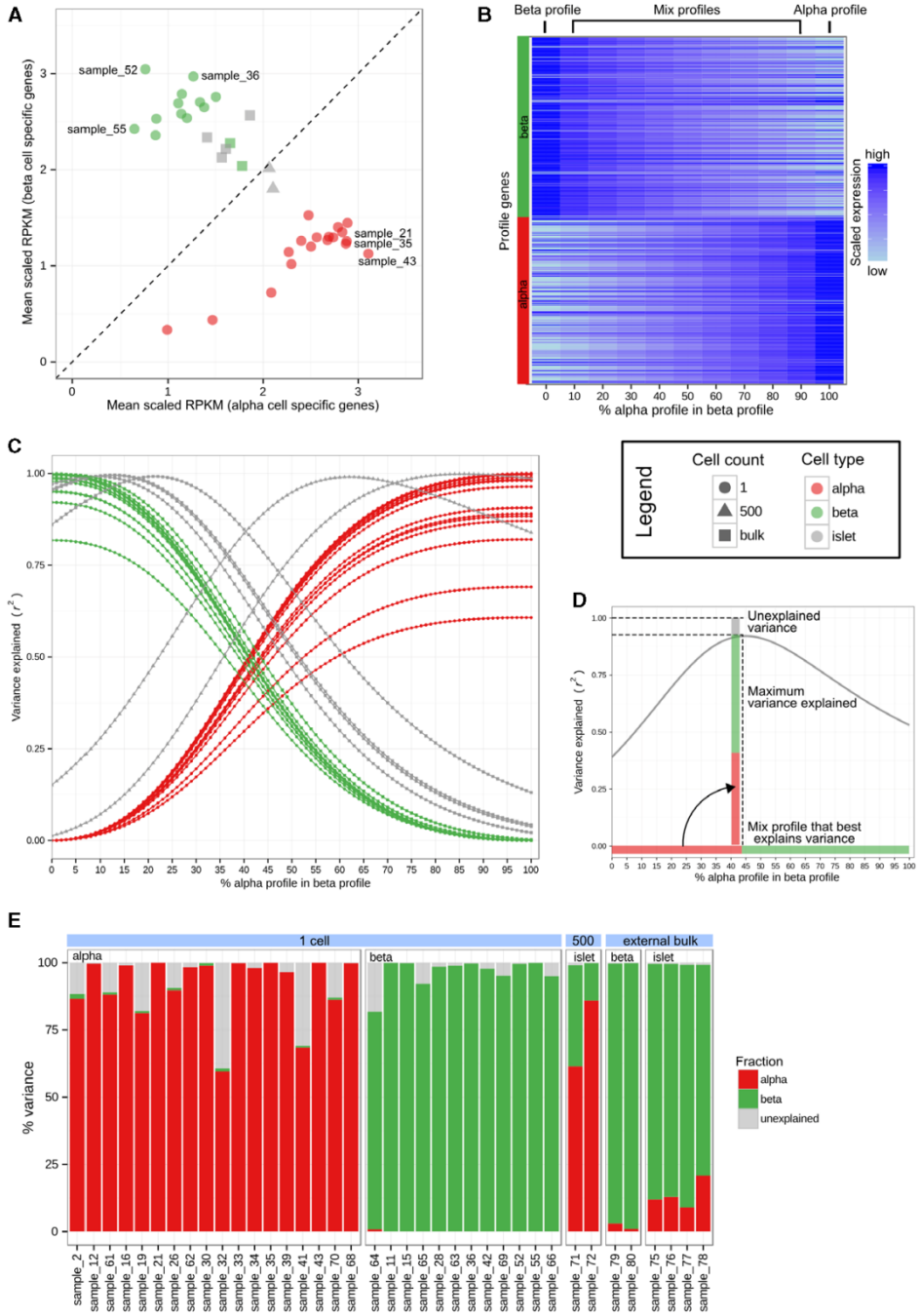

Figure EV2.

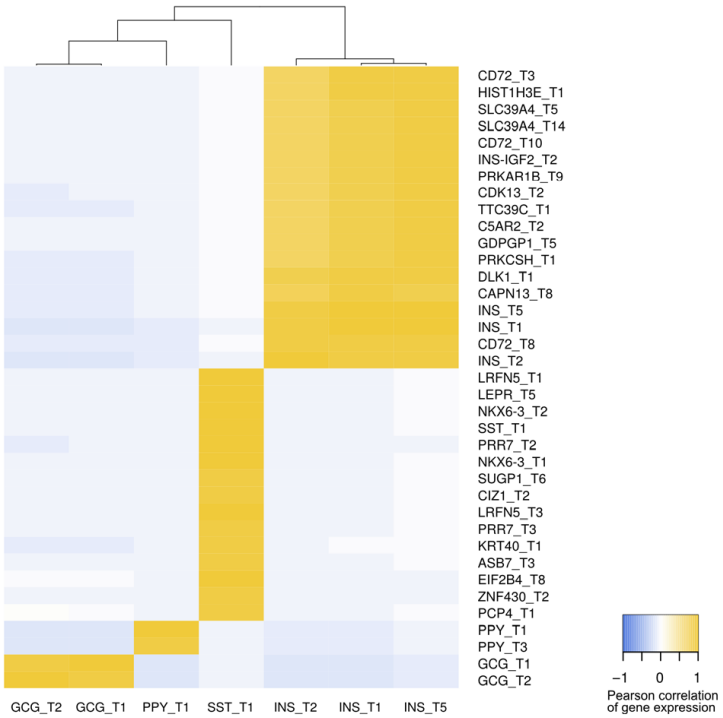

Figure EV3. Assessing pairwise correlation of endocrine marker genes.
Correlation matrix displaying all genes (*y*-axis) that are highly correlated ($r > 0.9$) with at least one of the endocrine marker genes (*x*-axis). Different transcripts of the same gene are indicated by "Tx".

Figure EV4. Specific expression of selected marker genes.
Relative expression (scaled RPKM value) of interesting genes across all single cells represented by bubble size and projected onto the MDS profile as displayed in Fig 1D.

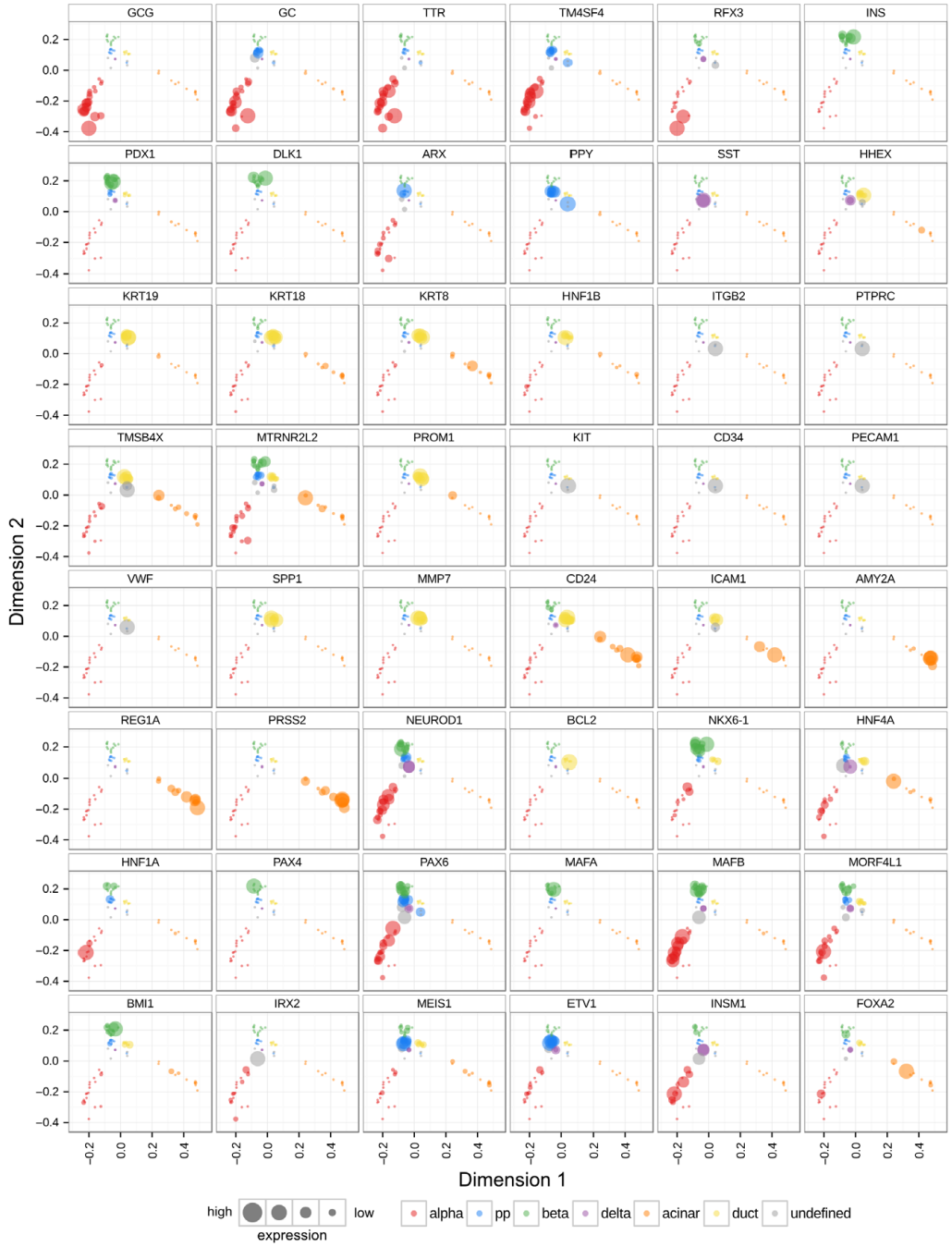

Figure EV4.

Figure EV5. Assessing cell type specificity of genes identified in diabetes-related GWAS.

A Cell type specificity for genes reported in diabetes-related GWAS. Each gene reported in a diabetes-related GWAS (search for "Diabetes" on GWAS Catalog) was assigned to the pancreatic cell type in which it was found to be most specifically expressed. Ranking was performed as described in Appendix Fig S7B and Dataset EV6.

B Heatmap showing mean expression values for the most cell type-specific diabetes-associated GWAS genes in the different here identified human islet cell types. Specifically, only genes with a specificity rank lower than 500 (dashed line in panel A) are listed, and genes with equal expression in multiple cell types are not shown. The numbers in the colored boxes indicate the number of studies in which the respective gene has been reported. Heatmaps are colored by mean ln(RPKM), the mean of the natural logarithm of the RPKM values across all cells of the respective cell type.

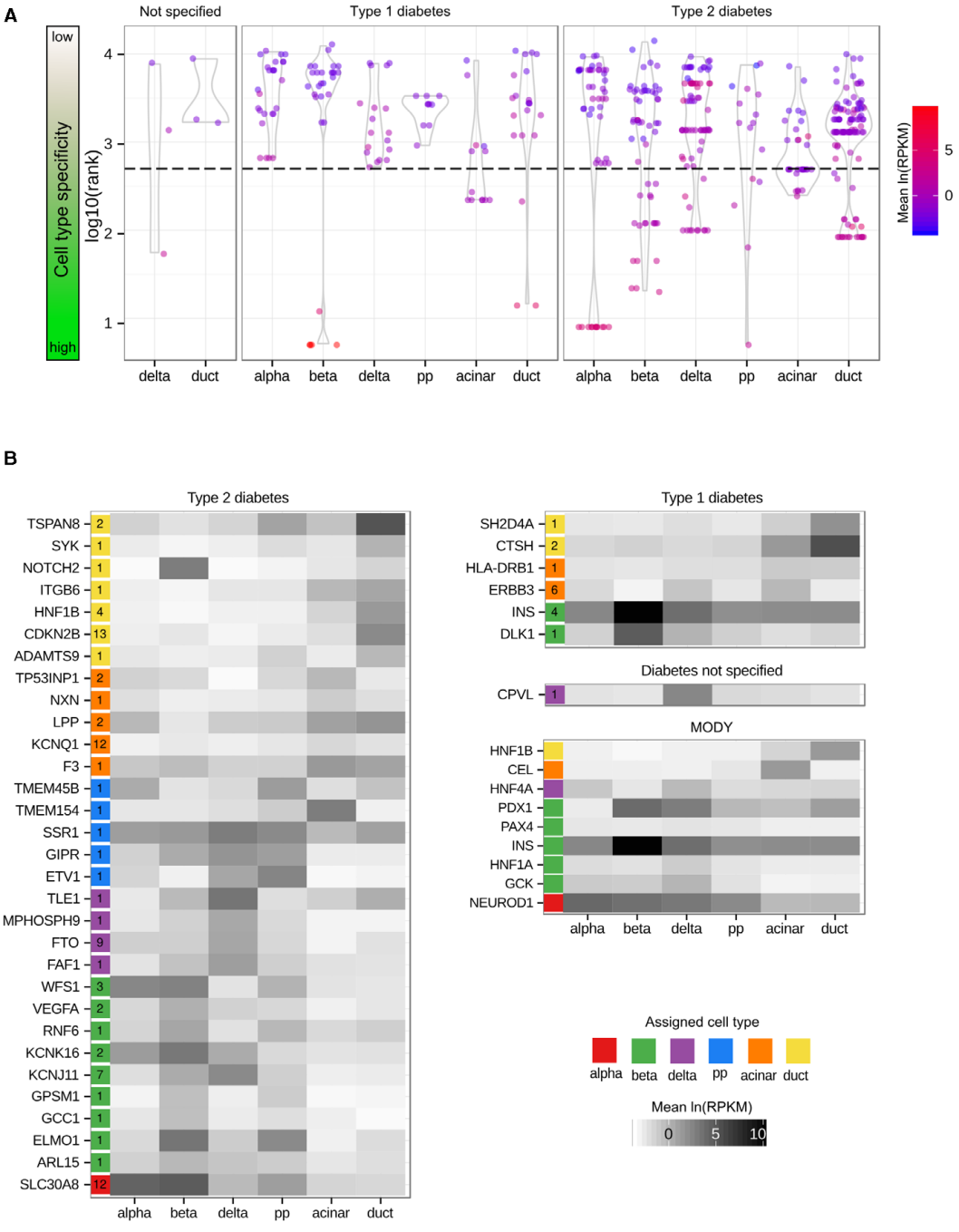

Figure EV5.

Appendix to "Single-cell transcriptomes reveal characteristic features of human pancreatic islet cell types"

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Key words: Single-cell RNA-seq / human pancreatic islets / beta cells / alpha cells / marker genes

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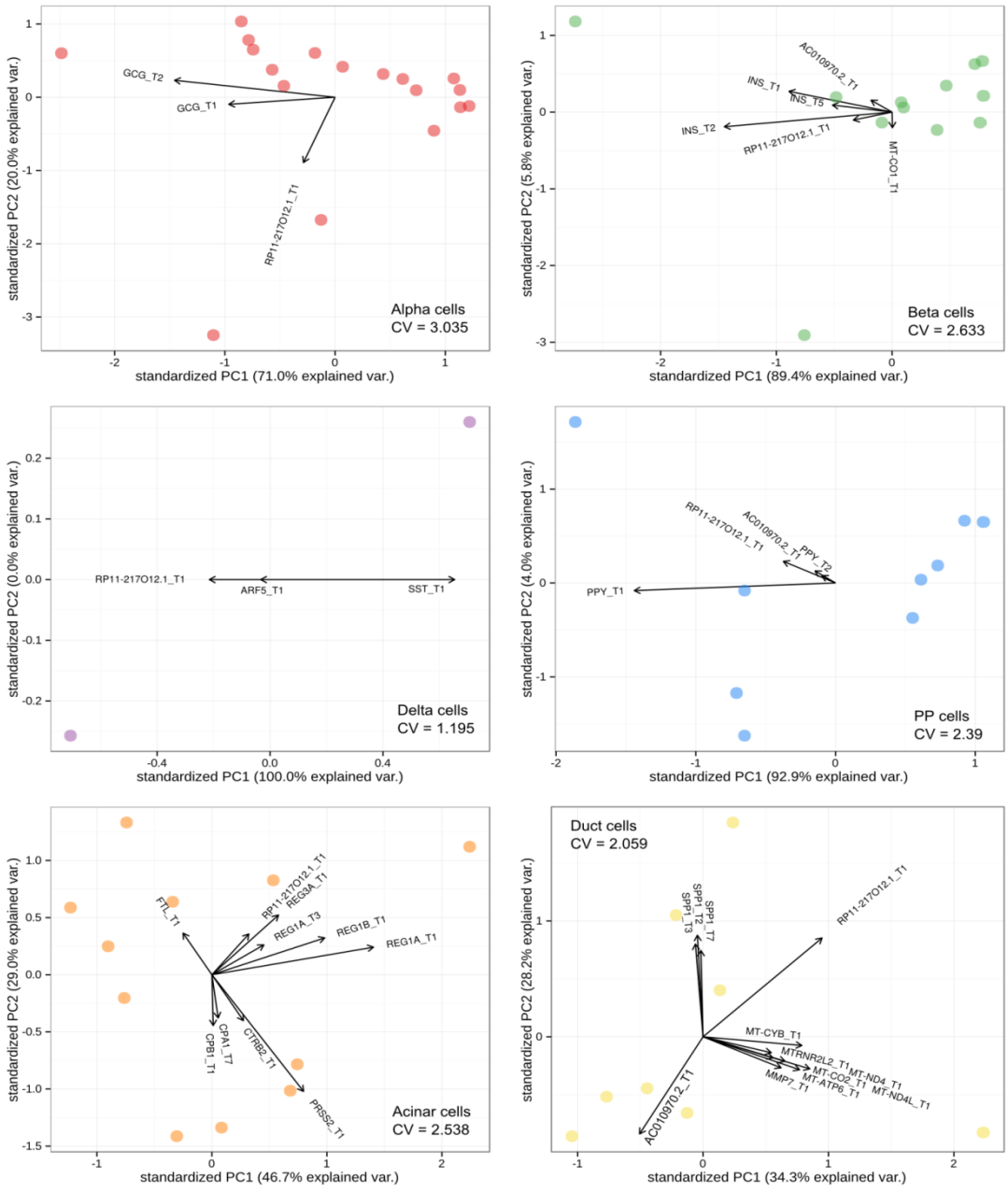
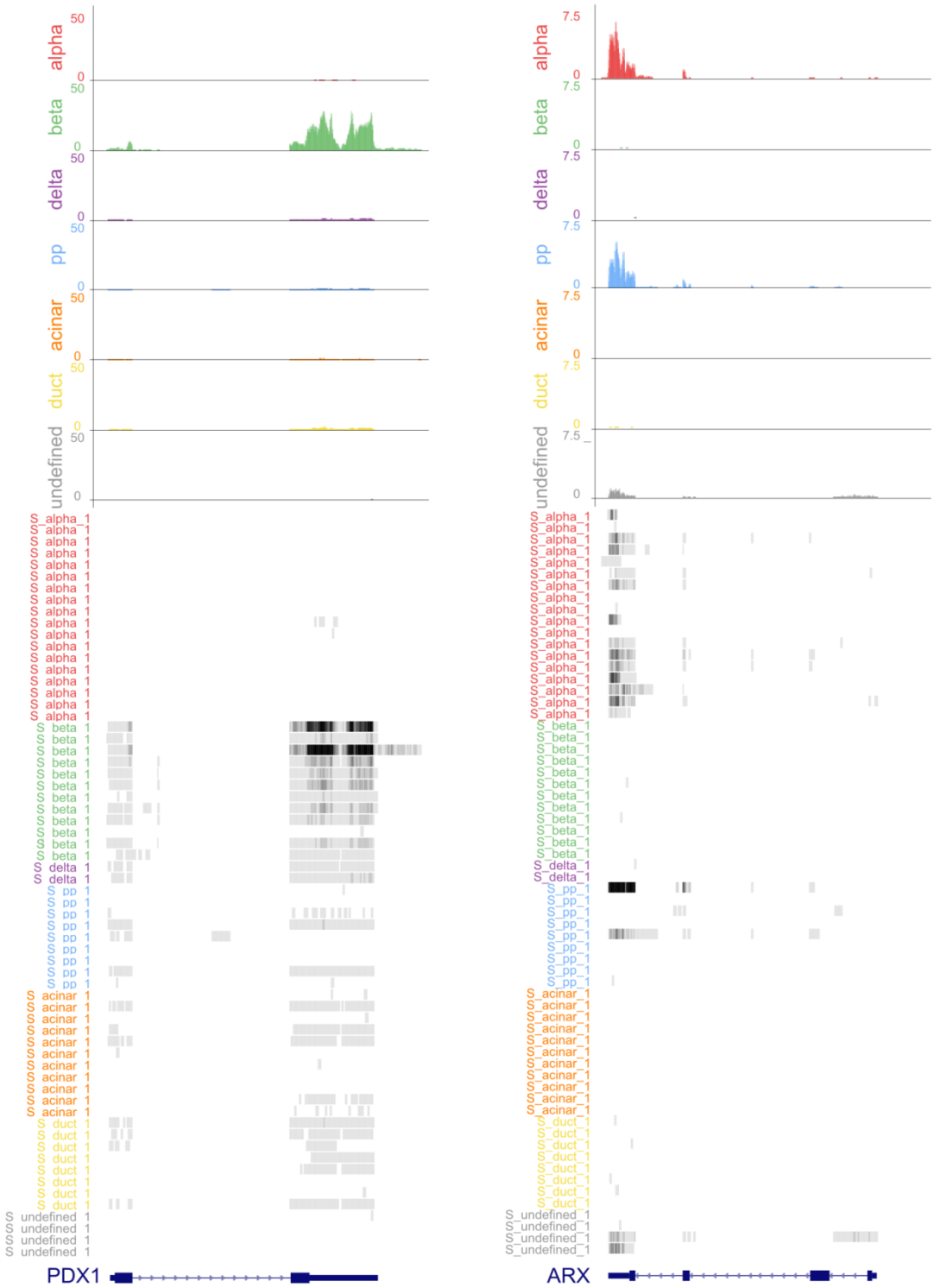
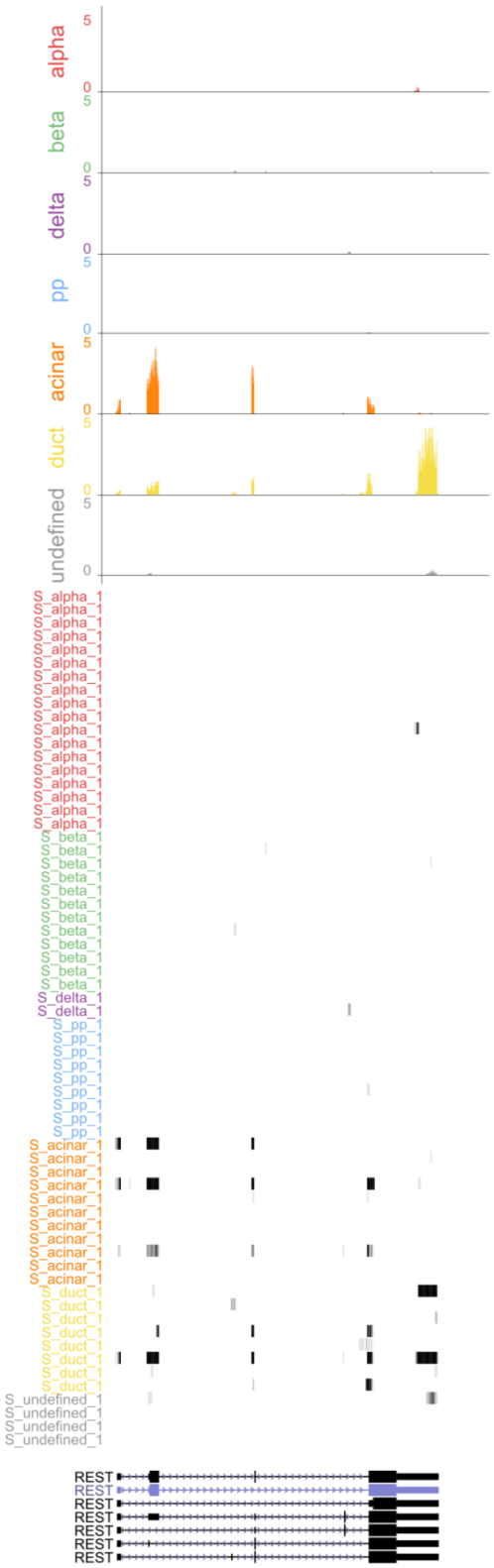


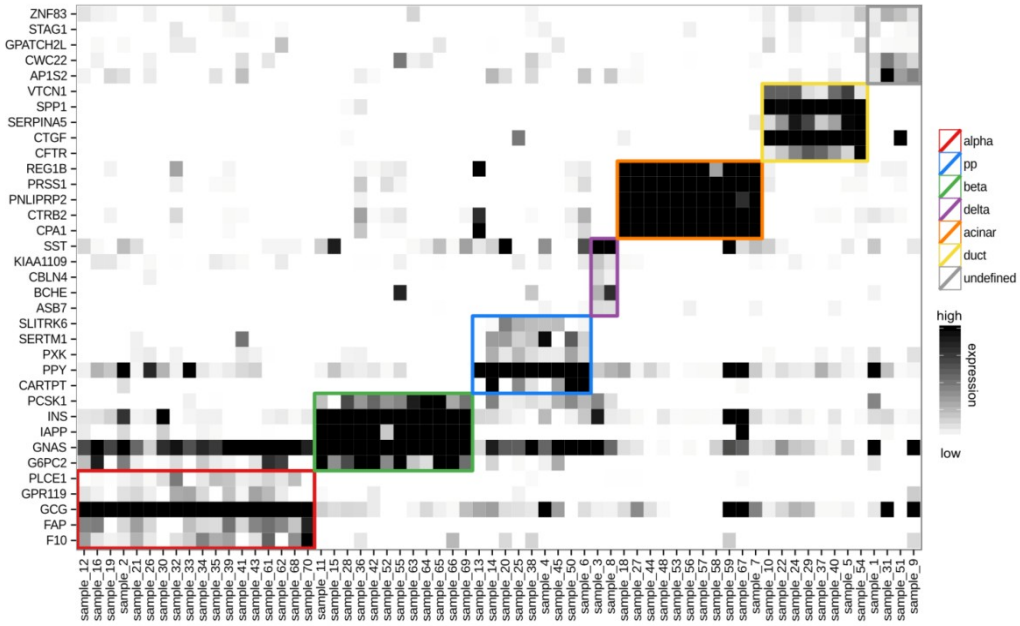
Appendix Figure S1: Assessment of heterogeneity within each identified human islet cell type
Separate biplots for each of the six identified human islet cell types show considerable variability within cell types. Only the most loaded eigenvectors (transcripts) are displayed. The coefficient of variance (CV) was calculated per cell type based on all transcripts that were expressed in at least one of the single-cells of the respective cell type.



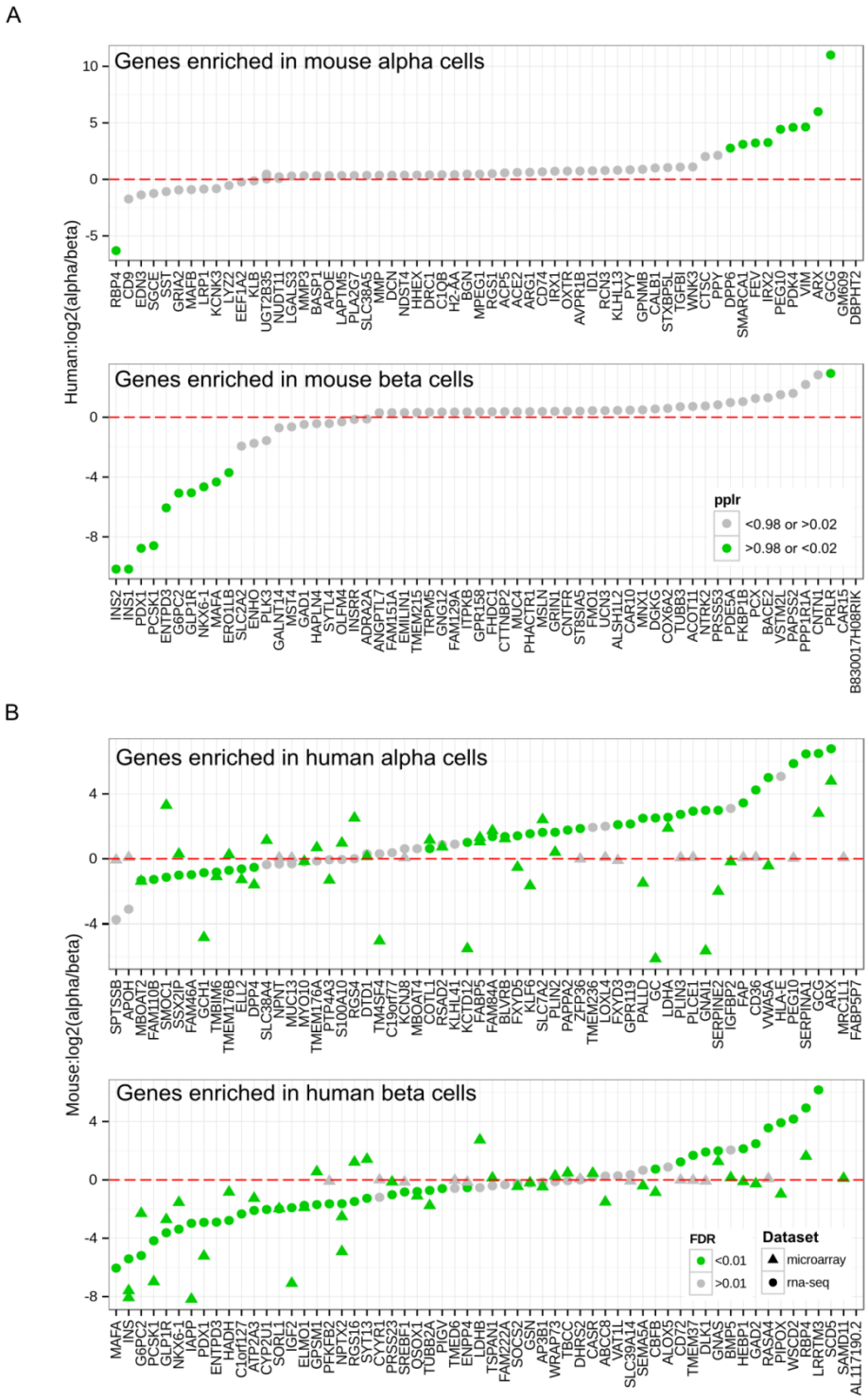
Appendix Figure S2: Single cell expression of PDX1 and ARX
UCSC Genome Browser tracks of PDX1 and ARX for all single-cells as well as merged tracks for each cell type. The scales for individual cells are 0-250 counts for PDX1 and 0-50 counts for ARX.



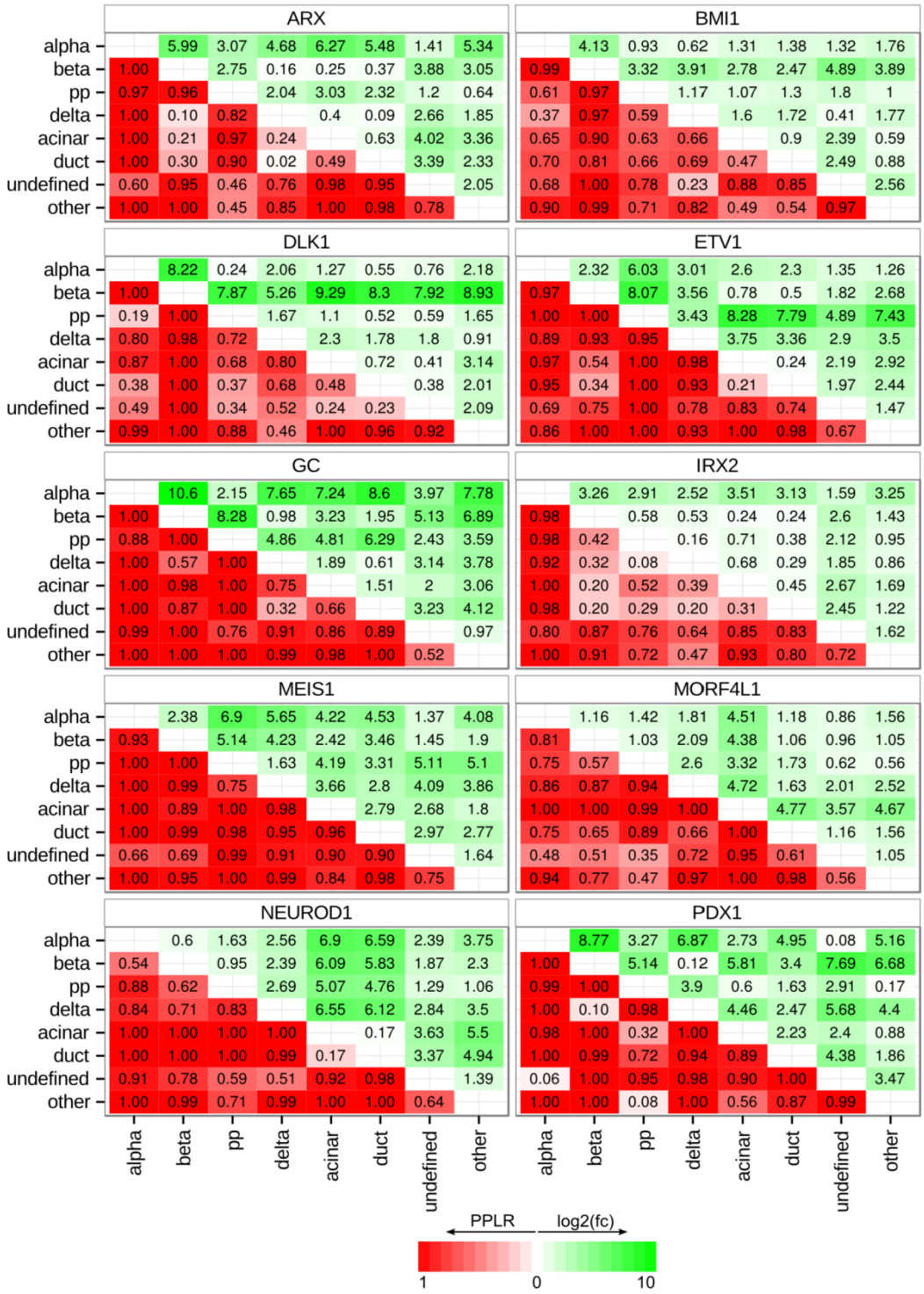
Appendix Figure S3: Single cell expression of REST
UCSC Genome Browser tracks of REST for all single-cells as well as merged tracks for each cell type. The scales for individual cells are 0-10 counts.



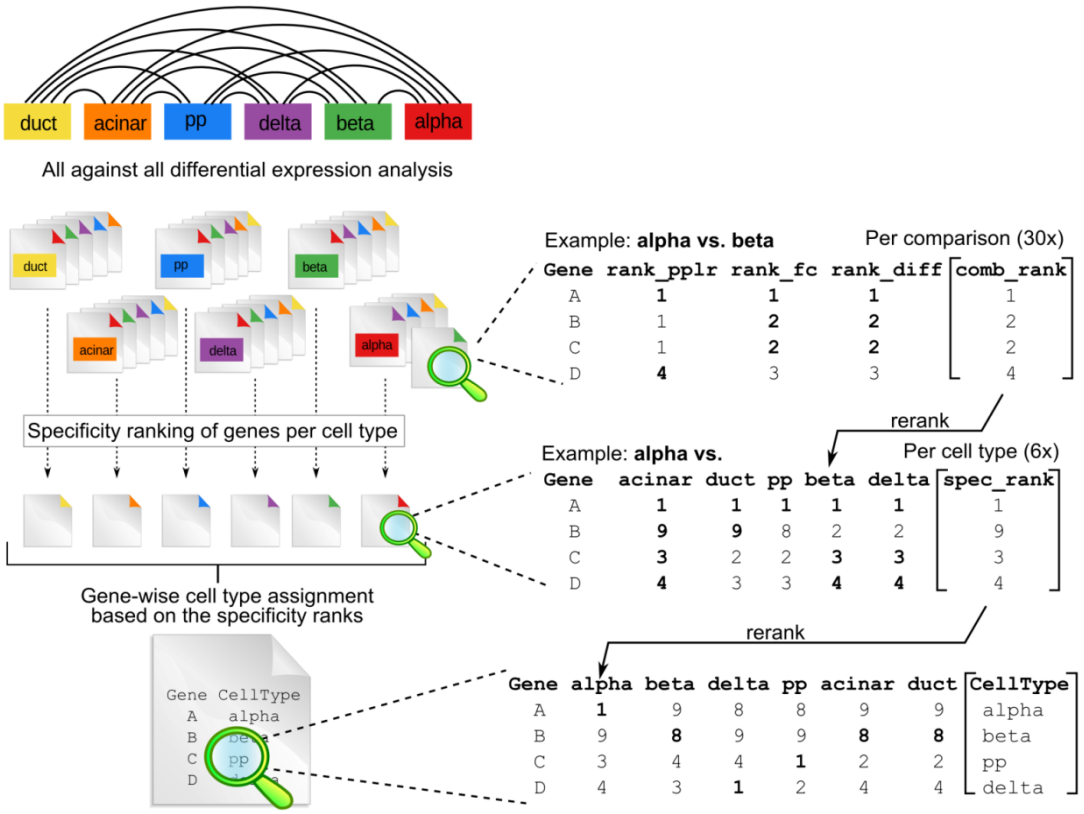
Appendix Figure S4: Identification of cell type specific transcripts
Heatmap displaying the expression patterns (scaled RPKM values) across all single cells for the top five cell type specific genes as determined by pairwise differential expression analysis.



Appendix Figure S5: Comparing alpha cell and beta cell specific genes between human and mouse
(A) Log fold change in RNA expression between human alpha and beta single-cells for genes identified as the 60 most alpha or beta cell specific genes in mouse. **(B)** Log fold change in RNA expression between mouse alpha and beta cells for genes identified as the 60 most alpha or beta cell specific genes in human single-cells. PPLR: probability of positive log ratio; FDR: false discovery rate.



Appendix Figure S6: Statistical significance of differential gene expression
Matrix showing the scaled probability of positive log-ratio (pplr: lower left) and log2 fold change (log2(fc): upper right) of pairwise differential expression analysis for individual genes displayed in Fig. 2C-F and Fig. 3D. "Other" signifies the comparison to all other cell types taken together.



Appendix Figure S7: Rank-based approach for defining cell type specific genes
Illustration of the rank based approach of defining cell type specific genes and assigning cell types to genes. PPLR: Probability of Positive Log Ratio; FC: Fold Change (expression); Diff: absolute difference (expression).

Artemisinins induce alpha to beta cell transdifferentiation by targeting GABA receptor signaling via gephyrin stabilization

The aim of this project is to discover chemicals which can induce alpha to beta cell transdifferentiation and with these chemicals to understand the mechanism of alpha to beta transdifferentiation. As preparatory work, a mouse cell line with inducible ARX misexpression was established. A chemical screen was performed on this cell line to look for functional ARX inhibitors. Artemisinins were identified as the top hits from the screening and their reprogramming effects were confirmed in mouse models and human islets. As molecular mechanism, artemisinins were found to bind to gephyrin which is a cofactor of GABA receptor. This interaction stabilizes gephyrin and enhances the GABA receptor signaling in mouse alpha cells. I used the single cell RNA-sequencing data established in the first project to investigate the cell type specific effects of artemisinins in human islets and observed artemisinins treatment induces loss of alpha cell identities and increase of GABA receptor subunits, consistent with the results observed in mouse alpha cells. In summary, from a chemical screen I discovered one group of approved drugs which can induce alpha to beta reprogramming. With chemical biology approaches I identified GABA receptor signaling is the critical part of this reprogramming.

Artemisinins induce alpha to beta cell transdifferentiation by targeting GABA receptor signaling via gephyrin stabilization

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Running Title: Artemisinins induce alpha to beta cell transdifferentiation

(195 words abstract, 3189 words in main text, 21804 characters in main text, 7 figures, 6 Supplementary figures, 52 references)

SUMMARY

Type 1 diabetes is characterized by the destruction of pancreatic beta cells, and generating new insulin-producing cells from other cell types has become a major aim of regenerative medicine. One promising approach is transdifferentiation of developmentally related pancreatic cell types including glucagon-producing alpha cells. In a genetic setting, overexpression of the master regulatory transcription factor Pax4 or loss of its counterplayer Arx are sufficient to induce the conversion of alpha cells to functional beta-like cells. Here we identify artemisinins as small molecules that functionally repress Arx and induce beta-cell characteristics in alpha cells. We show that the protein gephyrin is the mammalian target of these antimalaria drugs. Finally, we demonstrate that gephyrin-mediated enhancement of $GABA_A$ receptor signaling is the mechanism of action of these molecules in pancreatic transdifferentiation. Our results prove that gephyrin is a novel druggable target for the regeneration of pancreatic beta cell mass from alpha cells. Clinically approved antimalaria drugs artemether, artesunate, and dihydroartemisinin use this pathway to enhance beta cell characteristics in alpha cell lines and primary human pancreatic islets. These results should induce the clinical investigation of these drugs for regeneration of beta cell mass in C-peptide negative diabetes patients.

INTRODUCTION

Type 1 diabetes patients have often lost all functional beta cells, as indicated by the complete absence of insulin C-peptide from their serum. Replacing beta cell mass by pancreatic islet transplantation has been shown to be curative in essence, but is limited by the availability of donor islets, immunological complications and transplant survival (Shapiro et al., 2006). Therefore, attempts to regenerate patient-specific insulin-producing cells have been undertaken using different tissue sources, including ES, iPS, hepatic, exocrine, and other endocrine cells (Al-Hasani et al., 2013; Chera et al., 2014; Collombat et al., 2009; Kroon et al., 2008; Pagliuca et al., 2014; Sangan et al., 2015; Talchai et al., 2012; Zhou et al., 2008). In most cases, the approaches to increase beta cell mass have relied on the overexpression of master regulatory transcription factors involved in normal pancreas development, and in only a few cases small molecules or biologicals have been used (Fomina-Yadlin et al., 2012; Fomina-Yadlin et al., 2010; Kubicek et al., 2012; Pennarossa et al., 2013; Shen et al., 2013; Xie et al., 2013; Yi et al., 2013). Alpha cells are a particularly attractive starting point as they are developmentally closely related to beta cells. These cells have been shown to be able to replenish insulin-producing cells mass following extreme beta cell loss (Thorel et al., 2010; Ye et al., 2015). In a genetic model, overexpression of the transcription factor Pax4 converts mouse alpha cells to beta cells during development (Collombat et al., 2009) and when triggered in adulthood (Al-Hasani et al., 2013). Molecularly, the beta cell factor Pax4 acts by directly repressing the alpha cell master regulatory transcription factor Arx (Collombat et al., 2003) and loss of Arx alone is sufficient to convert alpha cells into beta cells (Courtney et al., 2013). So far, Pax4 and Arx-mediated alpha and

beta cell transdifferentiation events have been observed *in vivo* in the endogenous environment of a pancreatic islet of Langerhans. Thereby, it remains unclear whether they occur in a cell autonomous mechanism, or are dependent on other islet cell types and/or signaling from remote organs like the liver. To discriminate the two scenarios, we generated cell line models for inducible overexpression of pancreatic transcription factors and showed their applicability to identify small molecule modulators of the transdifferentiation process. From a representative library of approved drugs, we identified artemisinins as compounds that functionally counteract the alpha cell specific transcription factor ARX, thereby overcoming a major barrier for alpha cell transdifferentiation.

Artemisinins are among the most effective therapies for malaria, and based on their rapid toxicity to plasmodia are the preferred first-line treatment for the disease (Nosten and White, 2007). Despite being dispensed in more than 300 Million doses annually, the molecular mechanism of action of artemisinins is not clear. Different molecular targets have been proposed including heme binding and iron oxidation in human erythrocytes (Shukla et al., 1995), and the inhibition of the plasmodium-specific proteins sarco/endoplasmic reticulum Ca^{2+}-ATPase PfATP6 (Eckstein-Ludwig et al., 2003) or PI3K (Mbengue et al., 2015). Potent effects of these compounds are observed also observed on human cells, where artemisinins have been assigned to anti-inflammatory and anti-cancer properties (Ho et al., 2013, 2014). The effect of isolated artemisinins in the pancreas has not yet been evaluated, but limited evidence exist for positive effects of Artemisia extracts in animal models of type 1 diabetes (Ahmad et al., 2013) and in human patients (al-Waili, 1988).

A mammalian molecular target for artemisinins has remained elusive, and here we show that these compounds bind to gephyrin, a multifunctional protein primarily studied in neurons (Tyagarajan and Fritschy, 2014). Gephyrin exerts different functions including enzymatic activity in the synthesis of the molybdenum cofactor MoCo, regulation of mTOR signaling via direct interaction with mTOR (Sabatini et al., 1999), and structural roles in the transport of glycine and GABA receptors to the membrane (Fritschy et al., 2008). Interestingly, GABA has been proposed as a factor that can reverse diabetes by induction of beta cell proliferation (Soltani et al., 2011). We show that artemisinins increase GABA signaling and prevent glucagon secretion by alpha cells, resulting in the acquisition of beta cell characteristics. Long-term artemisinin treatment in mice results in increased islet size and beta cell mass, consistent with the expected phenotype. We show that these compounds induce the generation of insulin expressing from alpha cells in pancreatic islets from a linage tracing model. These effects are conserved in human islets of Langerhans, where alpha cells acquire beta cell characteristics after short-term compound treatment as proven by single cell transcriptomics. Functionally, these effects result in transcriptional repression of ARX and improved glucose-stimulated insulin secretion.

RESULTS

A cell line model for transcription factor mediated transdifferentation allows screening for functional ARX inhibitors

To discriminate cell-autonomous effects of transcription-factor mediated transdifferentiation from phenotypes that require paracrine and endocrine signaling in an islet microenvironment, we engineered mouse beta cell line Min6 to allow the inducible

overexpression of ARX (Fig. S1). Based on the known heterogeneity of the parental cell line (Nakashima et al., 2009), we isolated clonal derivatives with integrations of constructs for the doxycycline-controlled transcriptional activation of myc-tagged ARX (Fig. S1). While basal expression levels of alpha and beta cell genes varied in the different subclones, we observed that induction of ARX overexpression consistently activated the transcription of alpha cell genes and repressed beta cell genes in a time-dependent manner (Figure S2). These changes indicate that our cell lines faithfully model the beta to alpha fate switch upon ARX overexpression, which was previously only observed in animal models. Thereby, we have generated a cellular system that allows screening for functional repressors of ARX. To identify such compounds, we induced ARX expression and at the same time added compounds and then measured insulin levels after 72 hours. In control DMSO-treated samples we observed a 50% reduction in insulin levels compared to uninduced cells (Figures 1A and 1B). We then screened a library of 280 clinically approved small molecules selected for their structural and target diversity. Hit compounds were selected for their ability to maintain high insulin levels in the presence of ARX while not affecting cell viability (Figure 1C). All compounds that appear to counteract ARX in beta cells were tested for their ability to induce insulin expression in the alpha cell line aTC1-LT (Figure 1D). Interestingly, two artemisinins, artemether and its active metabolite dihydroartemisinin, not only fully inhibited the ARX overexpression phenotype in beta cells, but also induces insulin expression in pancreatic alpha cells (Figure 1E), as predicted for functional inhibitors of ARX. Importantly, Artemether treatment increased insulin expression in Min6-ARX induced cells and in alpha-TC1 cells in a dose-dependent manner (Figures S3A and S3B). To further understand the effects of artemether on alpha cells, we analyzed the changes in

the proteome of aTC1-LT cells treated for 72 h with artemether. Proteins with roles in oxidative phosphorylation and mitochrondrial localization were significantly altered, with some members showing increased, others decreased abundance. In line with artemisinins acting as functional Arx antagonists, glucagon was among the most dramatically decreased proteins after three days treatment (Figure 1F). Importantly, artemether treatment reduced the abundance of both proglucagon and intermedia processed glucagon peptides in alpha cells (Figure 1G).

Artemether induces insulin express in alpha cells by targeting gephryin

Based on these findings, we investigated the effects of additional artemisinin analogs in alpha cells. Several close structural analogs including arteether and artesunate show effects comparable artemether. In contrast, deoxyarteeether, an analog lacking the endoperoxide moiety, did not increase insulin expression in alpha cells (Figure S3C). To rule out the possibility that artemether induced insulin expression due to the generation of reactive oxygen species (ROS), we applied the ROS inducer elesclomol and detected no significant change of insulin expression in alpha cells (Figure S3D). However, the combination with the anti-oxidant N-acetylcysteine, that is known to suppress the anti-malarial properties of artemisinins (Arreesrisom et al., 2007), abolished the effect of artemether on insulin expression in alpha cells (Figure S3E).

To identify the molecular mechanism of artemisinins in pancreatic alpha cells, we first tested whether the proposed targets of the compounds in plasmodia, SERCA and PI3K, where involved. We used the SERCA inhibitor thapsigargin on alpha cells and did not observe increased insulin levels up to dose-limiting toxic concentrations (Figure S3F). Next we tested whether artemether-treatment of alpha cells resulted in reduced PI3P

and p-Akt levels, indicative of PI3K inhibition (Figures S3G and S3H). Rather than inhibition we observed a slight increase of both readouts, thereby ruling out a conserved mechanism of action based on the proposed plasmodium targets. Therefore, we turned to a chemical proteomics approach (Rix et al., 2012) to identify the proteins interacting with artemisinins in alpha cells. We coupled artesunate, an artemisinin active in alpha cells, to solid support and performed pull-down experiments in the presence and absence of competing free artemether (Figure 2A). Mass spectrometry identified Gephyrin as the most significantly enriched specific interactor (Figures 2B and S4A). Artemether increases gephyrin protein levels in alpha cells (Figure 2C and S4B), while mRNA expression levels remain unchanged (Fig S4C). To examine the interaction between gephyrin and artemisinins in a cellular context, we used a fluorescently labeled Artelinic acid probe. In alpha cells, this molecule shows highly significant costaining with gephyrin (Figure S4D). In a functional genomics experiment, knock-down of gephyrin in alpha cells abolishes the increase of insulin intensity by artemether treatment (Figures 2D-F).

Gephyrin-mediated increases of GABA signaling induces insulin in alpha cells

We next investigated if artemethers action on gephyring modulates its downstream signaling in MoCo synthesis, mTOR signaling and GABA receptor clustering. We observed increased MoCo synthesis in artemether-treated alpha cell extracts, indicative of enhanced gephyrin enzymatic activity (Figure S4E). A small proportion of endogenous gephyrin interacts with mTOR, and artemether inhibits this interaction (Figure 3A). For GABA receptor, which physically interacts with gephyrin, we observe increased mRNA and protein levels of several subunits (Figures 3B and S4C). RNA-Sequencing

experiments of alpha cells further underlined the effects of artemether on GABA receptor signaling. Gene set enrichment analysis identified the synaptic transmission process among the significantly altered pathways (Figure 3C), and we observed a significant upregulation of genes P2rx3, VAMp1 and Nrxn3 in the pathway (Figure S4F). To measure artemisinin effects on GABA signaling, we used automated patch-clamp technology. aTC1-LT cells pre-treated with artemether for 72 h alpha cells responded with an increased membrane current to stimulation with high dose GABA (Figure 3D). $GABA_A$ receptors are ligand-gated ion channels that open a chloride selective pore upon agonist binding. In line with activated GABA signaling we observe increased intracellular chloride levels in artemether treated alpha cells (Figure S4H). To prove that GABA receptor signaling plays a functional role in the mechanism of action of artemisinins in pancreatic alpha cells, we combined artemether with bicuculline or gabazine, two $GABA_A$ receptor antagonists. The presence of either compound inhibited the effects of artemether in aTC1 cells (Figures 3E and 3F). Importantly, the treatment with the GABAR agonist Thiagabine alone increased the insulin expression in alpha cells to levels comparable to those induced by artemisinins (Figure 3G).

Hormone secretion controls pancreatic cell type stability

GABA is normally co-secreted with insulin by beta cells, and suppressing glucagon secretion is one of the physiological functions of GABA signaling in alpha cells (Li et al., 2013; Rorsman et al., 1989; Wendt et al., 2004; Xu et al., 2006). Artemether treatment inhibits glucagon secretion by alpha cells in low glucose stimulation (Figure 4A). Interestingly, supplementing recombinant glucagon abolishes the effect of artemether on the insulin induction in aTC1-LT cells (Figure 4B). These data imply that maintaining the

proper concentration of glucagon in the close vicinity of alpha cells is critical for the stability of this cell type. In order to lower the extracellular glucagon concentration, we applied Decanoyl-RVKR-CMK (PCi), a highly potent, irreversible, cell-permeable inhibitor of proprotein convertases that inactivates prohormone convertase 2 at subnanomolar concentrations (Denault et al., 1995). Treatment of aTC1-LT cells with this compound resulted in a dramatic increase of intracellular glucagon (Figure 3C). At the same time, these alpha cells stained positive for insulin protein (Figure 3C). On the transcriptional level, the decrease of glucagon secretion repressed glucagon expression and significantly upregulated the transcription of beta cell related transcription genes Pax4 and Mnx1 (Figure 3D) These results suggest that artemether-mediated amplification of GABA receptor signaling prevents glucagon secretion, thereby reducing extracellular glucagon resulting in the loss of alpha cell identity.

Artemether induces alpha cells to express insulin expression in vivo

To characterize the physiological relevance of our finding, we examined the effects of artemether in a mouse models. We observe an increase in pancreatic islet size and beta cell mass following three months treatment with artemether in drinking water (Figures 4A and 4B). These changes occur in the absence of a dramatic shift in islet subpopulations or cell proliferation (Figure S5), thereby phenocopying Arx loss *in vivo* (Courtney et al., 2013). To prove the origin of new insulin-positive cells from alpha-cells we turned to a lineage tracing model. We isolated islets from mice harboring a construct for Cre recombinase under the control of the glucagon promoter and an integration of a floxed RFP allele in the Rosa26 locus, so that all glucagon-expressing alpha cells would permanently be RFP-positive. While in control DMSO treated islets we observed only a

background of approximately 1% of cells being double positive for insulin and RFP, 24 h treatment of these islets with artemether resulted in fourfold up-regulation of this cell population (Figure 4C and 4D). These data indicate that artemether induces a rapid induction of insulin expression in alpha cells within mouse islets, resulting in long-term expansion of the beta cell population and islet mass.

To understand the effects of artemether in alpha cell development, zebrafish larvae carrying the Glucagon-GFP and Insulin-mcherry reporters were treated with artemether from Day1 to Day5. Artemether treatment at 2.5 μM induced significant decrease of alpha cell mass at Day5 in zebrafish larvae (Figure 4E and 4F) without significant change of beta cell mass, indicating the suppressive effects of artemether on alpha cell development.

Single-cell transcriptomics reveals artemether effects in human alpha cells

We next tested the effects of artemether on human alpha cells in an islet microenvironment. Compared to control DMSO treated islets, 72 h artemether treatment increased the number of double-positive cells expressing both insulin and glucagon (Figure 6A and S6A,B). The effects of artemether or GABA on global transcriptional profiles were tested by bulk samples containing 500 cells. With Gene Set Enrichment Analysis (GSEA) (Mootha et al., 2003; Subramanian et al., 2005) we observed significant decrease of exocrine specific genes transcription with artemether or GABA (Figure S6C). When we calculated the overlaps of genes both upregulated by artemether or GABA (Core-upregulation) with endocrine or exocrine specific genes, we found a significant overlap between core-upregulation and endocrine gene set but not exocrine gene set (Figure S6D). The exocrine and endocrine gene sets were

established based our recent publication (Li et al., 2015). To investigate the cell type specific gene expression changes with artemether treatment, we performed single cell transcriptomics on human primary islets. We treated intact islets with artemether or DMSO, and after 72 h dissociated the islets, FACS-sorted single cells to individual wells of a 96-well plate containing lysis buffer and employed a Smart-Seq2 protocol (Li et al., 2015). We then represented the diversity transcriptomes of individual cells in a multidimensional scaling blot (Figure 6B). We observed that overall artemether did not induce a new cell type signature after that short treatment periods, and artemether treated alpha cells clustered with DMSO treated alpha cells. Then we compared the median expression of GABAA receptor genes in artemether vs. DMSO treated alpha cells. Consistent with the findings in mouse cell line, we identified strong increase of GABRB3 and GABRG2 (Figure 6C). To identify more subtle changes, we used GSEA to identify affected pathways on the same dataset (Figure 6D). We observed a strong and significant downregulation of cell type specific genes in artemether treated alpha cells (Figure 6E). This included genes EIF4A1, CRYBA2, PDK4 and MUC13 (Figure 6F). Similar analysis has been performed on beta cells. We observed significant upregulation of alpha cell genes in artemether treated beta cells (Figure S6E). One of the most significantly altered gene sets are catabolic processes. Interestingly, genes in these pathways are significantly higher expressed in beta cells compared to alpha cells. In line with conferring beta-cell character to alpha cells, artemether causes the upregulation of these genes in alpha cells, whereas it represses them in beta cells (Figures S7F and S7G).

Artemether improves insulin secretion in human islets

We next tested whether the reduction in alpha cell character and improved beta cell function would be detectable on intact human islets following artemether treatment. First we tested for the expression of alpha cell factor ARX (Figure 7A). While we observed donor-to-donor variability of the effect size, ARX expression was consistently reduced significantly. We then wanted to show whether the loss in alpha cell character corresponded to increase beta cells properties. We observed that normalized to total protein artemether treated islets contained more intracellular insulin compared to DMSO treated islets from the same donor (Figure 7B). Finally, we tested whether this insulin increase was functional and preformed glucose stimulated insulin secretion assays. While artemether treatment did not affect secretion in low glucose conditions, islets from all donors tested secreted significantly more insulin when challenged with high glucose concentrations (Figure 7C).

DISCUSSION

Artemisinin combination therapy is the treatment of choice for malaria and more than 300 million treatments are dispensed annually (Dondorp et al., 2010). Despite this large patient cohort, no clinical data on the effects of artemisinins on human pancreatic endocrine function have been published and for several reasons such changes might have gone unnoticed so far. The acute life-threatening condition of plasmodium infected patients together with the known propensity of plasmodium infection to cause hypoglycemia make blood glucose levels highly variable in the short term. Furthermore, in healthy individuals even a dramatic increase in beta cell number is not expected to cause a phenotype, as these cells only secrete insulin in a glucose-regulated manner. Unfortunately, currently no imaging methods are available to directly assess human beta cell mass. The ideal subject to study artemisinin effects on pancreatic function would be a type 1 diabetes patient with a complete absence of detectable insulin C-peptide, a condition that affects approximate 60% of T1D patients or one in 1500 children. We are currently trying to obtain blood samples from such patients who receive artemisinin-based combination therapies additionally for the treatment of malaria. If artemisinins also induce alpha to beta cell conversion in humans, we expect measureable C-peptide levels in post-treatment blood samples.

The short treatment cycle, negative effects of the plasmodium or other drugs in combination treatment or achievable artemisinin levels in the pancreas might still limit the clinical usefulness in this setting. However, even in that case our findings open up completely new avenues for drug discovery towards a treatment for type 1 diabetes by transdifferentiating alpha to beta cells. These could include structurally different gephyrin

stabilizers but also compounds that target other players in the GABA receptor signaling pathway.

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Materials and Methods

Reagents

Antibodies used in this project are insulin (Sigma 18510), glucagon (Abcam ab92517), Arx (R&D AF7068, Lot No. CFOM0211121), Myc (Cell Signaling Technology CST2276, Lot 19), Histone H2B (Cell Signaling Technology CST2934, Lot 1), Gephyrin (Abcam ab25784), GABA receptor subunit beta 3/4 (Millipore MAB341), GABA receptor subunit alpha 2 (Abcam ab72455), GABA receptor subunit gamma 2 (ABcam ab87328), Akt (Cell Signaling Technology CST4691), p-Akt (Cell Signaling Technology CST4060P). Artemether and primers were obtained from Sigma. The sequences of primers are published (Fomina-Yadlin et al., 2010). Cy-3–labeled donkey-α-guinea pig antibodyfrom Jackson ImmunoResearch. All other fluorescently labeled antibodies were purchased from Life Technologies Corporation.

Establishment of inducible cell lines

To generate inducible Min6 cells, we first transduced the cells with a lentivirus carrying TetR DNA and a neomycin resistance cassette (Gentarget Inc., cat.# LVP017-Neo) and then isolated a single clone expressing high amounts of TetR protein, validated with the Tet01 antibody from MoBiTec. This TetR expressing cell line was then transduced with virus particles coding for FlagMyc tagged (C-terminal) human ARX cDNA also expressing the puromycin resistance gene. The vector used for this work was the pSTK007, which is a modified version of the pLenti4.2 (Life technology). Then, a second round of clonal selection was performed where numerous clones were tested for maximum repression in the uninduced state and a high degree of induction upon treatment with doxycyline (1ug/ml).

All lentiviruses were produced in HEK293T cells using the Virapower plasmid mix (Life technology), according to the the manufacturer's protocol. All virus work was done in a Class II laboratory, and before transferring the cells to a regular lab the cells were proven to be virus free using a p24 ELISA kit (Cell Biolabs, cat.#VPK-108-H).

Cell culture.

Mouse pancreatic cell lines αTC1 were grown in low-glucose DMEM supplemented by 10% FBS, 50U/mL penicillin and 50 ug/mL streptomycin. Mouse pancreatic cell lines Min6 with Doxycycline inducible construct was grown in high-glucose DMEM supplemented by 15% Tet System Approved FBS (Clonetech 631106), 71 uM 2-mercaptoethanol, 50U/mL penicillin and 50 ug/mL streptomycin. The mouse islet from alpha cell linage-tracing mouse were kept in RPMI medium supplemented by 10% FBS, 50U/mL penicillin and 50 ug/mL streptomycin. The cell culture for human islets followed established protocols (Walpita et al., 2012).

High-through put screening

Compounds (50 nL) were transferred to black optical suitable 384-well plate (Corning 3712) from DMSO stock plates using acoustic transfer (Labcyte Inc.). Min6 cells (3000 cells per well) were plated in 50 ul media on top of the compounds. Three days after treatment, cells were fixed in 3.7% formaldehyde for ten minutes at room temperature. Following PBS washing, cells were fixed with cold pure methanol in -20 C for 10 minutes, permeabilized by 1% Triton X-100 in PBS for 30 minutes and blocked by 3% BSA in PBS for 30 minutes. Twenty microliters of primary anti-insulin antibody, diluted in 1:2000 in 1.5% BSA, was added per well and incubated in 4 °C overnight. After washing with PBS twice, 20 uL Cy-3–labeled donkey-α-guinea pig antibody diluted in 1:1000 and

10ug/mL Hoechst 3342 in PBS was added per well and incubated for 1 h. After two washes with PBS, plates were stored in 4 C in dark until analysis.

Images were taken by an automated microscope (Perkin Elmer Operetta) using a 20X objective. Images were exposed for 10 ms in Hoechst channel and 500 ms in AIexa Fluor 548 channel. Images were analyzed by Harmony software. Nuclei were identified by (Harmony Method C) and cytoplasm was defined based on the nuclei (Harmony Method C). In total 1152 wells were screened containing 280 compounds from CLOUD library, CeMM's collection of clinical approved drugs with unique structure in triplicates with control wells. Hits were selected based on the intensity of insulin in the Alexa Fluor 548 channel and cell numbers in the Hoechst channel.

RNA-seq

Cells were lysed and RNA isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The libraries for RNA-seq for 24h induction in Min6 cells were prepared with Ribo-zero Kit and Scriptseq v2 Kit obtained from Epicenter by following the manual from the provider. For the rest of the RNA-seq libraries the amount of total RNA was quantified using Qubit 2.0 Fluorometric Quantitation system (Life Technologies) and the RNA integrity number (RIN) was determined using Experion Automated Electrophoresis System (Bio-Rad). RNA-seq libraries were prepared with TruSeq Stranded mRNA LT sample preparation kit (Illumina) using Sciclone and Zephyr liquid handling robotics (PerkinElmer). Library amount was quantified using Qubit 2.0 Fluorometric Quantitation system (Life Technologies) and the size distribution was assessed using Experion Automated Electrophoresis System (Bio-Rad). For sequencing libraries were pooled and sequenced on Illumina HiSeq 2000 using 50 bp single-read. Reads were aligned with tophat (v2.0.4) with the --no-novel-juncs --no-novel-indels

options (Kim et al., 2013). Gene expression was calculated as Reads Per Kb per Millions of reads (RPKMs) using RPKM_count.py from RSeQC package (Wang et al., 2012) and the NCBI RNA reference sequences collection (RefSeq) downloaded from UCSC (Kent et al., 2002) .

Chloride staining

Alpha-TC1-LT cells were pretreated with artemether for three days and incubated with MQAE (Thermo Fisher Scientific E-3101) for 1 hour and washed with PBS. Live cell imaging were performed in an automated microscope (Perkin Elmer Operetta) using a 20X objective and analyzed by Harmony software.

Moco synthase assay

Alpha-TC1-LT cells were pretreated with artemether for three days and lysed with NP-40 buffer. The measurement of Moco synthase was performed with Xanthine Oxidase Assay Kit (Abcam ab102522).

RT-qPCR

After the RNA was isolated with RNeasy Mini Kit (Qiagen), it was reverse transcribed with random primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) on Lightcycler 480 qPCR machine (Roche).

Western blot

Whole cell extracts were generated by lysing cells in NP-40 buffer containing 150 mM sodium chloride, 1.0% NP-40 and 50 mM Tris, pH 8.0 supplemented by Protease Inhibitor Cocktail (Roche). The whole-cell lysate (30 µg) was loaded onto a SDS–polyacrylamide gel for electrophoresis at 30 mA per gel, and then transferred by electrophoresis to a nitrocellulose membrane (GE Healthcare Life Science). All the blots

were incubated with corresponding primary antibodies diluted in 1:1000 in 5% milk in 4 C overnight and in HRP-labeled 1:20000 secondary antibodies for 1 h. The signals were detected using ECL Prime Western blotting Detection Reagent (Amersham).

Immunoprecipitation

Primary antibody against mTOR(Cell Signaling Technology CST2972) was incubated with Dyabeads (Life technology 10765583) for 15 min and wash with PBST. Antibody-coupled beads were incubated with alpha-TC1-LT cell lysis overnight, washed with PBST for three times and eluted with SDS-loading buffer. The input, supernatant (5%) and elution were used for western blot to detect, p-mTOR (Cell Signaling Technology CST5536), mTOR and Gephyrin (Synaptic system 147-111).

PI3P assay

The measurement of PI3P was performed with PI(3)P Mass ELISA kit (K-3300, Echelon). In brief, alpha-TC1-LT cells were pretreated with artemether for three days and collected. The lipid extraction and ELISA assay were perfomed according to the manufacturer's guideline.

Chemical proteomics

Briefly, 10 mg alpha cell lysis was pre-incubated with 20 mM Artemether or DMSO for 30 min. After this, artesunate which was linked covalently to sepharose beads was added to cell lysis and incubated for two hours. The enriched proteins were eluted by formic acid, digested with trypsin and analyzed by gel-free one-dimensional liquid chromatography mass spectrometry (1D-LCMS). The details of chemical proteomics were described in S.M&M.

Whole cell proteomics

aTC1 cells were harvested after three day treatment and lysed with 2%SDS. The cell lysis was digested with FASP digest and labeled the individual channels with the different isobaric TMT-labels. Then all the channels are combined and fractionated into 50 fractions with offline SOP. The fractions are analyzed on the Masspec to give a relative quantification of the proteins individual samples. The technical details of FASP, TMT-labeling and offline SOP were described in S.M&M.

Glucagon secretion assay of mouse alpha cell line

Alpha-TC1-LT cells were pretreated with artemether for three days. Cells were incubated with high glucose medium (5 g/L glucose in KRBS medium) for one hour at first and with low glucose medium (0.5 g/L glucose in KRBS medium) for another hour. Supernatnat after low glucose challenge was collected to measure glucagon content. The ELISA assay for glucagon was performed by Glucagon ELISA Kit (Alpco 48-GLUHU-E01-AL).

Glucagon-GFP/Insulin-mcherry zebrafish larvae assay

Larvae of intercross Glcgn:GFP and ins:NTR-mcherry fish were treated 26hpf (fertilization) with artemether of the according drug; on day 5 they were sorted for potentially double positive larvae (stereo microscope), embedded into agarose and pictures were taken on the confocal at 25x. With this pictures 3D models of the islets were established and the GFP or mcherry positive cells were counted by a 3D imaging tool.

Insulin secretion assay and intracellular insulin content assay of human islet

Human islets were pretreated with artemether for three days. Cells were incubated with low glucose medium (0.5 g/L glucose in KRBS medium) for one hour at first and with high glucose medium (5 g/L glucose in KRBS medium) for another hour. Supernatnat

after low glucose and high glucose incubation was collected to measure insulin content. The ELISA assay for glucagon was performed by Insulin Elisa Kit (Alpco 80-INSHU-E01.1). The islet pellets after high glucose incubation were lysed by NP-40 buffer and insulin content was measured by the same kit.

Statistical methods

All the p-values were calculated by student t test, unless specified as other methods. The Gene ontology terms enrichment was operated by Gorilla (Eden et al., 2007; Eden et al., 2009).

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Figure Legends

Figure 1. A cell line model for transcription factor mediated transdifferentiation allows screening for functional ARX inhibitors

A. Representive images of Min6-ARX cells stained for insulin and Myc. **B.** Quantification of Insulin reduction upon ARX overexpression, detected by immunofluorescence. Scale bar 5μm. *, $p<0.001$ compared to uninduced cells. **C.** Overview of screening data in Min6-Arx cells. **D.** Validation of hits from the primary screen in aTC1 cells. **E.** Immunofluorescence showed increases of Insulin in alpha cells with Artemether treatment. Scale bar 10μm. **F.** Overview of quantitative whole cell proteome analysis. Only the significantly regulated proteins were present. **G.** Western blot validation of Glucagon protein change with Artemether treatment.

Figure 2. Artemether induces insulin express in alpha cells by targeting gephryin

A. Outline of the chemical proteomics experiment. **B.** Volcano blot showed the top candidates identified by chemical proteomics. Both p-value and fold-change were calculated based on spectro counts of two biological replicates. **C**. Western blot for gephyrin in alpha cells treated with multiple doses of artemether. **D.** Western blot for validation of Gephyrin knock-down by shRNA. Ponceau S staining is used as loading control. **E.** Insulin immunofluorescence on alpha cells treated with artemether with or without Gephyrin knock down. Scale bar 10μm. **F**. Quantification of Insulin and Gephyrin intensity in **E**.

Figure 3. Gephyrin-mediated increases of GABA signaling induces insulin in alpha cells

A. Immunoprecipitation *o*f mTOR and western blot for mTOR and Gephyrin in alpha cells treated with artemether. **B.** Western blot for GABA receptor subunits in aTC1 cells treated with artemether. **C.** GO term enrichment of RNA-seq data in alpha cells with Artemether treatment. **D.** GABA currents measured by patch clump assay in alpha cells. *, p=0.036. **E and F.** Quantification of Insulin intensity in alpha cells treated with GABAR antagonist Bicuculine (**E**) or Gabazine (**F**) in combination with Artemether on aTC1 cells. Scale bar 10μm. *, p=0.010, **, p<0.001 compared to Artemether treated cells.

Figure 4. Hormone secretion controls pancreatic cell type stability

A. Secreted glucagon in artemether-treated alpha cells aTC1 measured by Glucagon ELISA kit. *, p=0.03. **B.** Quantification of insulin intensity immunofluorescence in alpha cells with artemether with/without glucagon supplement versus control. *, p<0.001. **C.** Representative images of Insulin and Glucagon immunofluorescence in alpha cells treated with PCi. Scale bar 3μm. **D.** RT-qPCR of islet related genes in alpha cells upon PCi treatment. All data was normalized to DMSO control. *, p=0.010, **, p=0.019, ***, p=0.002, ****, p<0.001,

Figure 5. Artemether induces alpha cells to express insulin expression in vivo

A. Stainings for insulin and glucagon in mouse pancreas following three months treatment of Artemether by drinking water. **B.** Quantifiction of islets size in mouse. *, p<0.001. **C.** Co-staining of RFP and Insulin in RFP-labeled lineage tracing mouse islets. **D.** Quantification of RFP/Insulin double positive cells in mouse islet. *, p=0.019. **E.** Representative islet from zebrafishes with or without artemether treatement. **F.** Quantification of Glucagon-GFP positive cells and Insulin-mcherry positive cells in zebrafish islets. *, p=0.007, **, p=0.088, ***, p=0.004, ****, p=0.003.

Figure 6. Single-cell transcriptomics reveals artemether effects in human alpha cells

A. Increase of Insulin/Glucagon double positive cells (labeled by arrows) following Artemether treatment. Scale bar 5μm. **B.** MDS blot showing the overview of cell clusters from single cell RNA-seq on human islets. **C.** Expression pattern of GABA-A receptor subunits in human alpha cells with artemether treatment. **D.** Gene Set Enrichment Analysis on the artemether treated human alpha cell dataset. **E and F.** Downregulation of human alpha cell specific genes by artemether treatment.

Figure 7. Artemether improves insulin secretion in human islets

A. RT-qPCR assay for ARX expression in human islets. **B.** Measurement of Insulin content intracellularly in human islet treated with artemether. **C.** Measurement of secreted Insulin with high glucose challenge in human islets treated by artemether. *, $p<0.001$; **, $p=0.002$; ***, $p=0.03$; ****, $p=0.03$; *****, $p=0.05$. **D.** Summary of the proposed mechanism. Artemther, through interaction with Gephyrin, enhances GABA signaling in alpha cells, which leads to decrease of Glucagon secretion. Decrease Glucagon concentration in the close vicinity of alpha cells induces loss of alpha cell identity and increase of Insulin expression.

Figure S1. A. Western blot for Myc-tag in ARX-inducible overexpression Min6 cell lines. Histone H2B is used as a loading control. **B.** Western blot for ARX in inducible Min6 cell line. Histone H2B is used as a loading control. **C.** Expression of ARX in inducible Min6 cells measured by RNA-seq.

Figure S2. A, B and C. Time course of gene expression changes genes upon ARX overexpression.

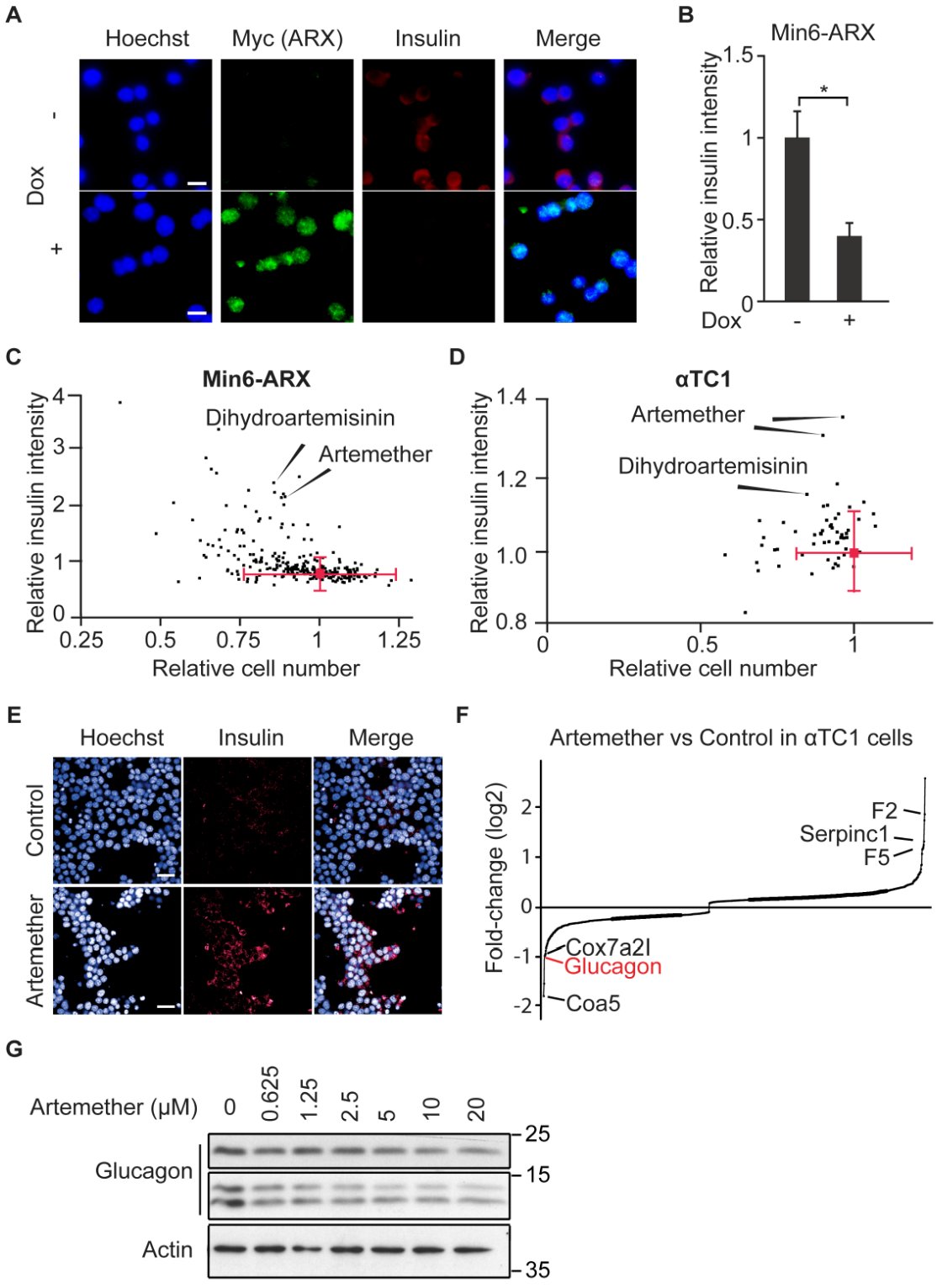
Figure S3. A and B. Dose-response curve of artemether on Min6-ARX cells (**A**) and alpha cells aTC1 (**B**). **C.** Insulin intensity in aTC1 cells following treatment of DMSO (0), Artesunate (1), Deoxyarteether (2), Arteether (3), n-hydroxy-11-azaartemisinin (4), artemistene (5), artemisone (6), artemisiten (7) and anhydro dihydro artemisinin (8). **D-F.** Quantification of insulin intensity in aTC1 cells following treatment of ROS inducer Elesclomol. (**D**), artemether in combination with N-Acetyl-Cysteine (**E**) and SERCA inhibitor (**F**). *, p=0.034. N.S., p>0.05. **G.** Western blot for p-Akt and AKT of alpha-TC1 cells treated with multiple doses of Artemether. **H.** Measurement of PI3P in alpha-TC1 cells treated with artemether. *, p=0.007. **, p=0.002.

Figure S4. A. Summary of proteins identified in the chemical proteomics assay. **B.** Immunofluorescence of Gephyrin in alpha cells treated with artemether. Scale bar 10 μM. **C.** Expression of GABA-A receptor subunits detected by RNA-seq. **D.** Costaining of gephyrin and fluorescently-labeled artelinic acid in alpha cells. **E.** Moco systhesis activity and intracellular chloride concentration in alpha cell with artemether treatment. *, p=0.04. **F.** Fold-change of genes related to synaptic transmission. **G.** Measurement of intracellular chloride intensity of live cell fluorescence. *, p<0.001.

Figure S5. Immunofluorescence of Sst, Pp, and BrdU co-stained with Insulin or Glucagon in mouse pancreas with or without Artmether treatment.

Figure S6. A and B. Quantification of cell fractions in human islets treated with artemether by individual donor (**A**) or summary (**B**). *, p<0.001, n=5. **C.** GSEA on artemether or GABA treated human islet bulk samples. **D.** Overlap between core-upregulation gene set and exocrine or endocrine gene set. Fisher's exact test is used for the significance test. **E.** Expression of alpha cell specific genes in human beta cells treated by artemether from single cell RNA-seq assay. The Venn diagrams were made

http://www.cmbi.ru.nl/cdd/biovenn/. **F.** Gene Set Enrichment Analysis on the artemether treated human beta cell dataset. **G.** Expression of genes related to Protein catabolic process in human alpha and beta cells treated by artemther.



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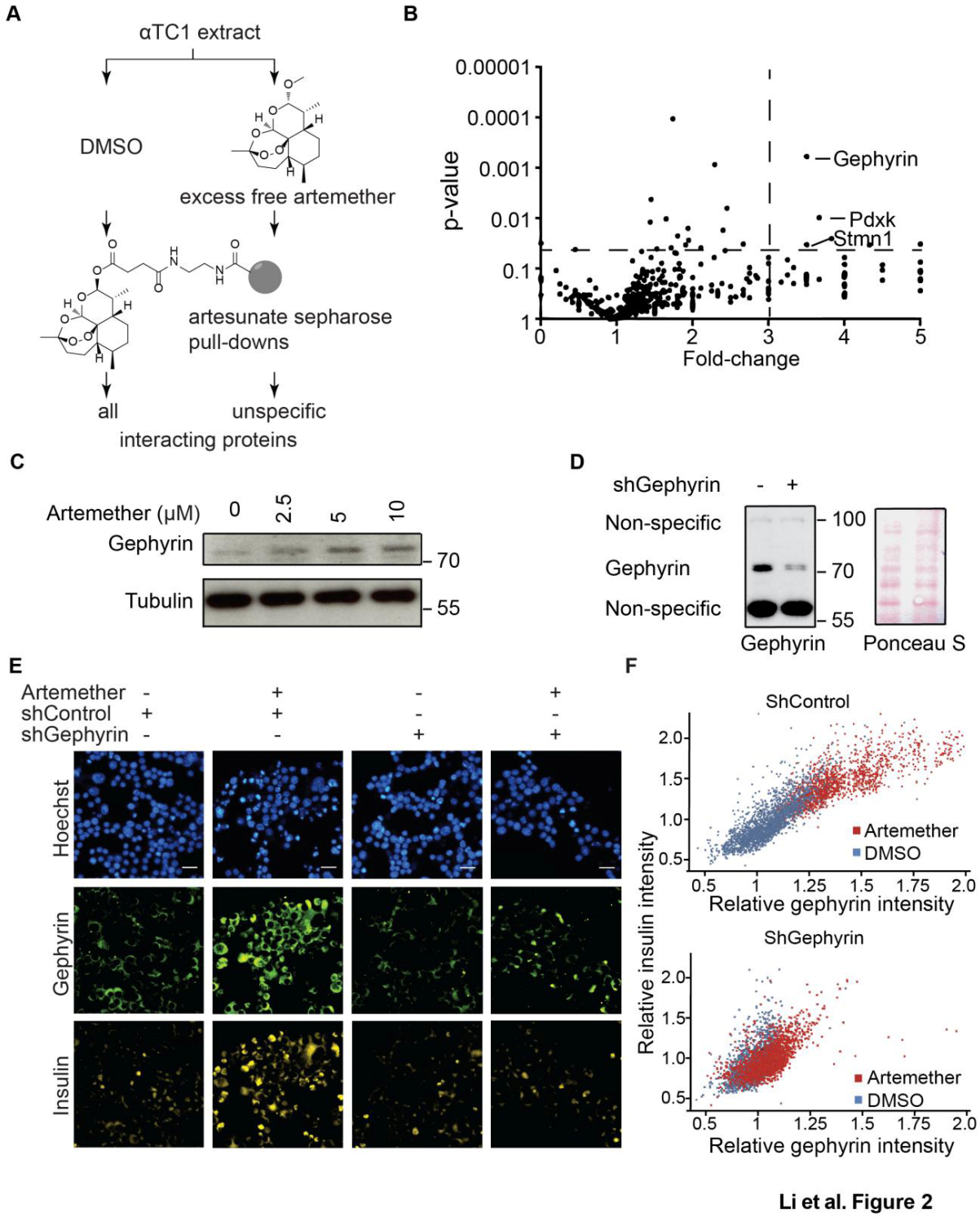

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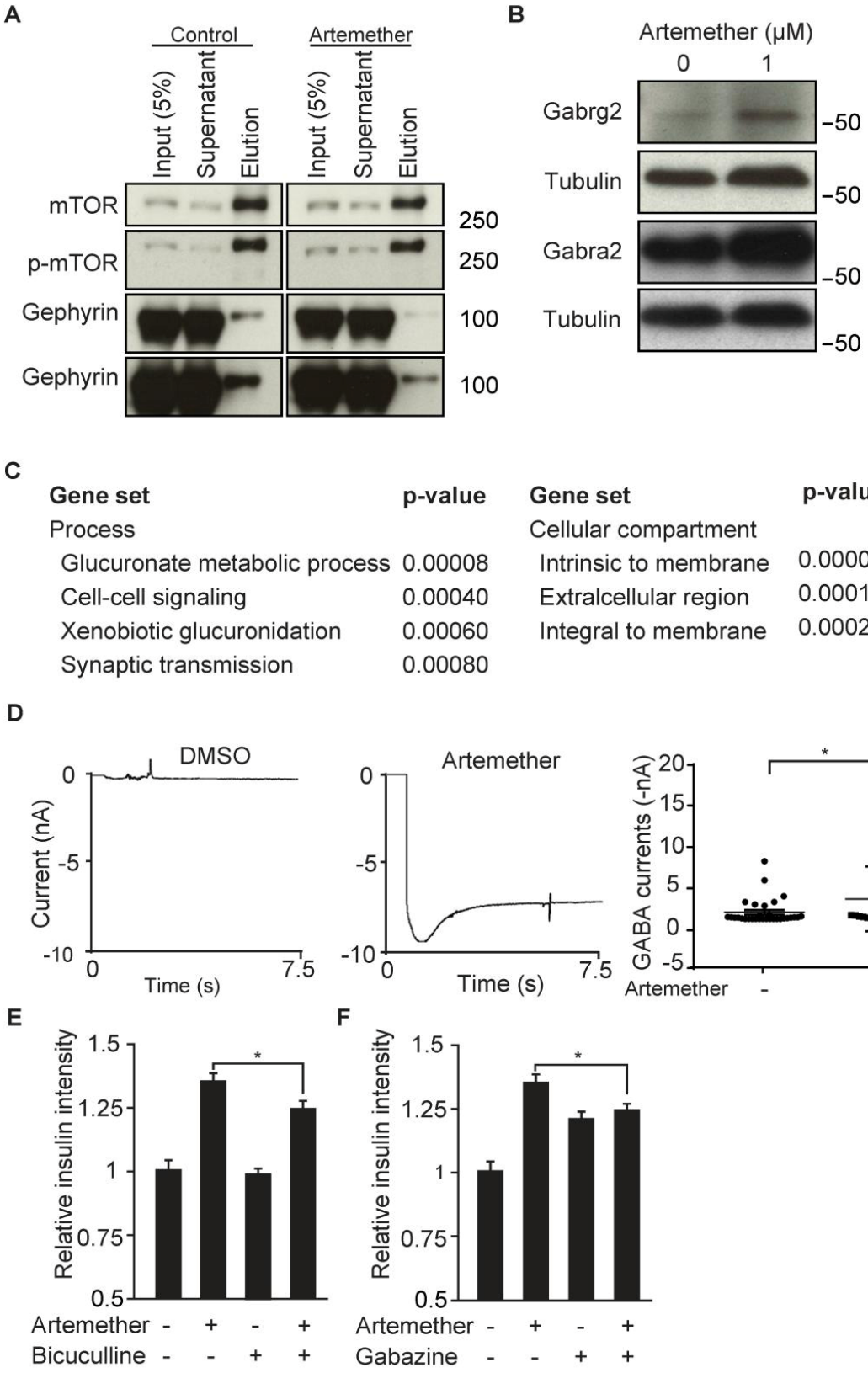

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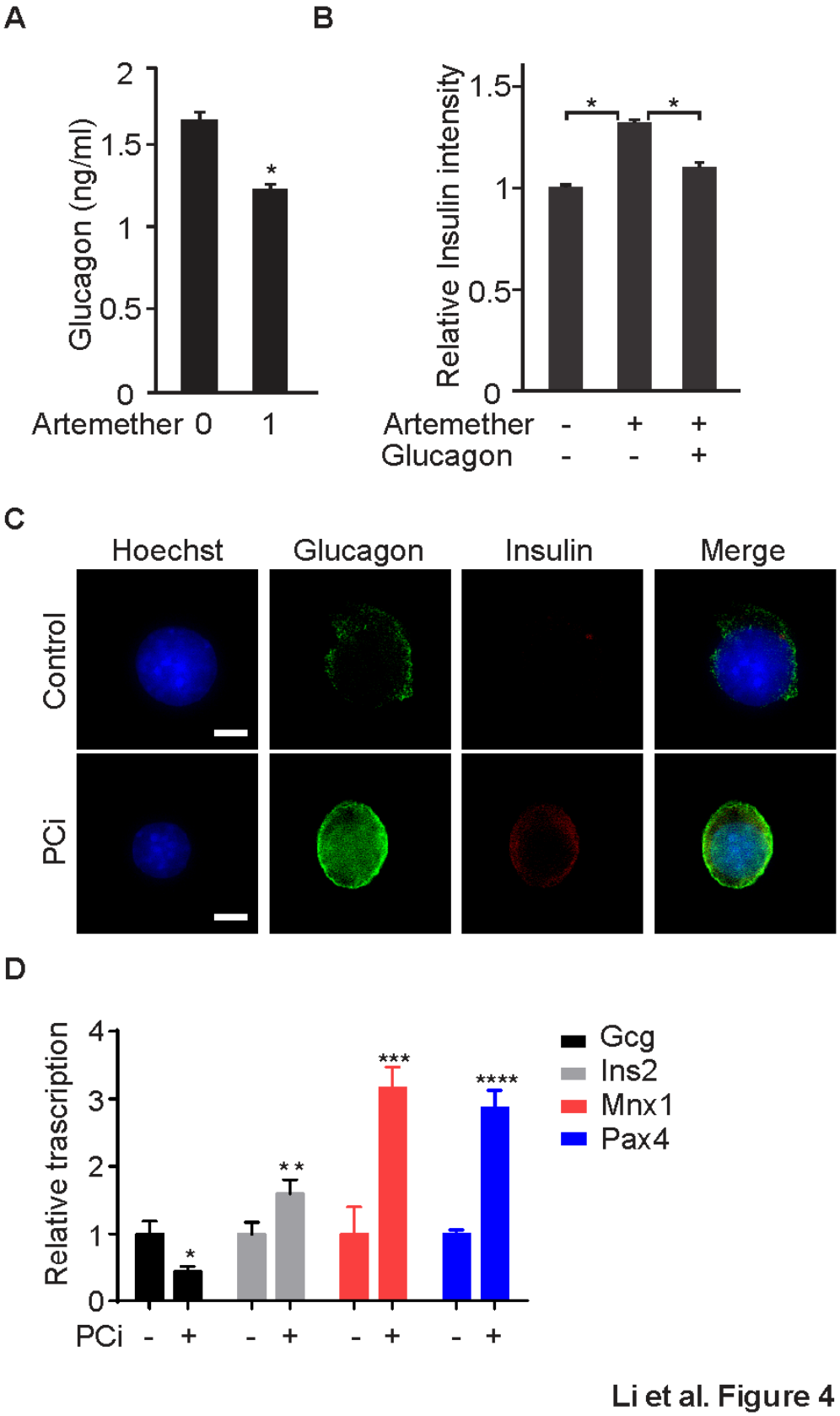

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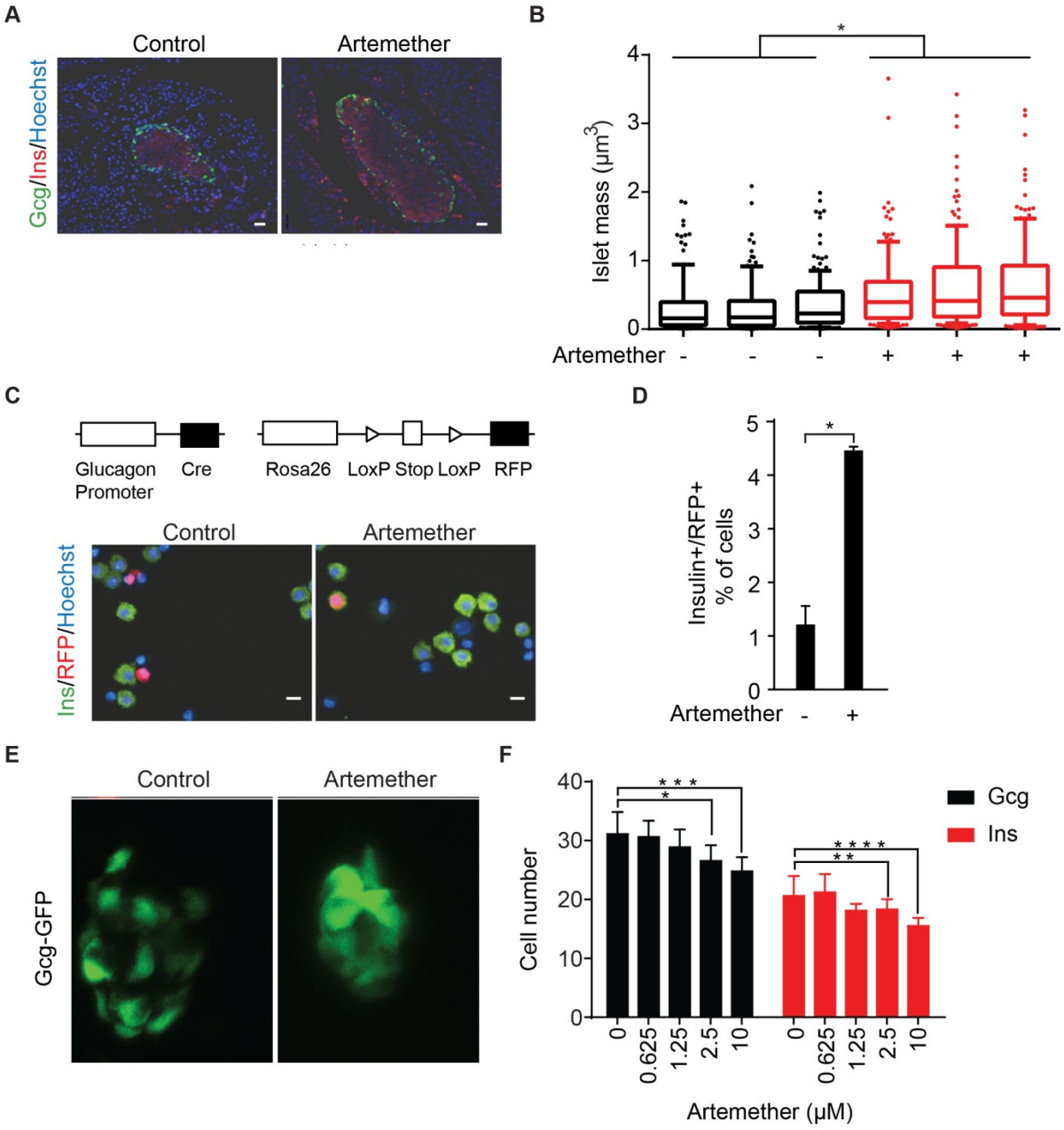
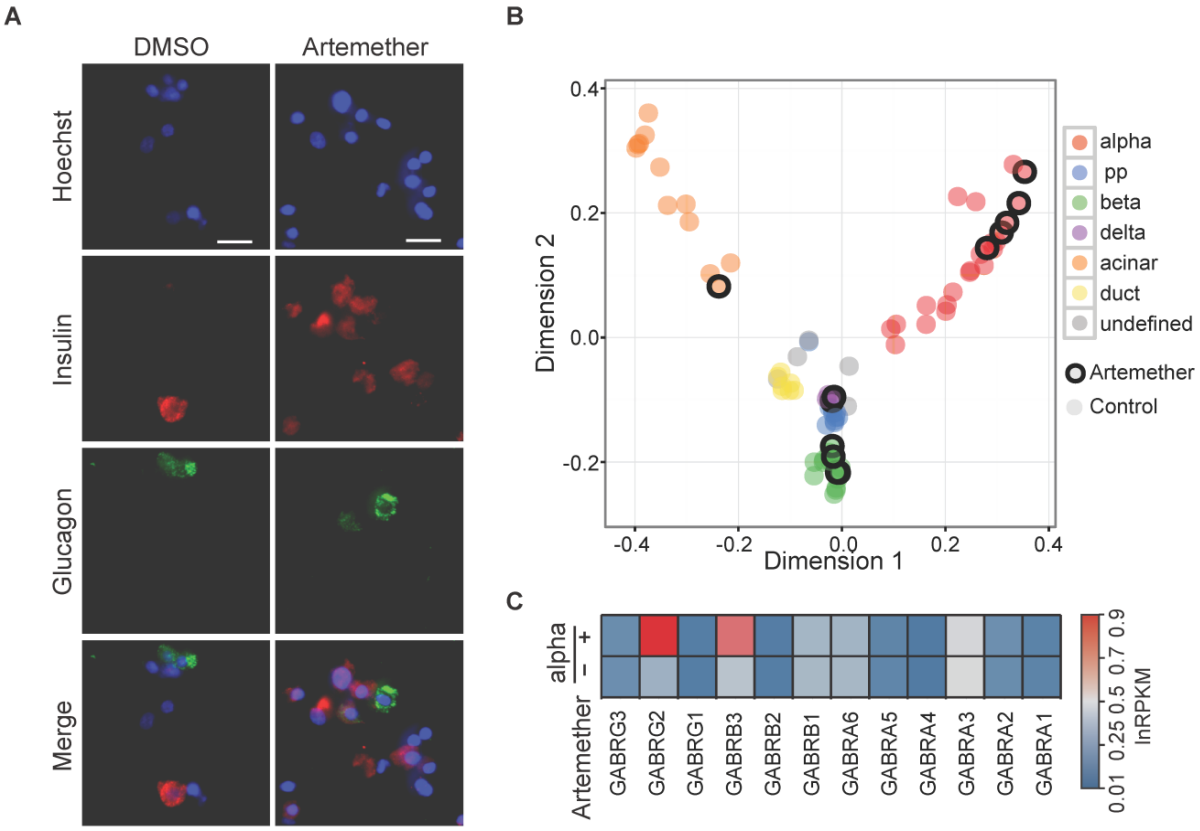
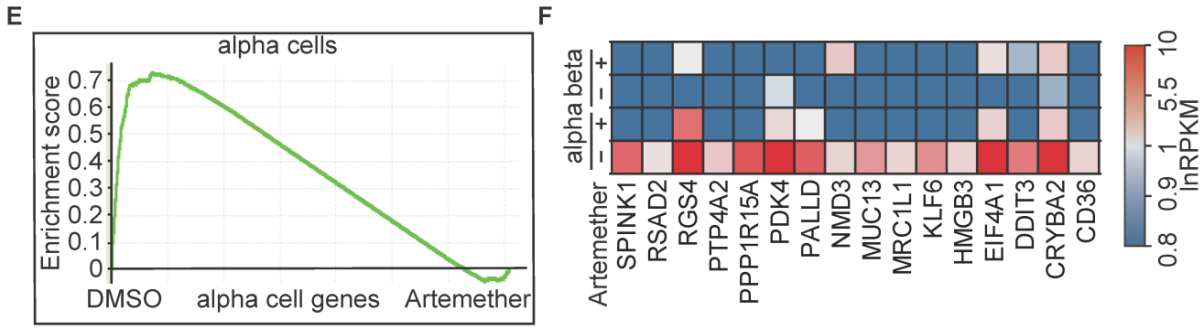


Li et al. Figure 5



D

GSEA of downregulated genes in alpha cells	p-value	FDR
Alpha cell specific gene	$<10^{-6}$	$<10^{-6}$
Structural consistuent of ribosome	$<10^{-6}$	$<10^{-6}$
Nuclear envelope endoplasmic reticulum network	$<10^{-6}$	$<10^{-6}$
Structural molecule activity	$<10^{-6}$	0.009316
Endoplsmic reticulum part	$<10^{-6}$	0.011269
GSEA of upregulated genes in alpha cells	p-value	FDR
Potassium ion transport	$<10^{-6}$	0.211741
Ion channel activity	$<10^{-6}$	0.218214
Voltage gated cation channel activity	$<10^{-6}$	0.21854
Substrate specific channel activity	$<10^{-6}$	0.225567
Metal ion transport	$<10^{-6}$	0.226705



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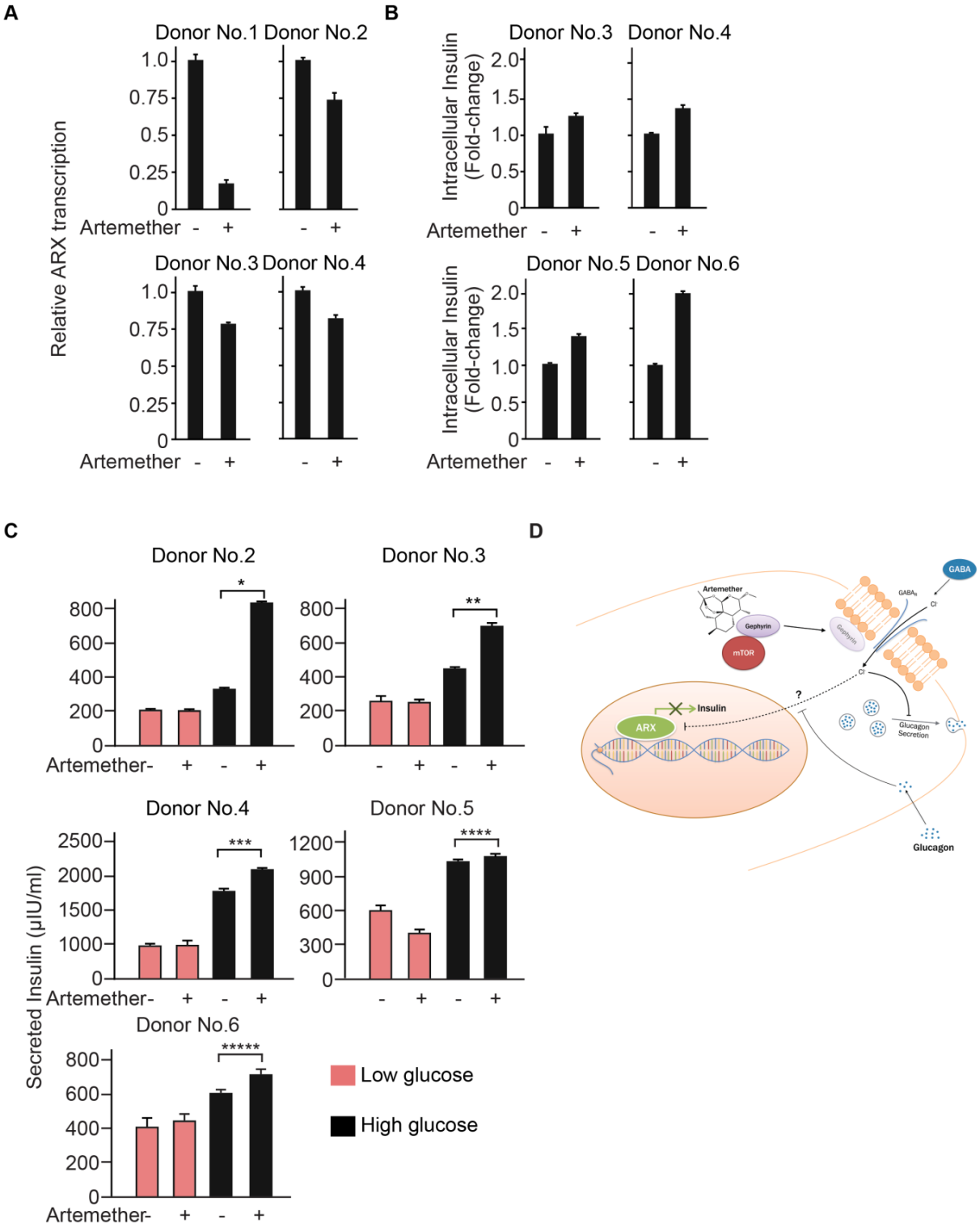

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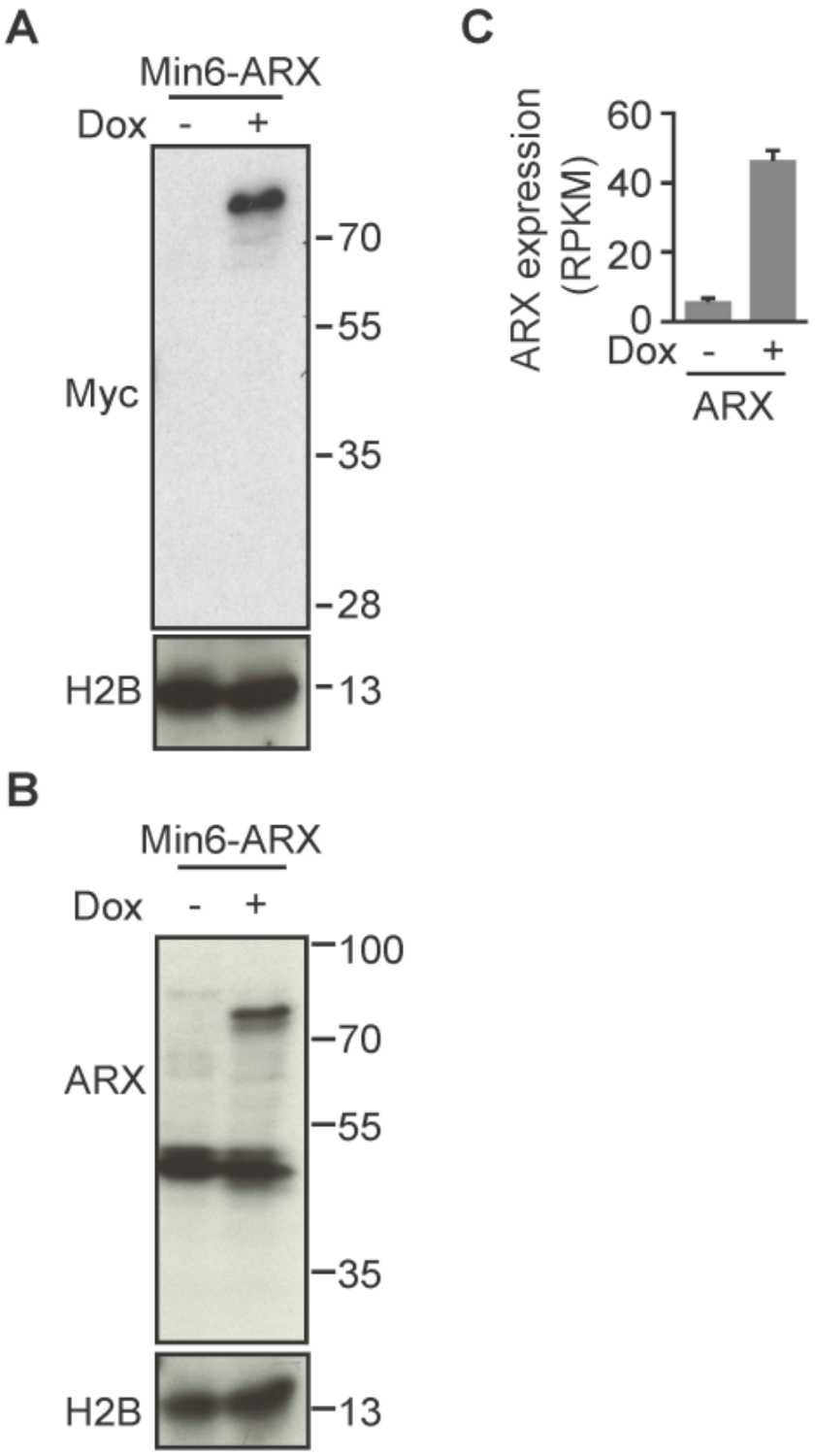

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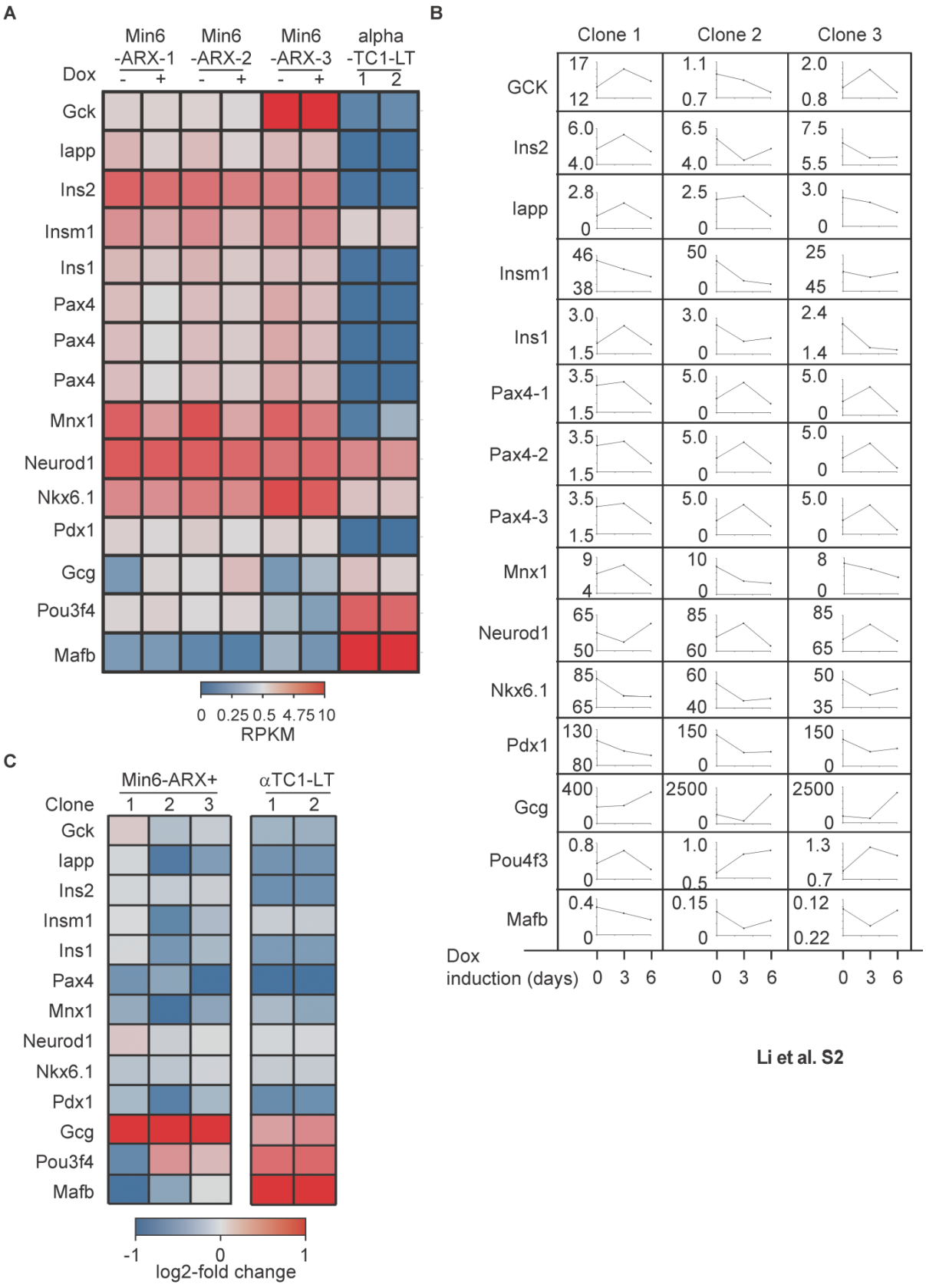
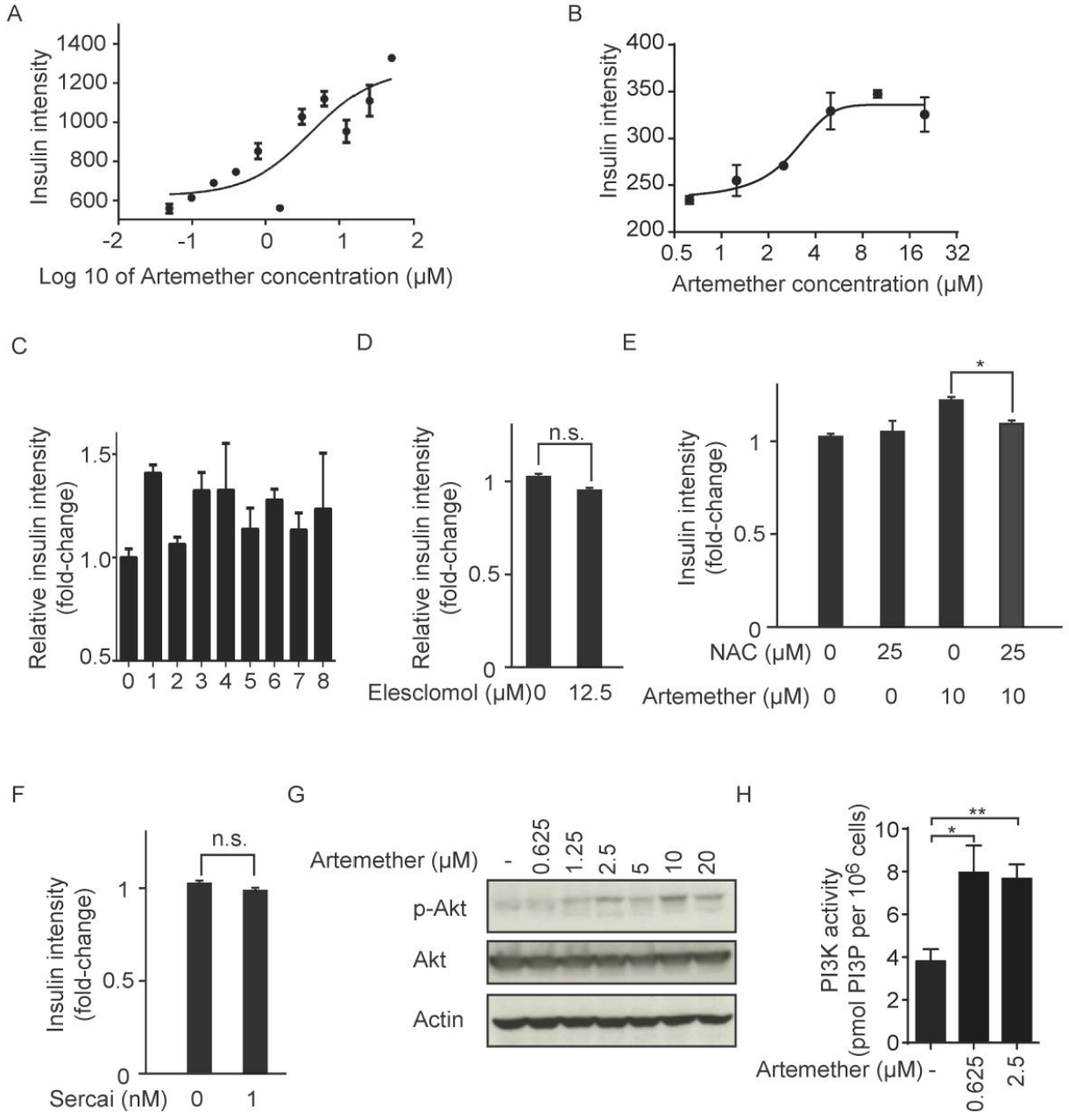

A
Min6 -ARX-1
Min6 -ARX-2
Min6 -ARX-3
alpha -TC1-LT
Dox
- + - + - +
1 2
Gck
Iapp
Ins2
Insm1
Ins1
Pax4
Pax4
Pax4
Mnx1
Neurod1
Nkx6.1
Pdx1
Gcg
Pou3f4
Mafb
0 0.25 0.5 4.75 10
RPKM
B
Clone 1
Clone 2
Clone 3
GCK
Ins2
Iapp
Insm1
Ins1
Pax4-1
Pax4-2
Pax4-3
Mnx1
Neurod1
Nkx6.1
Pdx1
Gcg
Pou4f3
Mafb
Dox
induction (days) 0 3 6
C
Min6-ARX+
αTC1-LT
Clone
1 2 3 1 2
Gck
Iapp
Ins2
Insm1
Ins1
Pax4
Mnx1
Neurod1
Nkx6.1
Pdx1
Gcg
Pou3f4
Mafb
-1 0 1
log2-fold change

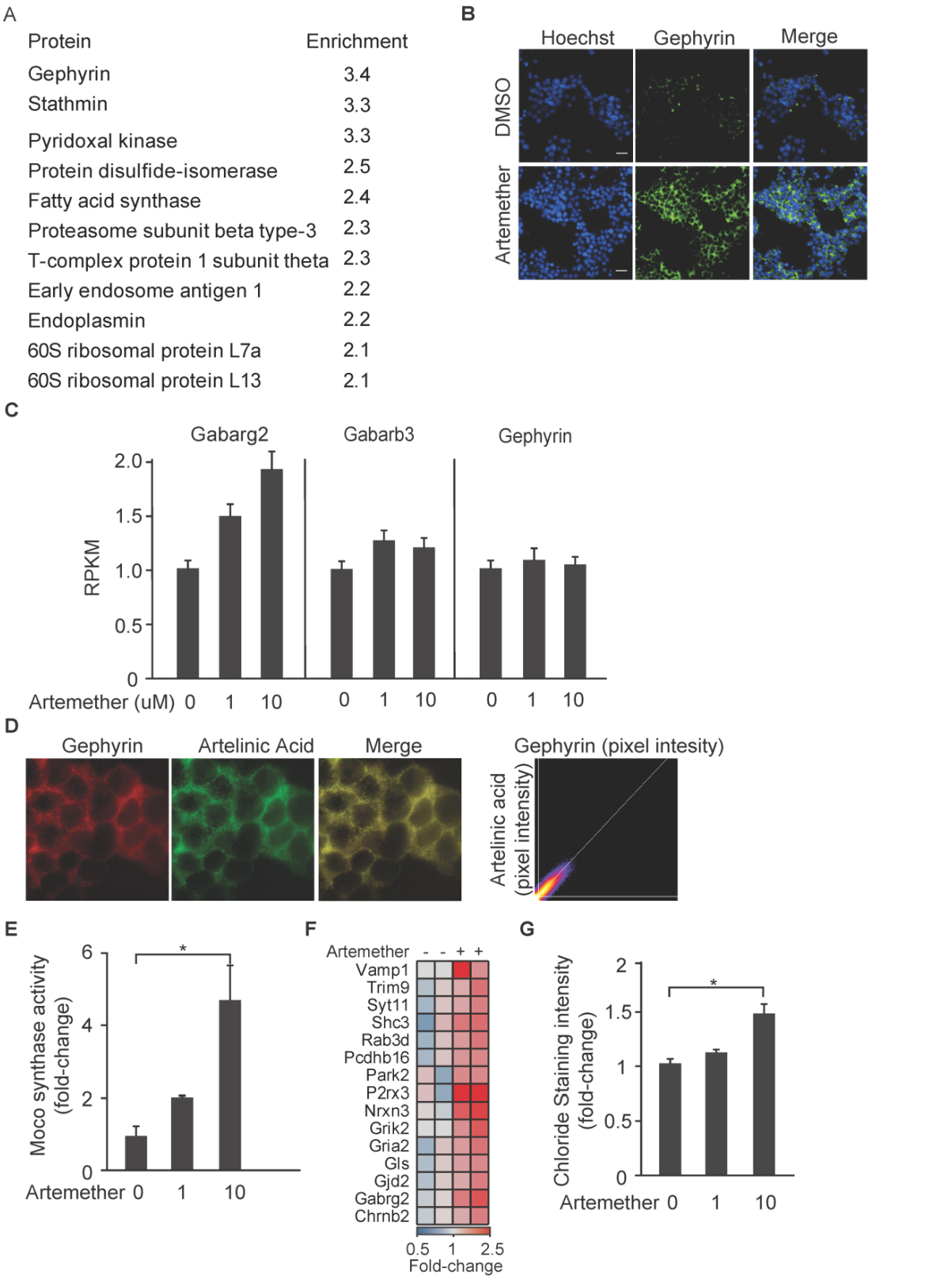
Li et al. S2



Li et al. Figure S3

A

Protein	Enrichment
Gephyrin	3.4
Stathmin	3.3
Pyridoxal kinase	3.3
Protein disulfide-isomerase	2.5
Fatty acid synthase	2.4
Proteasome subunit beta type-3	2.3
T-complex protein 1 subunit theta	2.3
Early endosome antigen 1	2.2
Endoplasmin	2.2
60S ribosomal protein L7a	2.1
60S ribosomal protein L13	2.1



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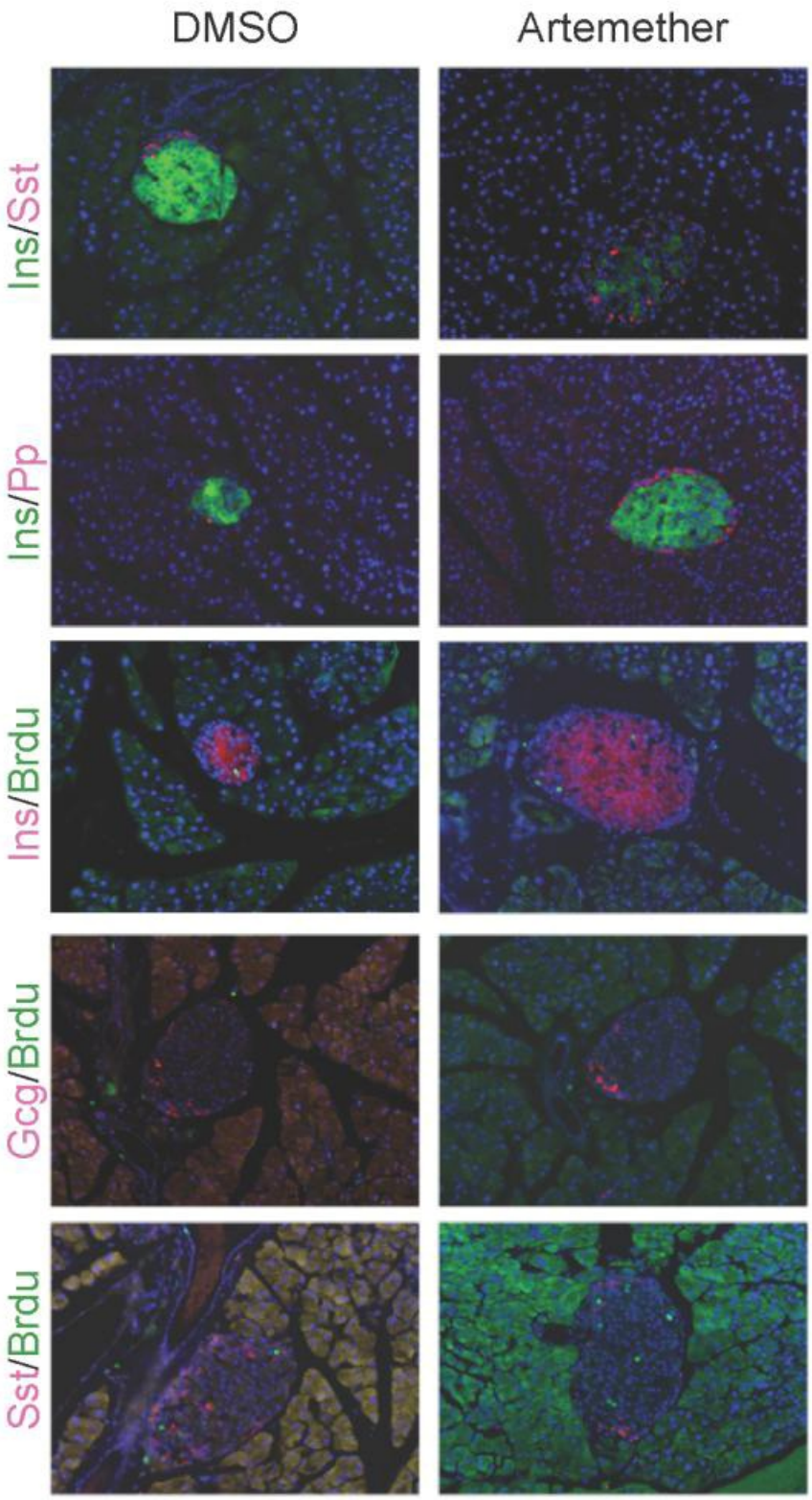
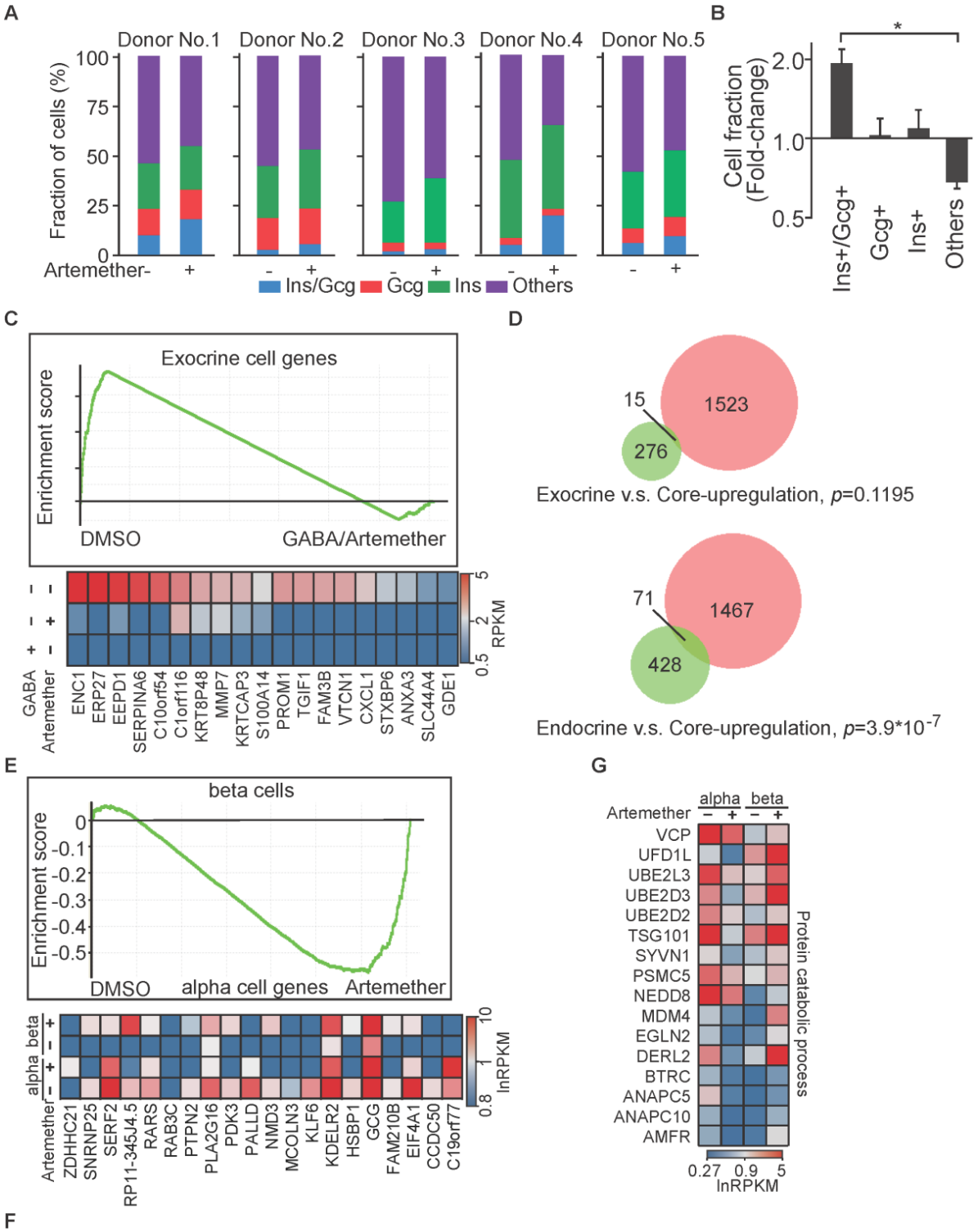


Li et al. S.Figure 5



F

GSEA of upregulated genes in beta cells	p-value	FDR
Protein catabolic process	$<10^{-6}$	0.002031
Cellular protein catabolic process	$<10^{-6}$	0.003058
Ubiqutin protein ligase activity	$<10^{-6}$	0.003717
Small protein conjugating enzyme acitivity	$<10^{-6}$	0.004288
Small conjugating protein ligase member	$<10^{-6}$	0.004441

Li et al. Figure S6

Prevention of beta cell dedifferentiation by PAX4 and approved drugs

To establish a research model of beta cell dedifferentiation for further study on the mechanism and therapies, I used a chemical inhibitor of FoxO proteins on Min6 cells which successfully phenocopied the changes observed in FoxOs deleted mouse models. The Min6 cells treated with FoxO inhibitor loose insulin expression and gain glucagon expression. With this model I studied beta cell dedifferentiation in three different aspects:

1. I hypothesize that overexpression of PAX4, a master regulator of beta cell linage specification, can inhibit beta cell dedifferentiation. Indeed, PAX4 overexpression does not only suppress glucagon transcription in beta cells treated with FoxO inhibitor but also induces upregulateion of genes which are downregulated by FoxO inhibitor in the genome-wide scale.
2. With comprehensive analysis on the transcriptional profiles of dedifferentiated Min6 cells. Genes related to lysosomal acidification were identified as top hits. Neutralization of lysosomal acidification by chloroquine inhibits glucagon transcription in both dedifferentiated beta cells and differentiated alpha cells. These effects could be prevented by supplementing recombinant glucagon.
3. I discovered loperamide, an approved drug for diarrhea treatment, could rescue beta cell dedifferentiation. Further studies suggest loperamide inhibits beta cell dedifferentiation through mobilization of intracellular calcium ions and upregulating FoxO1 expression. The effects of loperamide were confirmed in multiple models, including zebrafish larvae, C.elegans and human islets.

Prevention of beta cell dedifferentiation by PAX4 and approved drugs

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Running Title: Prevention of beta cell dedifferentiation

Key words : Diabetes/Beta Cell Dedifferentiation/FoxO1 inhibitor/Loperamide

200 words in abstract, 2552 words in main text, 6 figures, 4 supplementary figures, 31 references

Abstract

Loss of FoxO1 signalling in response to metabolic stress contributes to the etiology of type 2 diabetes by causing the dedifferentiation of pancreatic beta cells to a cell type reminiscent of endocrine progenitors. During normal development, differentiation of beta cells from endocrine progenitors is dependent on the expression of the master regulatory transcription factor Pax4. Here we show that FoxO inhibition in beta cells represses Pax4 target genes and induces the progenitor marker Ngn3, indicative of a dedifferentiation phenotype. Under these conditions, a subset of beta cells initiates the expression of the alpha cell marker glucagon, which remains stable even after the withdrawal of the FoxO inhibitor. In contrast, PAX4 overexpression reinforces a beta-cell phenotype and can overcome FoxO inhibitor mediated beta cell dedifferentiation. Mechanistically, FoxO inhibitor treatment increases lysosomal acidification and neutralization with chloroquine decreases the transcription of glucagon. With a high-content chemical screen we also identify the approved drugs loperamide to counteract dedifferentiation and glucagon expression by mobilizing intracellular free calcium and the NFAT signaling. Loperamide also counters the fully differentiated state of alpha cells, and in human pancreatic islets loperamide specifically inhibits the transcription of glucagon. Importantly, activation of intracellular calcium and neutralization of lysosomal acidification synergize to prevent the FoxO inhibitor-induced glucagon transcription in beta cells. Our study provides novel screenable assay systems, molecular targets and drug candidates that might contribute to prevent beta cell failure in diabetes.

Introduction

In Type II diabetes initial peripheral insulin resistance often progresses to insulin deficiency caused by beta cell dedifferentiation and apoptosis. A key regulator of beta cell dedifferentiation is the transcription factor FoxO1. Normally, FoxO1 resides in the cytoplasm of beta cells, but upon metabolic stress it translocates to the nucleus where it regulates the expression of its target genes (Kitamura et al, 2002; Kitamura et al, 2005; Meur et al, 2011; Okamoto et al, 2006). Prolonged and severe hyperglycemia then results in the loss of FoxO1 expression and beta cell failure (Gupta et al, 2013; Kluth et al, 2011). In a mouse model, knock-out of FoxO1 induces a loss of beta cell function following metabolic stress and these cells start expressing the pancreatic endocrine progenitor marker Ngn3 (Talchai et al, 2012), consistent with a dedifferentiation process. Importantly, some of the dedifferentiated beta cells start expressing the alpha cell marker glucagon which confirms the multi-lineage potential of these progenitors (Talchai et al, 2012). In contrast, the silencing of FoxO1 in alpha cells impaired preproglucagon transcription and glucagon secretion (McKinnon et al, 2006). Interestingly, beta cell dedifferentiation can also be induced by a mutation leading to ATP-insensitive potassium channel and it can be rescued by insulin therapy (Wang et al, 2014).

During normal development, master regulatory transcription factors control the formation of the differentiated endocrine cell types from a common pancreatic progenitor. For the development of beta cells, Pax4 is one of the defining transcription factors. Mice lacking Pax4 develop severe diabetes as a result of reduced beta cell mass, whereas alpha cell numbers are increased (Sosa-Pineda et al, 1997). In addition to beta cells, also somatostatin expressing delta cells are lost in $Pax4^{-/-}$ mice, suggesting close developmental relationship between these two cell types. The overexpression of Pax4

alone is sufficient to convert alpha cells into functional beta cells, confirming the major role of this transcription factor in beta cell lineage definition (Al-Hasani et al, 2013; Collombat et al, 2009).

Due to their respective roles, we hypothesize that FoxO inhibition and Pax4 expression are counteractors in the regulation of a fully differentiated beta cell state. We show that pharmacologic inhibition of FoxO in beta cell lines mimics many aspects of *in vivo* dedifferentiation, including the loss of insulin expression, the upregulation of Ngn3 and the emergence of a glucagon positive cell population. Interestingly, FoxO inhibition represses a gene set that is upregulated by PAX4. In line with our hypothesis, PAX4 overexpression can counteract FoxO inhibitor mediated beta cell dedifferentiation.

The dedifferentiation of beta cells by inhibition of FoxO family proteins has been linked to the inflexibility of beta cell regarding energy metabolism (Kim-Muller et al, 2014). To understand the mechanism of beta cell dedifferentiation in detail, we analyzed the FOXOi induced transcription changes and observed that FoxO inhibitor treatment upregulated multiple vacuole ATPases. These proteins drive the influx of hydrogen ions to acidify lysosome. Neutralization of lysosomal acidification by chloroquine inhibits glucagon transcription in both dedifferentiated beta cells and differentiated alpha cells.

In contrast to previous studies that were all performed *in vivo*, our *in vitro* system is readily amendable to screening approaches for small molecule modulators of beta cell dedifferentiation. We developed a high content assay for insulin and glucagon expressing cells following FoxO inhibition and screened a representative library of approved drugs. Treatment with loperamide resulted in an increase in insulin staining and a reduction in the number of glucagon positive cells. The repressive effects of loperamide on glucagon transcription are also observed in mouse alpha cell lines and

human islets. After ruling out effects by loperamide's activity on its canonical target, μ-opioid receptor, we discovered that loperamide suppresses glucagon transcription by increasing intracellular free calcium and FoxO1 transcription. It is known that loss-of-function mutation in FoxO in C.elegans leads to defects in dauer formation. Importantly, *in vivo* test of loperamide on C.elegans enhances the dauer formation under low temperature, which validates the effects of loperamide in a model organism (Gottlieb & Ruvkun, 1994; Jensen et al, 2010). Loperamide treatment significantly decreases glucagon transcription in mature human islets and increases both alpha and beta cell mass in the development of zebrafish larvae. With these results, our study provides new starting points for preventing beta cell dedifferentiation by transcription factor overexpression and small molecule treatment.

Results

PAX4 overexpression reinforces a beta cell phenotype

To study the role of PAX4 in beta cell differentiation, we generated Min6 cell lines that allow the inducible overexpression of FLAG-myc tagged versions of this master regulatory transcription factor (Min6-PAX4) and control GFP (Min6-GFP) (Fig.1A). Following doxycycline treatment, these cells respond with a strong induction at the protein level (Fig. 1B). On the mRNA level, expression of the overexpression constructs is apparent in the uninduced state, but doxycycline results in a further ~10-fold increase of PAX4 transcript (Fig. 1C). We then used RNA sequencing to study the consequences of PAX4 overexpression in beta cells. Compared to control GFP, PAX4 overexpression results in a significant and more than 2-fold increase of 333 genes, and repression of 371 genes. Gene set enrichment analysis reveals strong activation of genes expressed

in beta cells compared to alpha cells (Fig. 1D). These genes include Hnf4a and Stat6 (Fig. 1E). Interestingly, the low level expression of Ngn3 present in Min6 beta cells is repressed by PAX4 (Fig. 1F).

A cellular model for beta cell dedifferentiation

Beta cell dedifferentiation has previously been characterized with genetic knock-out of the transcription factor FoxO1 in a mouse model (Talchai et al, 2012). Furthermore, a small molecule FoxO inhibitor has been shown to cause the conversion of delta to beta cells *in vivo* (Chera et al, 2014). We hypothesized that pharmacologic FoxO inhibition in beta cell lines could provide a model system for studying the beta cell dedifferentiation process. Therefore, we treated the uninduced beta cell line Min6-PAX4 as well as the parental Min6 cells with the FoxO inhibitor AS1842856 (FoxOi) (Nagashima et al, 2010) for 48 hours. We studied the expression levels of key pancreatic marker genes by qRT-PCR. We observed a ~3-fold reduction in insulin expression, accompanied by an upregulation of the pancreatic progenitor marker Ngn3 and a strong induction of the alpha cell marker glucagon (Fig. 2A). At the single-cell level, all cells show reduced levels of insulin expression, whereas a subset of cells starts expressing glucagon (Fig. 2B and C, Supplementary Fig. 1A). The increase of glucagon is further confirmed by western blot (Fig. 2D). Importantly, FoxO1 target genes were affected by the addition of FoxOi (Supplementary Fig.1B). In the rescue assay, the overexpression of constitutively active FoxO1 decreases the fraction of glucagon positive cells from 23% to 14% (Fig. 2E and F). While wash-out of the inhibitor for another 48 hours causes the cells to reestablish insulin levels, the glucagon positive subpopulation remains stable and converts to a double-positive phenotype (Fig. G). To better understand the dedifferentiation process, we addressed the global gene expression changes caused by

FoxO1 inhibition. Compared to control cells, 441 transcripts are significantly upregulated more than 2-fold by FoxO inhibtion, whereas 219 transcripts are downregulated. In addition to insulin, other beta cell specific genes, including glucokinase, islet amyloid polypeptide and Pdx1 are downregulated, whereas alpha cell markers Mafb and glucagon are upregulated (Fig. 2H). Gene set enrichment analysis shows that genes specifically expressing in human (left) and mouse (right) beta cells are downregulated by FoxO1 inhibition (Fig. 2I).

PAX4 overexpression counteracts FoxO inhibitor mediated dedifferentiation

Considering the loss of a mature beta cell phenotype following FoxO inhibition and the repression of Ngn3 by PAX4 overexpression, we hypothesized that PAX4 can prevent dedifferentation. Gene set enrichment analysis indicates that PAX4 upregulated genes are strongly repressed by FoxO inhibition (Fig. 3A). This gene set includes genes specifically expressed in beta cells (Hdac9, Dio1, Kcnmb2) and other pancreatic cell types (Sst), and genes important for beta cell proliferation and function (Egfr, Cck, Casr) (Fig. 3B). We then tested the effects of PAX4 overexpression on the beta cell dedifferentiation phenotype. In the presence of elevated PAX4 levels, 48 hour FoxOi treatment failed to repress insulin and activate glucagon (Fig. 3C). On transcriptional level, PAX4 overexpression suppressed the induction of glucagon but failed to rescue the decrease of insulin (Fig. 3D). Importantly, PAX4 overexpression abolished the FoxOi induced increase of Bcl2l1, which is a canonical target of FoxO1. This experiment indicates that PAX4 functionally antagonizes FoxO inhibition to maintain beta cells in a differentiated state.

FoxO inhibitor treatment enhances acidification of lysosomes in beta cell

Following treatment of beta cells with FoxOi, we found the majority of lysosomal hydrogen transporter family members to be upregulated in the gene expression dataset (Fig. 4A). Accordingly, we observed increased intracellular acidification following FoxO inhibition. The increase of intracellular acidification was abolished by the lysosomal acidification neutralizer chloroquine (Fig. 4B). By inhibiting the lysosomal acidification, chloroquine prevents glucagon transcription in both dedifferentiated beta cells (Fig. 4C and Supplementary Fig. 1C) and differentiated alpha cells (Fig. 4D and Supplementary Fig. 1D). In alpha cells, decreased glucagon transcription results in reduced glucagon protein, measured by western blot (Fig. 4E). The disruption of lysosomal acidification by chloroquine for 24h treatment suppresses up to 50% of glucagon secretion in low glucose condition (Fig. 4F). Interestingly, the supply of recombinant glucagon peptide partially rescues the downregulation of glucagon transcription in both dedifferentiated beta cells and differentiated alpha cells, highlighting the importance of extracellular glucagon to maintain alpha cell identity (Fig. 4G).

A high content screen identifies inhibitors of beta cell dedifferentiation

Having shown that beta cell dedifferentiation can be prevented by transcription factor overexpression, we wanted to test the suitability of the cellular system in the identification of small molecule dedifferentiation inhibitors. To do so, we used an immunofluorescence assay for glucagon and insulin, following 48 hour FoxO inhibition, on an automated microscope in 384-well plates. First we tested an ALK5 inhibitor that was recently published as a tool compound to prevent beta cell dedifferentiation in islet culture (Blum et al, 2014). Indeed, we observed that this compound represses FoxOi mediated insulin reduction and glucagon increase (Supplementary Fig. 2A). To identify

approved drugs with similar properties, we tested a library of 283 representative compounds covering the diversity of clinically used compounds with regards to their chemical structure and molecular targets. We find that a subset of these compounds causes an increase in insulin staining and reduction of glucagon intensity in FoxOi treated cells (Fig. 5A and B). Among them, loperamide treatment antagonizes the action of FoxOi and prevents beta cell dedifferentiation (Fig. 5C). Importantly, the evaluation of insulin and glucagon intensities at the single cell level suggests that loperamide-treated cells are highly similar to the control cells but different from the FoxOi-treated cells (Supplementary Fig. 2B). The effects of loperamide were further investigated at the transcriptional level by RT-qPCR at different time points. Surprisingly, loperamide decreased both insulin and glucagon transcription, with or without the presence of FoxOi (Fig. 5D, Supplementary Fig. 2C and D). The suppression of glucagon by loperamide could be washed off, while the inhibition of insulin expression persisted after removal of the compound (Supplementary Fig. 2E). Therefore, the increase in insulin levels following loperamide treatment is likely caused by the inhibition of insulin secretion. Meanwhile, we measured the abundance of mRNA for transcription factors which are essential for beta cell development, including Mafa, Mnx1 and Pax4. Comparing FoxOi treatment, the combination of FoxOi and loperamide rescues the loss of these transcription factors indicating loperamide treatment is preventing the dedifferentiation of beta cells (Fig. 5E).

Loperamide is a mu-opioid receptor agonist (Giagnoni et al, 1983) which has been used for the treatment of diarrhea since the 1970s (FDA application No. 017694). In contrast to other opioid receptor agonists, loperamide does not have effects on the central nervous system. To test if activation of mu-opioid receptor can inhibit beta cell

dedifferentiation, herkinorin, a novel mu-opioid receptor agonist was used in combination with the FoxOi (Groer et al, 2007; Xu et al, 2007). Like loperamide, herkinorin prevented the decrease of insulin levels following FoxO inhibition. However, herkinorin did not inhibit the upregulation of glucagon in beta cells (Supplementary Fig. 3A). To further investigate on- vs. off-target effects of loperamide, the opioid receptor antagonist naltrexon was used in combination with loperamide. While naltrexon did not prevent the loperamide-mediated increase in insulin levels, the beta cell master regulatory transcription factor Pdx1 responded as predicated (Supplementary Fig. 3B). In summary, loperamide's effects on dedifferentiation can only partially be explained by the activation of opioid receptors.

Loperamide inhibits glucagon transcription through activation of intracellular calcium

The effects of loperamide on glucagon expression were tested in alpha cells by RT-qPCR and western blot assay. Loperamide treatment suppressed the production of glucagon in alpha cells at both the mRNA (Supplementary Fig. 4A and B) and protein level (Fig. 5F).

Previous studies of loperamide in beta cells suggest the compound to increase the free intracellular calcium by mobilization of storage in ER (He et al, 2003). We did observe the mobilization of free intracellular calcium with Fura-2 staining in beta cells with loperamide treatment (Supplementary Fig. 4C). Indeed, treatments with the calcium ionophores calcimycin induced the similar phenotypic changes in FoxOi-treated beta cells as loperamide (Fig. 5G). Calcimycin treatment also decreased the transcription of glucagon in alpha cells (Fig. 5H). Importantly, the combination of loperamide with the calcium chelator BAPTA-AM partially rescued the decrease of glucagon transcription in

alpha cells (Fig. 5I). This result hints that loperamide's inhibitive effect on glucagon transcription is dependent on intracellular calcium.

Loperamide treatment enhances the expression of FoxO1 in multiple organisms

It has been described that FoxO1 exerted protective effects on beta cells in mouse islets. Therefore we measured the protein level of FoxO1 in both Min6 cells and alpha cells treated with loperamide. Loperamide treatment increased FoxO1 mRNA and protein in alpha cells, beta cells and 293T cells suggesting loperamide enhance FoxO1 expression in different cell types (Fig. 6A and Supplementary Fig. 4D). Since FoxO protein promotes dauer formation in C.elegans, it was used as a model organism to test the effects of FoxOi and loperamide in this study. Indeed the chemical inhibition of FoxO suppresses the dauer formation rate (Fig. 6B, left) and loperamide increases the dauer formation rate (Fig. 6B, right) in C.elegans. These results confirmed that the effects of loperamide on FoxO biology are conserved in different organism. Interestingly, the calcium ionophore calcimycin also increases the FoxO1 proteins in both alpha and beta cells (Supplementary Fig. 4E). It hints the increase of intracellular calcium in pancreatic endocrine cells can enhance FoxO1.

Loperamide treatment exerts different effects on immature and mature islets

Having observed the inhibition of glucagon expression by loperamide in mouse alpha and beta cell lines, intact human islets were treated with loperamide for 48 h to test the compound's effects on mature human alpha and beta cells. Compared to the control population, loperamide treatment decreased the number of alpha cell and increased beta cells number and double positive cell number (Fig. 6C and D). We further investigated the transcriptional changes of endocrine factors in these human islets. Similar to the changes in the mouse alpha cells, loperamide treatment dramatically

decreased glucagon transcription without a significant effect on insulin transcription (Fig. 6E). The effects of loperamide in the development of islets were investigated on zebrafish larvae carrying double reporters: Gcg-GFP and Ins-mCherry. The treatment started at 26h pf and stopped at 5 days pf. Loperamide dramatically increased both Insulin and glucagon positive cells (Fig 6F and G), which suggested different effects on immature zebrafish islets to mature human islets.

Discussion

Beta cell dedifferentiation can partially explain the loss-of-function of beta cells during the late stages of Type II diabetes. Interfering with the process might prevent, or at least delay, the progression of diabetes. We developed a cell system that allows studying the dedifferentiation process in more detail than the *in vivo* models currently available. In our cellular system, beta cell dedifferentiation can easily be induced by two day treatment with a FoxO inhibitor. We observe that FoxO inhibition represses PAX4-induced genes. Testing the hypothesis that FoxO and PAX4 contribute to a fully differentiated beta cell phenotype, we show that PAX4 overexpression can antagonize FoxO inhibition. Thereby, increasing PAX4 levels in pancreatic islets might have beneficial effects for diabetes patients, not only by causing transdifferentiation of alpha cells, but also by preventing beta cell dedifferentiation. With the analysis of the transcriptional profiles we observed increased lysosomal acidification in beta cells following FoxO inhibition. Neutralizing of lysosomal acidification with chloroquine prevents the beta cell dedifferentiation, highlighting the importance of proper lysosomal pH for maintaining beta cell identity.

Having established an *in vitro* model allowed us to systematically test for compounds that prevent a beta cell dedifferentiation phenotype. We identified loperamide to prevent

glucagon expression in this dedifferentiation model. We observed that loperamide also repressed glucagon expression in the context of alpha cells and human islets, suggesting an impact on islet biology beyond beta cell dedifferentiation. Interestingly, the effects of loperamide on glucagon are unlikely related to mu-opioid receptor but likely relying on the mobilization of intracellular calcium. In one hand, it has been shown that the activation of calcium-dependent kinase Camkii enhances FoxO1 in hepatocytes (Ozcan et al, 2012). The increase of FoxO1 induced by loperamide treatment may be mediated by Camkii. In the other hand, the mobilization of intracellular calcium by calcium ionophores activates NFAT signaling and induces beta cell proliferation in human islets (Shen et al, 2015). In the opposite direction, loss of NFAT in beta cell induces beta cell apoptosis and loss-of-function (Heit et al, 2006). It is interesting to test if there is a cross-talk between NFAT and FoxO proteins and if loperamide regulates NFAT signaling. However, the treatment of loperamide on zebrafish larvae achieves a larger islet with more alpha and beta cells, which points out the difference effects of loperamide on immature and mature islets.

The treatment of loperamide on zebrafish larvae results in a larger islet with more alpha and beta cells. Species differences or the distinction between developmental and mature models could explain the differences to the observations in human and mouse islet cells. Loperamide has been shown to have protective effects on diabetic rats treated with streptozotocin to induce beta cell damage (Liu et al, 1999; Tzeng et al, 2003). However, the plasma concentration of loperamide in patients with diarrhea (taken orally) is in mid-nano-molar range, too low to activate intracellular calcium and lower glucagon transcription. An alternative administration of loperamide is needed in order to translate it into clinical application.

In summary, we established a cellular model for beta cell dedifferentiation which is suitable for transcriptional study and high-through put screening. With this model we identified Pax4, chloroquine and loperamide to prevent the dedifferentiation with different mechanism. This model will accelerate the study of understanding the mechanism of beta cell dedifferentiation and looking for therapies.

Materials and Methods

Reagents

Antibodies used in this project are directed against Insulin (Sigma 18510), Glucagon (Abcam ab92517), Pdx1 (Abcam ab47383), Pax4 (R&D AF2614), Arx (R&D AF7068), Myc (Cell Signaling Technology CST2276), Histone H2B (Cell Signaling Technology CST2934), FoxO1 (Cell Signaling Technology CST2880P). The FoxO inhibitor AS1842856 was obtained from Calbiochem. The ALK5 inhibitor was obtained from Enzo life science (ALK-270-445). Herkinorin was ordered from Abcam (ab120147). Loperamide (L4762) and primers were obtained from Sigma. The sequences of primers are the same of the primers used before (Fomina-Yadlin et al, 2010) except mouse Ngn3 (Forward : TCCGAAGCAGAAGTGGGTGACT ; Reverse : CGGCTTCTTCGCTGTTTGCTGA). All the fluorescently labeled secondary antibodies were purchased from Life Technologies Corporation.

Cell culture

Min6 and alpha-TC1 cell lines were obtained from ATCC. Min6 cells and inducible subclones were grown in high-glucose DMEM supplemented by 15% Tet System Approved FBS (Clonetech 631106), 71 uM 2-mercaptoethanol, 50 U/mL penicillin and 50 ug/mL streptomycin. The cells were kept in the continuous presence of 4ug/ml puromycin and 4ug/ml blasticidin. The overexpression of PAX4, ARX, or GFP was induced by 1ug/ml doxycycline. The mouse pancreatic cell line alpha-TC1 was grown in low-glucose DMEM medium supplemented with 10% FBS, 50U/ml Penicilin and 50ug/ml streptomycin.

Human islets (10,000 IEQs) were provided through the JDRF award 31-2012-783 (ECIT: Islet for Research program). Islets were cultured in CMRL medium (Life technology) supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Intact islets were treated with different drugs for two day. Half of the intact islets were collected for RT-qPCR. The other half of the islets were indubated in Accutase (Life technology) in 37 °C for 20 min, neutralized by CMRL medium, seeded to 384 well plate and continued to immunofluorescence assay.

Establishment of inducible cell lines

To generate inducible Min6 cells, we first transduced the cells with a lentivirus carrying TetR DNA and a neomycin resistance cassette (Gentarget Inc., cat.# LVP017-Neo) and then isolated a single clone expressing high amounts of TetR protein, validated with the Tet01 antibody from MoBiTec. This TetR expressing cell line was then transduced with virus particles coding for FlagMyc tagged (C-terminal) eGFP, human ARX or human PAX4 cDNA also expressing the puromycin resistance gene. The vector used for this work was the pSTK007, which is a modified version of the pLenti4.2 (Life technology). Then, a second round of clonal selection was performed where numerous clones were tested for maximum repression in the uninduced state and a high degree of induction upon treatment with doxycyline (1ug/ml).

All lentiviruses were produced in HEK293T cells using the Virapower plasmid mix (Life technology), according to the the manufacturer's protocol. All virus work was done in a Class II laboratory, and before transferring the cells to a regular lab the cells were proven to be virus free using a p24 ELISA kit (Cell Biolabs, cat.#VPK-108-H).

High-content screening

Compounds (25 nl in 10mM) were transferred to 384-well plates (Corning 3712) from DMSO stock plates using acoustic transfer (Labcyte Inc.). Min6 cells (3000 cells per well) incubated with 10uM FoxO inhibitor were plated in 50 ul media on top of the compounds. Two days after treatment, cells were fixed in 3.7% formaldehyde for ten minutes at room temperature. Following PBS washing, cells were fixed with cold pure methanol in -20 C for 10 minutes, permeabilized by 1% Triton X-100 in PBS for 30 minutes and blocked by 3% BSA in PBS for 30 minutes. Twenty microliters of primary anti-Insulin antibody and anti-Glucagon antibody, both diluted in 1:2000 in 1.5% BSA, was added per well and incubated in 4 °C overnight. After washing with PBS twice, 20 ul fluorescence labeled seconday antibody diluted 1:1000 and 10ug/mL Hoechst 3342 in PBS was added per well and incubated for 1 h. After two washes with PBS, plates were stored in 4 °C in dark until analysis.

Images were taken by an automated microscope (Perkin Elmer) using a 20X objective. Images were exposed for 10 ms in Hoechst channel and 400 ms in Alexa Fluor 488 and 548 channel. Images were analyzed by Harmony software. Nuclei were identified by Harmony Method C and cytoplasm was defined based on the nuclei (Harmony Method C). In total, 283 compounds were screened from the CLOUD library, CeMM's collection of clinical approved drugs with unique structure. Hits were selected based on the intensity of Insulin in the Alexa Fluor 548 channel, intensity of Glucagon in the Alexa Fluor 488 channel, amount of DNA and cell numbers in the Hoechst channel. Cells whose Hoechst intensity were lower than 1000 were treated as dead cells and removed from the screening. All the other immunofluorescence assays were done and analyzed in the same way unless specified.

RNA-seq

Cells were lysed and RNA isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The libraries for RNA-seq for 24h induction in Min6 cells were prepared with Ribo-zero Kit and Scriptseq v2 Kit obtained from Epicenter by following the manual from the provider. For the rest of the RNA-seq libraries the amount of total RNA was quantified using Qubit 2.0 Fluorometric Quantitation system (Life Technologies) and the RNA integrity number (RIN) was determined using Experion Automated Electrophoresis System (Bio-Rad). RNA-seq libraries were prepared with TruSeq Stranded mRNA LT sample preparation kit (Illumina) using Sciclone and Zephyr liquid handling robotics (PerkinElmer). Library amount was quantified using Qubit 2.0 Fluorometric Quantitation system (Life Technologies) and the size distribution was assessed using Experion Automated Electrophoresis System (Bio-Rad). For sequencing libraries were pooled and sequenced on Illumina HiSeq 2000 using 50 bp single-read. Reads were aligned with tophat (v2.0.4) with the --no-novel-juncs --no-novel-indels options (Kim et al, 2013). Gene expression was calculated as Reads Per Kb per Millions of reads (RPKMs) using RPKM_count.py from RSeQC package (Wang et al, 2012) and the NCBI RNA reference sequences collection (RefSeq) downloaded from UCSC (Kent et al, 2002) . The enrichment calculation was done by Gene Set Enrichment Analysis (Mootha et al, 2003; Subramanian et al, 2005). The basal expressions of genes in alpha and beta cells were taken from microarray data in literature (Kubicek et al, 2012).

RT-qPCR

After the RNA was isolated with the RNeasy Mini Kit (Qiagen), it was reverse transcribed with random primers using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed with Power SYBR Green PCR Master

Mix (Applied Biosystems) on Lightcycler 480 qPCR machine (Roche). All the results are normalized to beta-Actin.

Intracellular PH measurement

To measure the intracellular PH change in Min6 cells, they were pretreated with FoxOi with or without chloroquine for two days. The measurement was performed by pHrodo® Red AM Intracellular pH Indicator (Life technologies) following the manufacturer's guideline. The images were taken by the Operetta and the relative intensity was quantified by Harmony.

Calcium staining

The calcium staining was done in live Min6 cells with Fura-2 kit (F-1201, Life technologies). Min6 cells are seeded and pretreated with loperamide for 18h before the assay. The staining was performed according to the manufacturer's guideline.

Dauer formation

C. elegans strain CB1370 was seeded by egg-lay onto the plates with FoxO Inhibitor and loperamide at different concentrations. For testing the effect of Foxo1 Inhibitor on the suppression of dauer formation, worms were grown at 23 °C. For the induction of dauer formation to test the effect of Loperamide, worms were grown at 22°C. Animals were evaluated after 7 days by scoring their survival after 30 min treatment with 1% (w/v) SDS.

Zebrafish larvae assay

Larvae of intercross Glcgn:GFP and ins:NTR-mcherry fish were treated 26hpf (fertilization) with loperamide; on day 5 they were sorted for potentially double positive larvae (stereo microscope), embedded into agarose and pictures were taken on the

confocal at 25x. With this pictures 3D models of the islets were established and the GFP positive cells and mcherry positive cells were counted by a 3D imaging tool.

Statistical methods

All the p-values were calculated by student t test, unless specified as other methods.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Conception and design: SK and JL. Cell line establishment: SK, TF and JH. Next generation sequencing: TP and CB. RNA-seq analysis: FP and JL. Human islet prepartions: CB and EB. Zebrafish assays: CT and MD. Dauer formation assay: CR and IS. Performing experiments: JL, CHL, and TC.

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Figure Legends

Figure 1. PAX4 overexpression reinforces a beta cell phenotype.

A. Schematic overview of the Min6 beta cell model. **B.** Western blot for Myc in two inducible Min6 cell lines after 24 hour induction of Myc-GFP and Myc-PAX4 by 1 ug/ml doxycycline (Dox). Histone H2B is used as a loading control. **C.** Expression of PAX4 in inducible Min6 cells measured by RNA-seq as Reads Per Kilobase per Million mapped reads (RPKM). **D.** Result from Gene set enrichment analysis. Significantly upregulated genes in induced PAX4 samples were enriched in beta cell specific genes. **E.** Expression of Hnf4 and Stat6 in induced Min6-GFP and Min6-PAX4 cells. **F.** Expression of Ngn3 in induced Min6-GFP and Min6-PAX4 cells.

Figure 2. A cellular model for beta cell dedifferentiation.

A. Representative images of Min6-Pax4 cells stained for insulin and glucagon after two day treatment. Scale bars 10 um. **B.** Transcriptionss of Ins2, Gcg and Ngn3 after two day FoxO inhibitor (FoxOi, 10 uM) treatment measured by qPCR. **C.** Measurement of insulin and glucagon intensity in Min6 cells treated with FoxO inhibitor in single cell level. **D.** Western blot showing the increase of glucagon protein in beta cells treated with FoxO inhibitor **E.** Quantification of each single cell treated with FoxO inhibitor and transfected with FoxO1 overexpression construct. Blue, cells without FoxO1 overexpression and glucagon positive; Yellow, cells with FoxO1 overexpression and glucagon positive. **F.** Western blot showing the overexpression of FoxO1. **G.** Representative images (left panel) and single cell evaluation (right panel) of Min6-Pax4 cells stained for insulin and glucagon after 48 h FoxOi treatment followed by 48 h washing off the compound. Scale

bars 10 um. **H.** Expression change of pancreatic endocrine factors in 2-day FoxOi treated cells versus control DMSO-treated Min6-PAX4 cells. **I.** GSEA showing downregulation of mouse (left) and human (right) beta cell specific genes with FoxO inhibitor treatment.

Figure 3. PAX4 overexpression counteracts FoxO inhibitor mediated dedifferentiation.

A. Result from Gene set enrichment analysis. Significantly downregulated genes in FoxOi treated samples were enriched in genes upregulated by PAX4 overexpression. **B.** Gene set upregulated by PAX4 and repressed by FoxOi. Color scheme indicates the fold-change of induced Min6-PAX4 vs.control induced Min6-GFP cells (top row) and FoxOi-treated vs. control DMSO-treated uninduced Min6-PAX4 cells (lower row) **C.** Representative images of uninduced and induced Min6-Pax4 cells (after two days induction and simultanous FoxOi treatment) stained for insulin and glucagon. Scale bars 10 um. **D.** Expression of Gcg, Ins2 and Bcl2l1 in uninduced and induced Min6-PAX4 cells (after two days induction and simultanous FoxOi treatment) measured by qPCR.

Figure 4. FoxO inhibitor treatment enhances acidification of lysosomes in beta cell

A. FoxO inhibitor treatment upregulates the transcription of lysosomal hydrogen ion transporter in beta cells, measured by RNA-seq. **B.** Intracellular PH indicator showed increase of acidification with FoxO inhibitor treatment in beta cells and decrease with chloroquine. **C.** Chloroquine inhibits FoxOi-induced glucagon transcription in beta cells. **D.** Chloroquine suppresses glucagon transcription in alpha cells. **E.** Western blot showing decrease of glucagon protein by chloroquine treatment in alpha cells. **F.** Secretion of glucagon in alpha cells in low glucose medium pretreated with chloroquine

(5uM) for 24h. **G.** Increase of glucagon transcription by supply of recombinant glucagon with chloroquine in dedifferentiated beta cells (left) and alpha cells (right).

Figure 5. Loperamide inhibits glucagon transcription by activation of intracellular calcium.

A. Overview of the compound screening results. All the results were normalized to the DMSO control. Each dot represents the mean value of three replicates and was colored by relative cell number. Results whose standard deviations were more than 20% of the mean values were removed. **B.** Summary of hits identified from the screening. **C.** Representative images of beta cells treated with/without FoxO inhibitor in combination with loperamide. Scale bar, 10 um. **D.** Loperamide suppresses glucagon transcription induced by FoxO inhibitor in beta cells. **E.** Loperamide rescues the transcription of beta cell specific transcription factors. **F.** Western blot showing decrease of glucagon protein in multiple doses of loperamide. **G.** Calcimycin suppresses glucagon transcription induced by FoxO inhibitor in beta cells. **H.** Calcimycin inhibits glucagon transcription in alpha cells. **I.** Combination of loperamide and Calcium chelator BAMPT-AM rescues the decrease of glucagon transcription in alpha cells with loperamide treatment alone.

Figure 6. Diverse effects of loperamide on different organism

A. Western blots showing the increase of FoxO1 protein with loperamide treatment. **B.** Effects of FoxOi and loperamide on dauer formation of C.elegans. **C.** Representative images of human islet stained for insulin and glucagon. Scale bars 10 um. **D.** Summary of population change in human islet. **E.** Measurement of INS and GCG transcription in human islets by RT-qPCR. All the data points are normalized to DMSO control, n=3. **F.** Representative images of insulin positive cells in zebrafish larvae. **G.** Quantification of Insulin or Glucagon positive cell numbers with loperamide treatment in zebrafish larvae.

Supplementary Figure 1.

A. Representative images of Min6 cells stained for Insulin and Glucagon. Erlotinib was used in 10 uM for two day. Scale bars 10 um. **B.** Downregulation of FoxO1 targeted genes by FoxO inhibitor measured by RNA-seq. **C.** Dose curve of suppression on glucagon transcription by chloroquine in beta cells treated with FoxO inhibitor. **D.** Dose curve of suppression on glucagon transcription by chloroquine in alpha cells.

Supplementary Figure 2.

A. Representative images of beta cells stained for insulin and glucagon after two day compound treatment. Alk5i stands for Alk5 inhibitor (10 uM). Scale bars 10 um. **B.** Single cell evaluation of beta cells stained with Insulin and Glucagon. **C.** Dose curve of suppression on glucagon transcription by loperamide in beta cells treated by FoxO inhibitor. **D.** Measurement of insulin transcription over time-course in beta cells by RT-qPCR. **E.** Measurement of insulin and glucagon transcription before and after washing off the compounds according to the indicated time scheme.

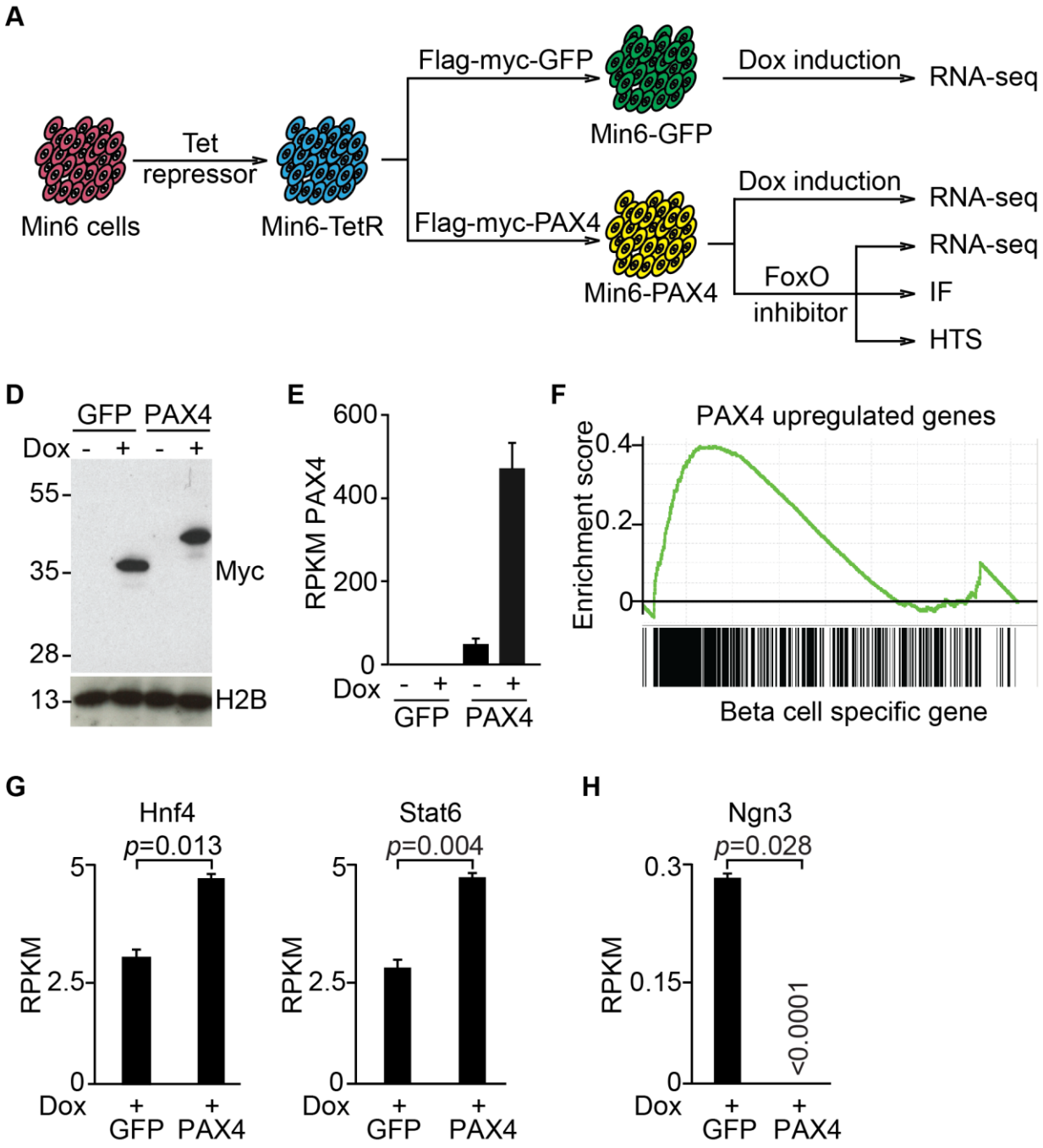
Supplementary Figure 3.

A. Representative images of beta cells stained for Insulin and Glucagon. Herkinorin was used at 15 uM for two day. Scale bars 10 um. **B.** Representative images of beta cells stained for Insulin and Glucagon. Naltrexon was used at 20 uM for two day. Scale bars 10 um.

Supplementary Figure 4.

A. Measurement of insulin and glucagon transcription in alpha cells by RT-qPCR. **B.** Dose curve of suppression on glucagon transcription by loperamide in alpha cells. **C.** Fura-2 staining in alpha cells pre-treated with loperamide for 16h. **D.** Transcriptions of

FoxO1 in beta cells treated with loperamide or calcimycin, measured by qRT-PCR. **E.** Western blot showing increase of FoxO1 protein by calcimycin in alpha and beta cells.



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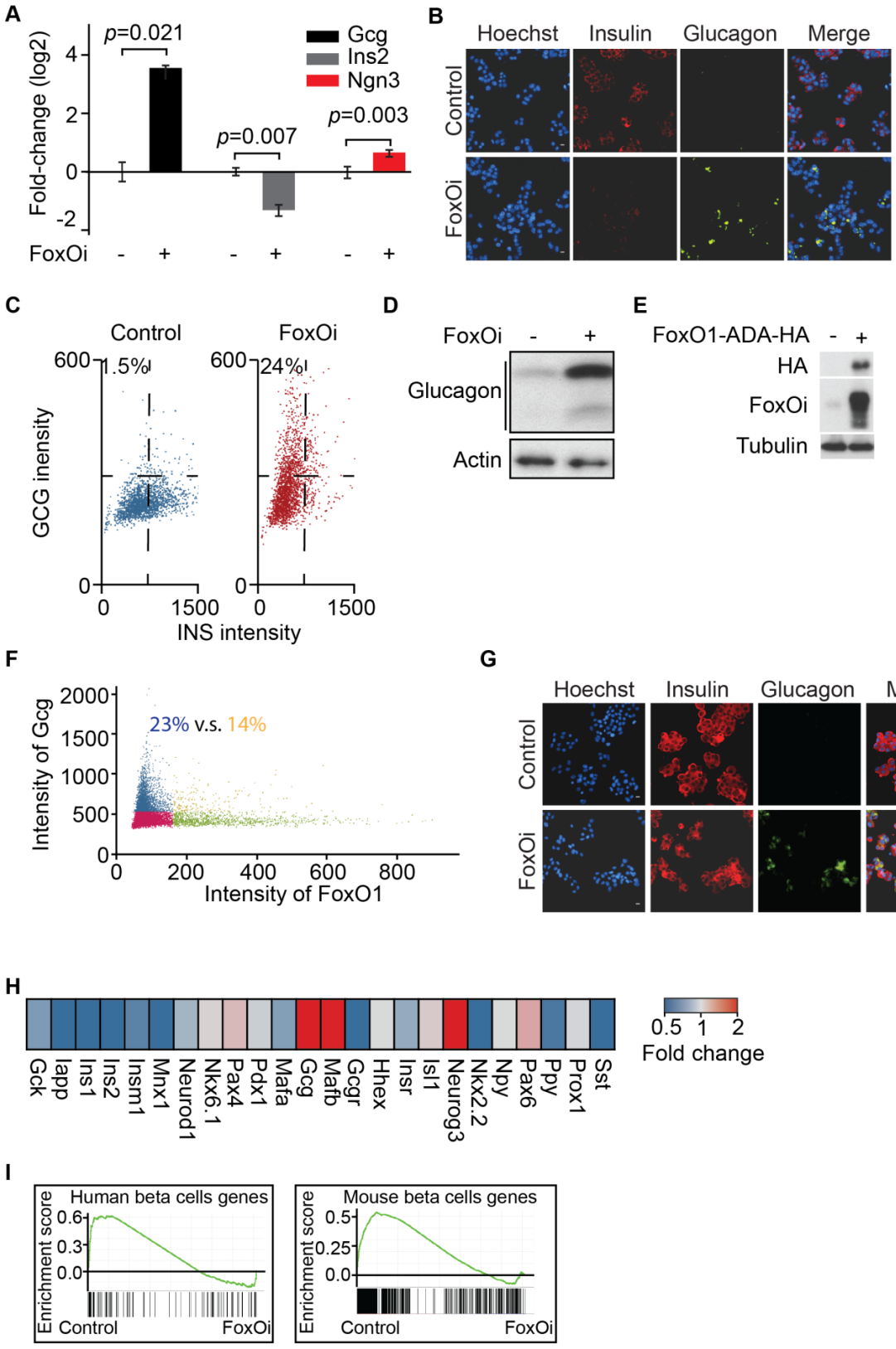

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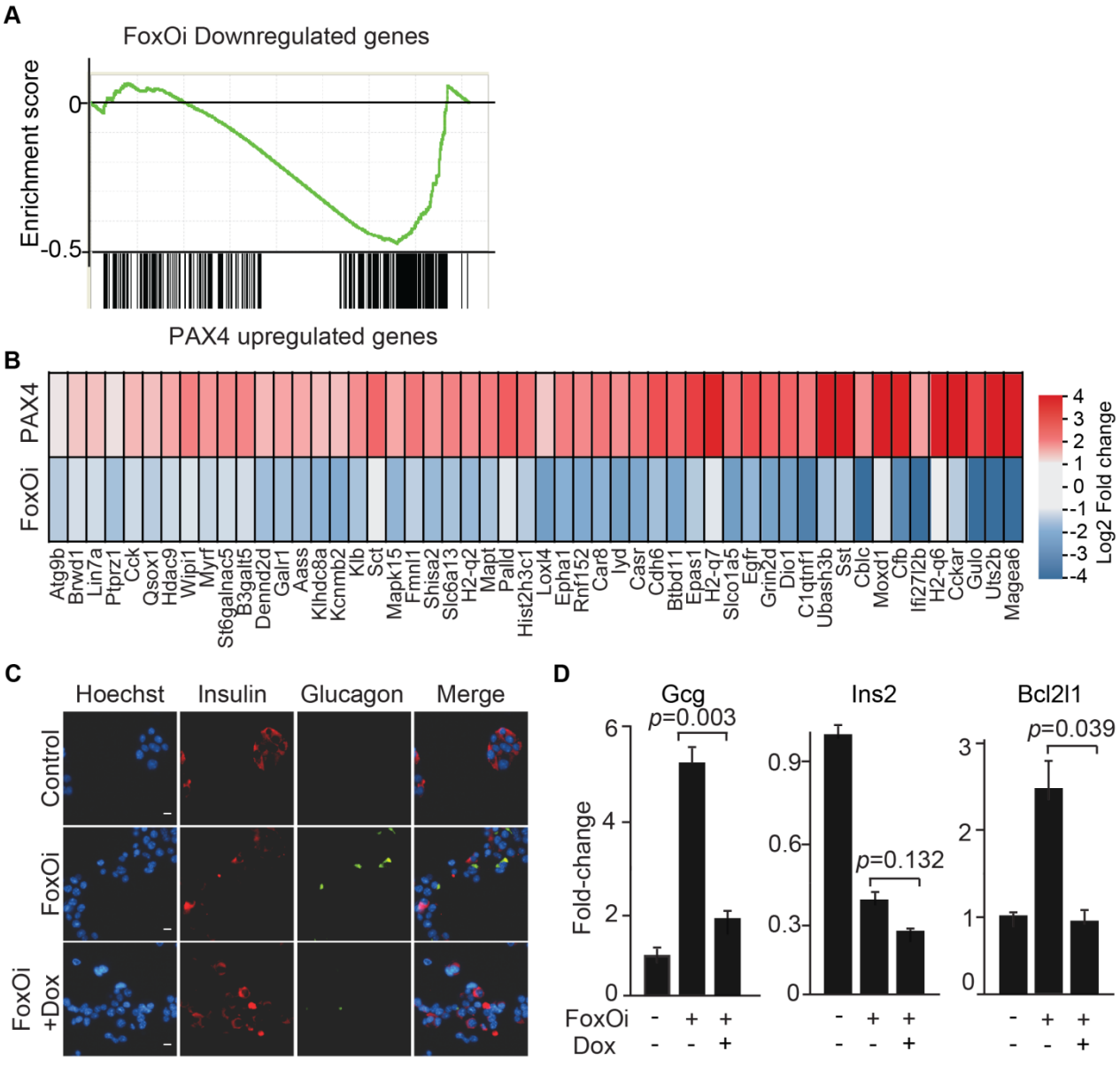

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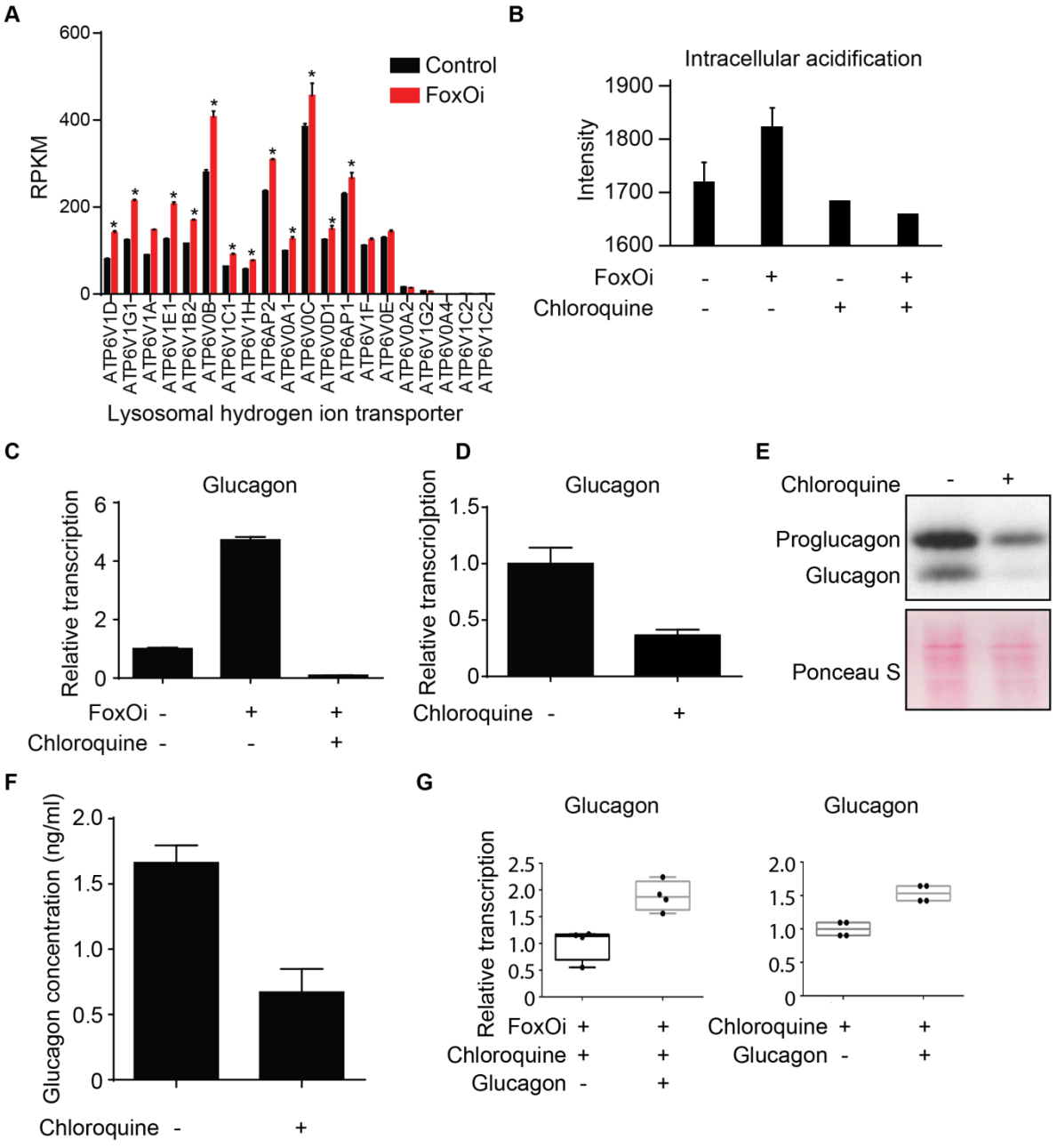

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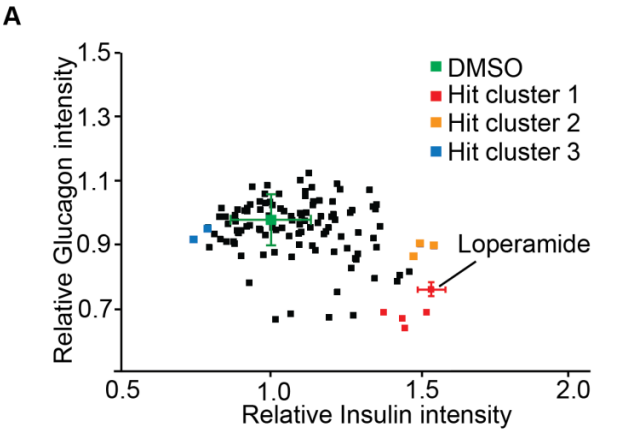
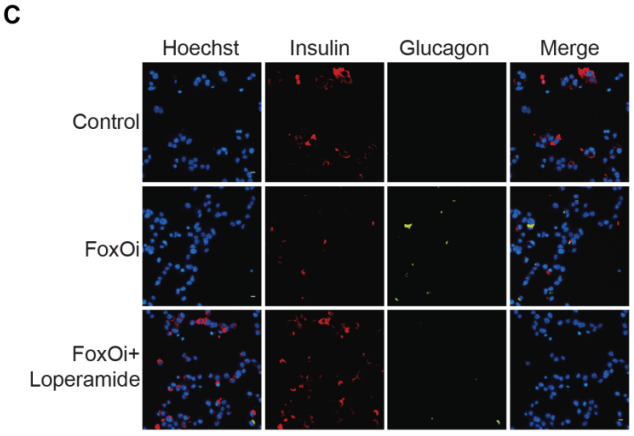
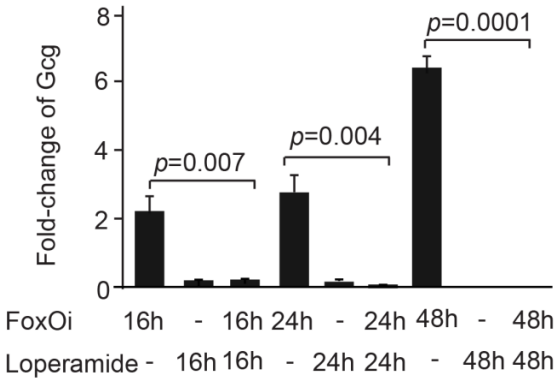
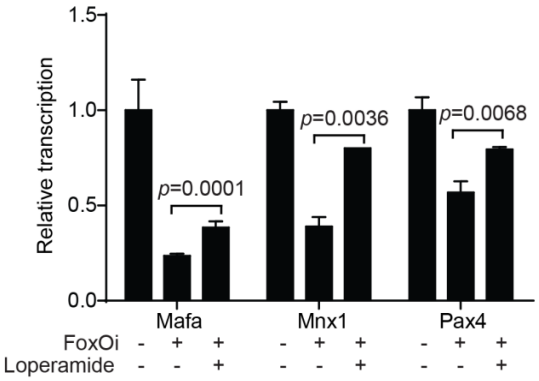


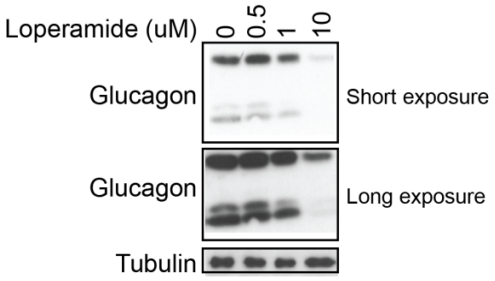
B

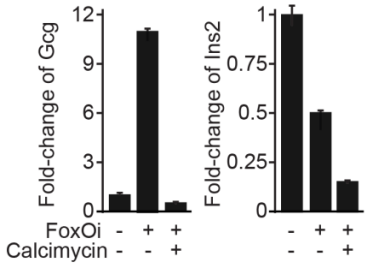
Drug	Insulin	Glucagon
Loperamide	1.53	0.77
Miglustat	1.51	0.70
Tolvaptan	1.44	0.65
Hydralazine	1.43	0.68
Gefitinib	1.37	0.70
Cisapride	1.54	0.91
Imipramine	1.49	0.92
Trihexyphenidyl	1.47	0.88
Pamidronic Acid	0.79	0.97
Methyldopa	0.74	0.93

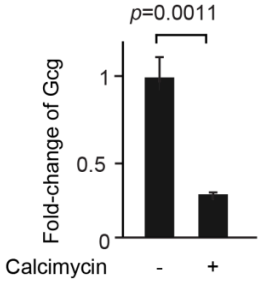


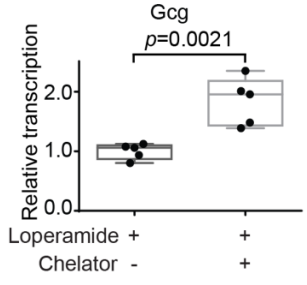












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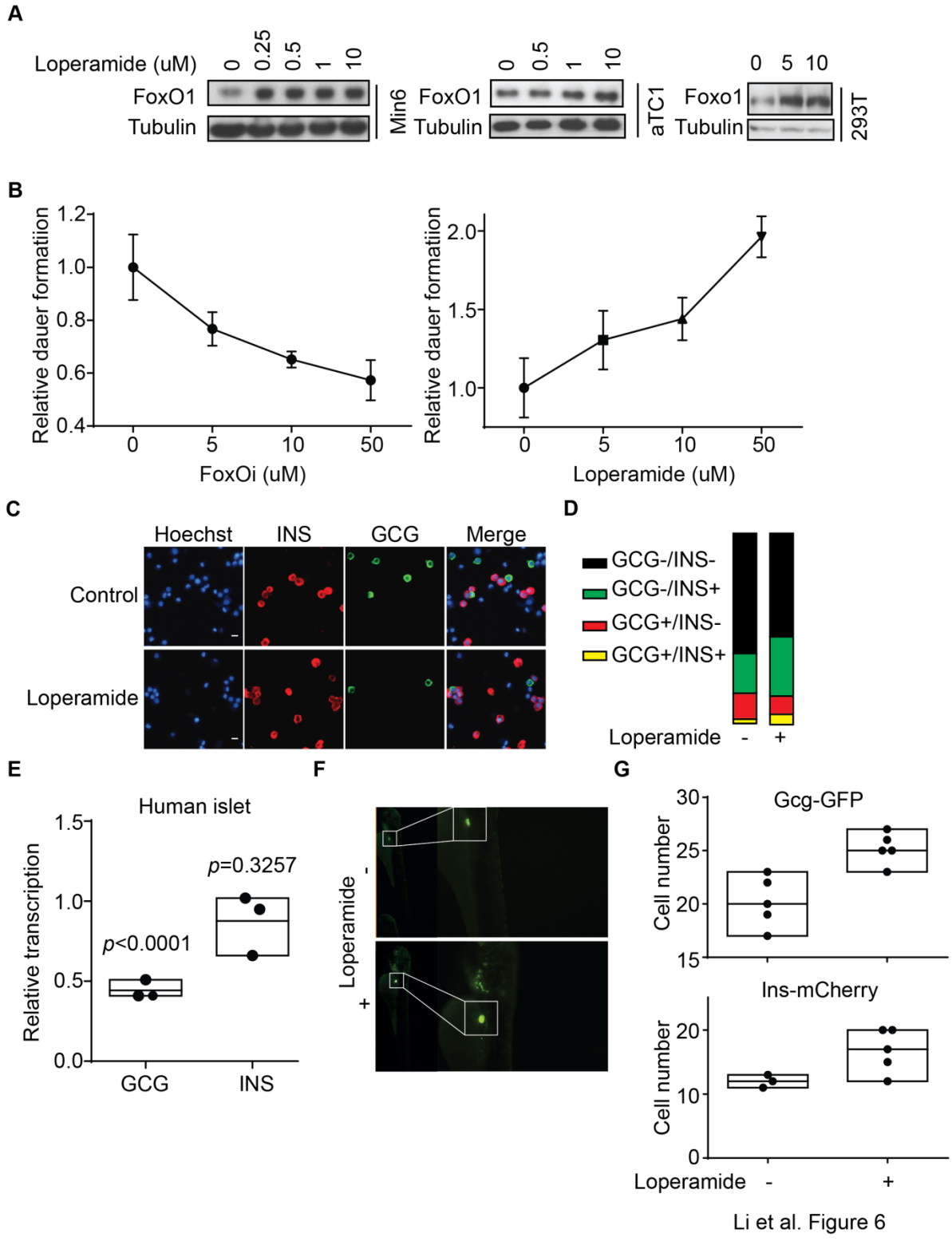

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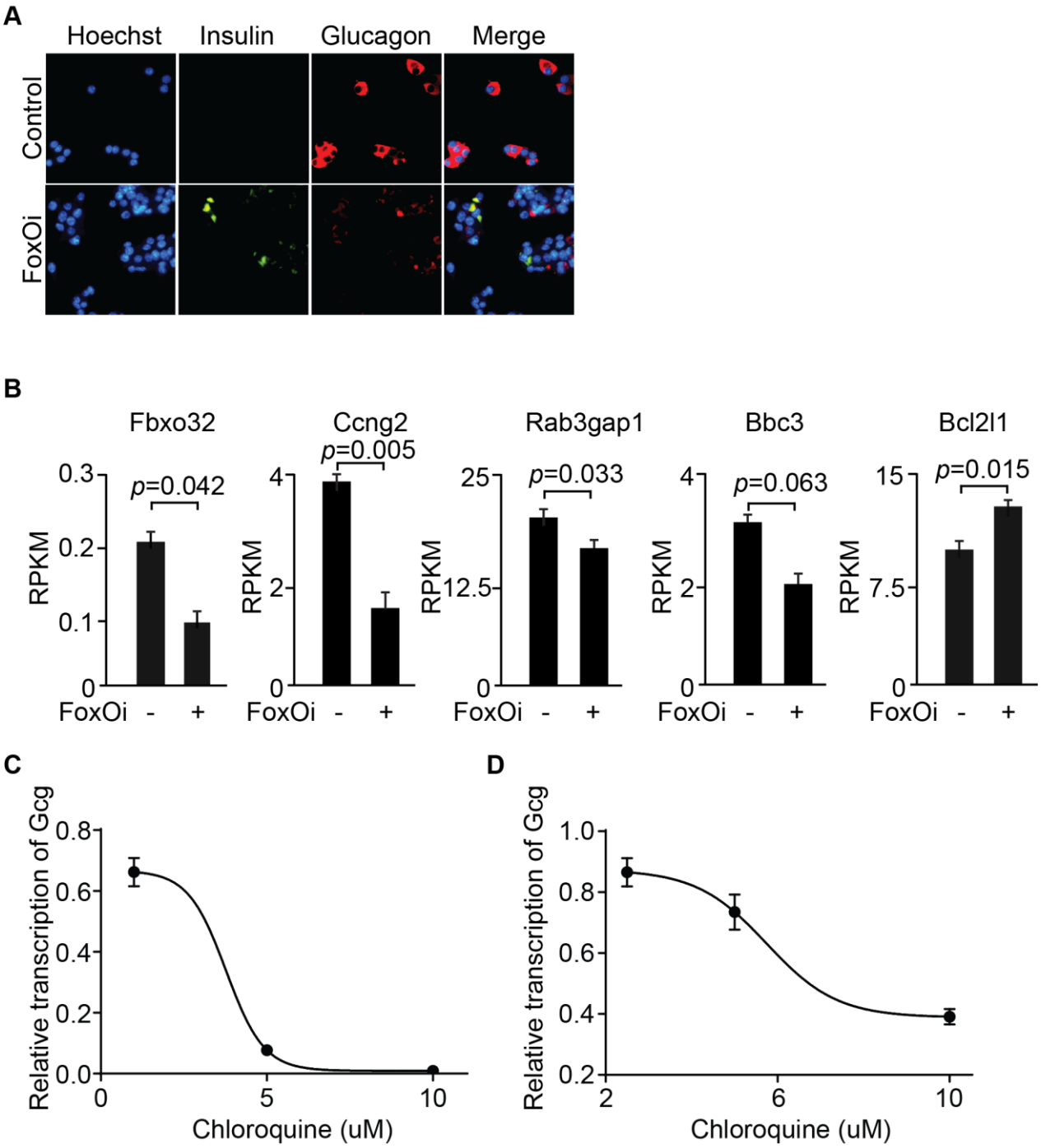
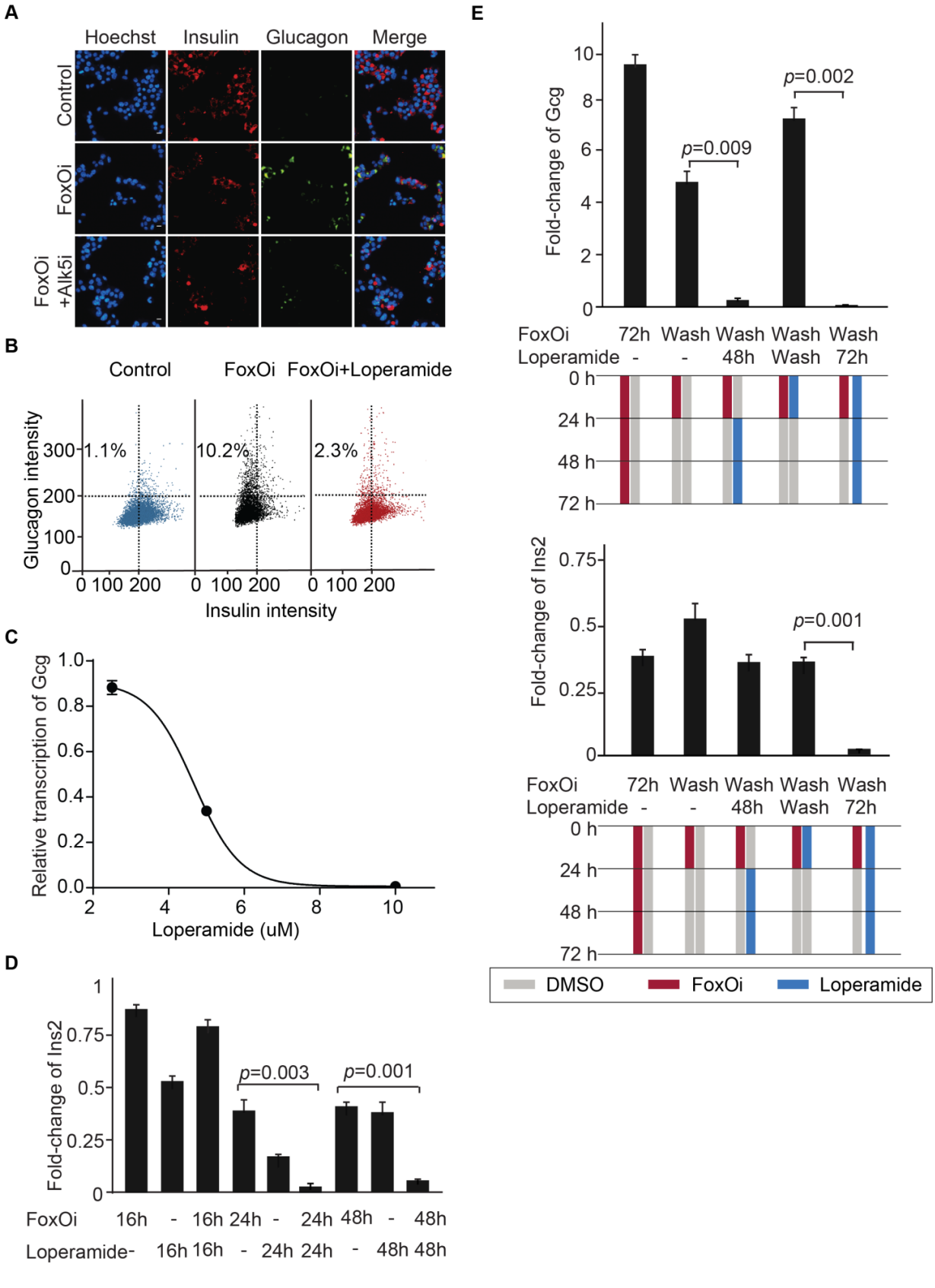


Li et al. Supplementary Figure 1



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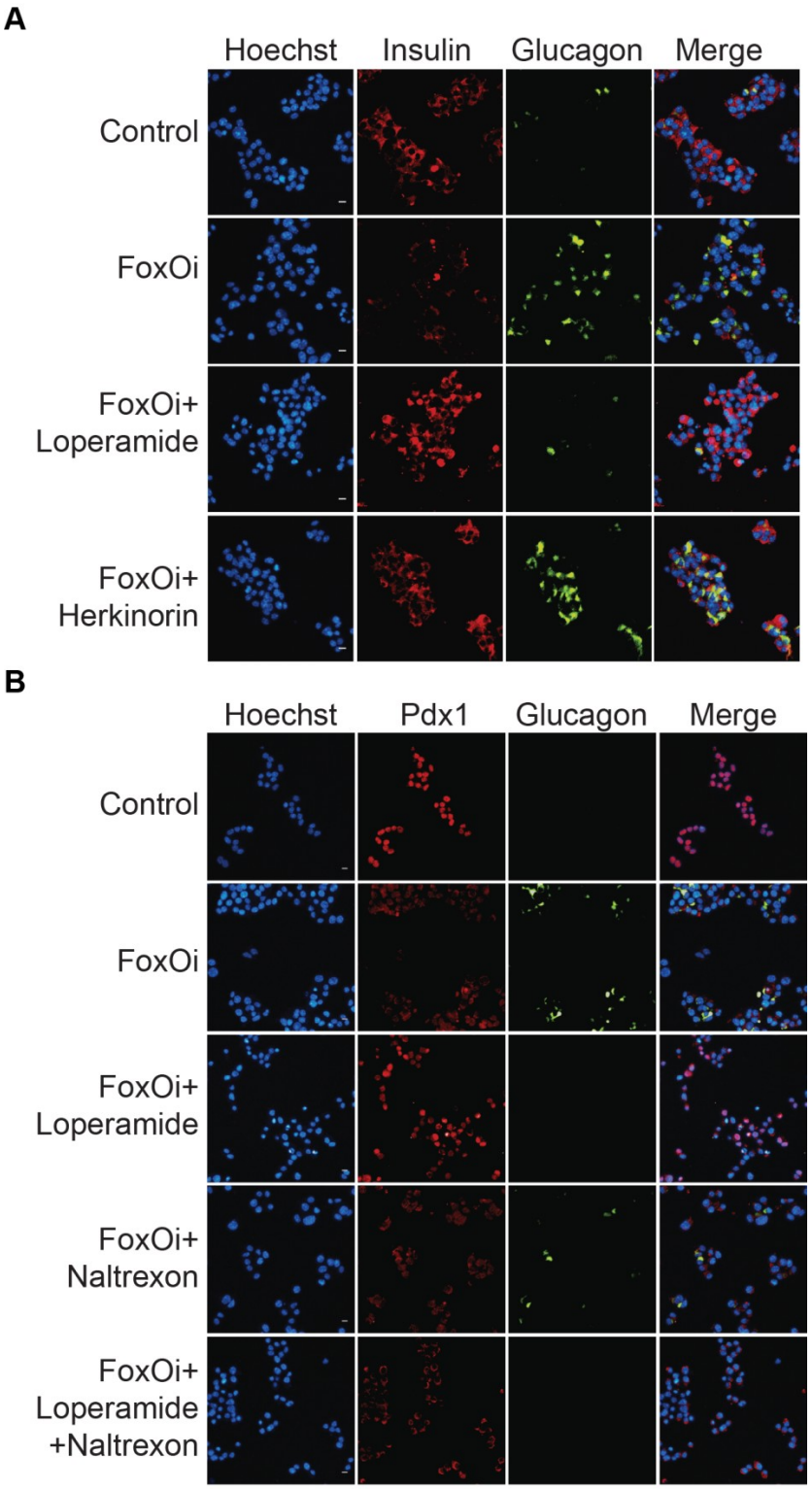

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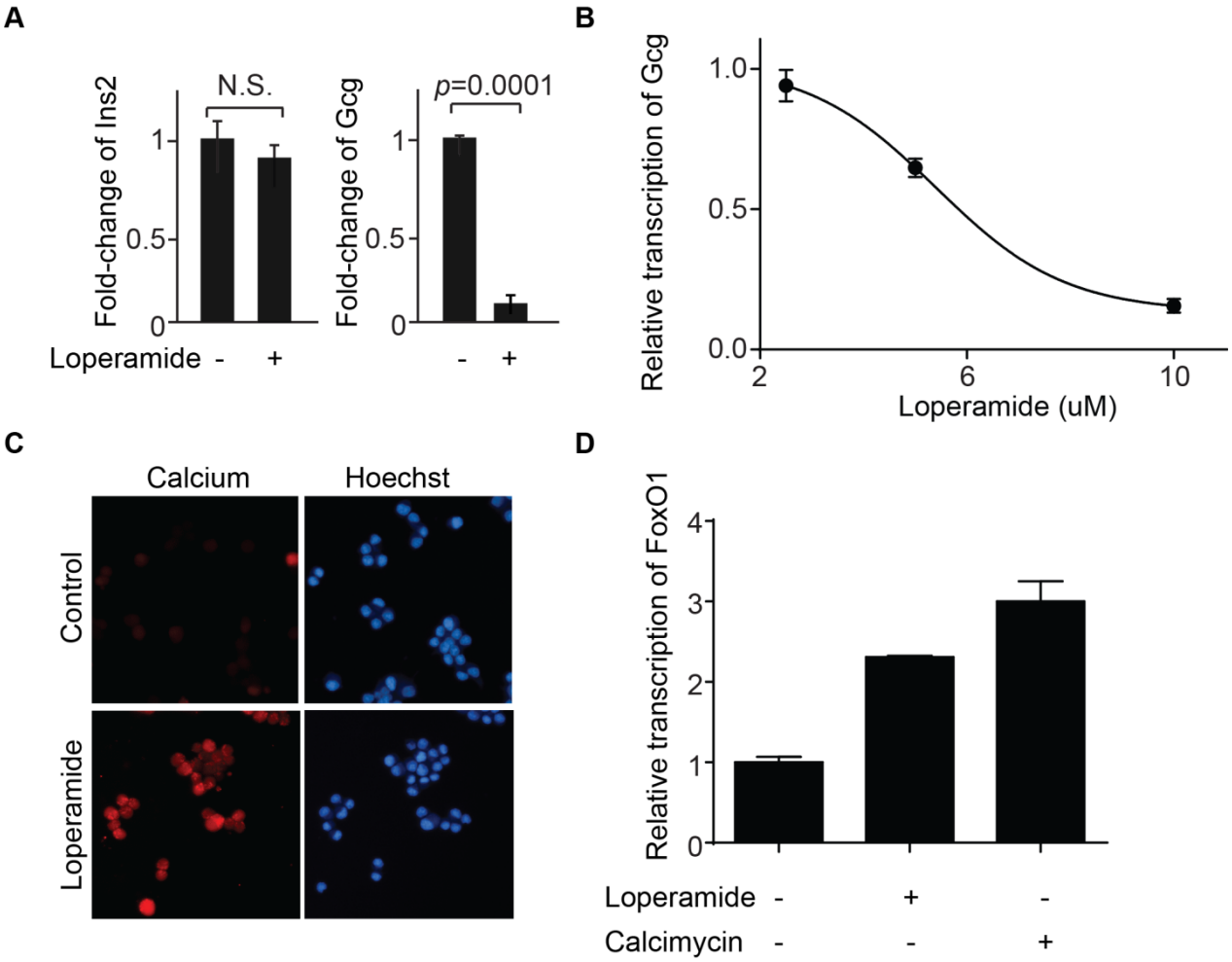


Li et al. Supplementary Figure 4

Discussion

Diabetes is the one of the major health problems worldwide. None of current therapies provide a solution for the loss of beta cells in type I, type II diabetes and MODY. Insulin therapy is so far the most effective therapy for diabetes, but it may induce severe side effects such as nocturnal hypoglycemia which can be lethal to patients. With islet transplantation diabetic patients can restore the insulin production transiently, but the recurrence of insulin deficiency after the transplantation and the shortage of donors are the major bottle necks of transplantation. To my knowledge the ideal therapy for insulin deficiency is to generate new beta cells from endogenous tissues of the same patient by regeneration of beta cell or reprogramming of other cell types into beta cell. In this way the immune attack to foreign tissues could be avoided and the secretion of insulin could be controlled by physiological factors. The existence of auto-antibodies may have negative effects for the newly generated beta cells. However, taking the experience from islet transplantation the autoimmunity is not a big problem if there are enough insulin-producing cells and the patients receive proper medical cares.

For this goal, scientists have tried to reprogram different cell types into insulin producing cells. Many cell types including hepatocytes, intestinal cells, fibroblasts[144-146], pancreatic duct, acinar, alpha and delta cells could be reprogrammed into beta cells. These reprogramming processes are mainly direct transdifferentiation, meaning cells do not go through a stem cell-like cells state before becoming insulin producing cells. The neo-generated beta cells can perform proper function as normal beta cells and rescue insulin deficiency in multiple animal models. These studies highlight the possibilities to generate beta cells from other cell types *in vivo*.

However, none of these reprogramming methods could be tested in patients because they are all based on genetic manipulations. Typically scientists manipulate certain key transcription factors

in vivo, like overexpressing Pdx1-Mafa-Ngn3 in hepatocytes or deleting Arx in pancreatic alpha cells. Unlike kinases or receptors, transcription factors are proteins which are believed to be undruggable. For this particular reason, proteins like Arx cannot be used as a target for drug discovery.

One issue which hinders the progress of pharmacological study for diabetes is the lack of reference transcriptome of each cell types in human islet. To address this fundamental question of islet biology, in collaboration with my colleagues I applied the single cell RNA-seq technology on human islets and established high-quality transcription profiles which cover all the cell types in mature human islets. With these profiles I confirmed the current knowledge of human islets, such as the cell type specific expression of hormones and transcription factors. This data set provides a reference transcriptome for human islets which could be applied in many different studies in islet biology. Furthermore, these profiles were provided in a user-friendly style, with a table containing expression level of all the known transcripts for each cell and visualization of the data in UCSC genome browser.

With the initial analysis of the single cell RNA-seq data set, *REST* was discovered as the critical factor to discrete exocrine cells from endocrine cells. *REST* is a suppressive transcription factor to inhibit the neuronal genes' transcription in non-neurons. Pancreatic endocrine cells have high expression of genes containing REST binding motifs and almost absence of *REST* transcription comparing to exocrine cells. Notably, the activity of REST depends on its interacting histone demethylase LSD1 and LSD1 could be inhibited by a highly specific antagonist GSK2879552[147]. Therefore REST/LSD1 complex is a promising target for reprogramming pancreatic exocrine cells to endocrine cells.

Another usage of the single cell RNA-seq data set is to look for alpha cell or beta cell specific proteins which could be used as cell-type specific markers. In this line *vitamin D binding protein*

(*GC*) was discovered as a highly specific alpha cell genes and this observation is validated by immunofluorescence in histological samples of human pancreas. GC belongs to albumin family which can be found in body fluid. GC is responsible to transport and maintain the stability of vitamin D in serum. Loss of GC results in shorter half-life of vitamin D, decrease of circulating vitamin D[148] and osteopathy[149]. Importantly vitamin D deficiency has been linked to the occurrence of diabetes[7,9,150]. Studies on rodent models shows vitamin D deficiency accelerates the development of diabetes[151]. The supplementation of vitamin D protects the animals from diabetes[16,17,152]. As far as I know the effects of GC on the islets is unknown. It is reasonable to hypothesize that GC, as a human alpha cell-specific protein, has dramatic impacts on the development of diabetes. It is interesting to investigate the changes of islet with either deletion or misexpression of GC.

With the single cell RNA-seq data set as a reference transcriptome we got the right tool for the transcriptional study of islet reprogramming. To perform chemical screening looking for drugs transdifferentiating alpha cells to beta cells, I used an inducible beta-to-alpha reprogramming cell line as a screening model to identify chemicals which can prevent this reprogramming. I hope in this way there are higher chances to find hits comparing a direct screening on alpha cells. With this idea in mind Min6 cells were engineered to harbor an inducible overexpression construct of ARX. After the full characterization of the Min6-ARX cell line a chemical screening was done with a library containing representative approved drugs by immunofluorescence assay. Artemisinins were discovered from the screening to rescue the loss of insulin in beta cells upon ARX misexpression. The reprogramming effects were confirmed in multiple animal models, including a wild type mouse model, an alpha cell linage tracing mouse model and human islets. RNA-seq on bulk samples of human islets showed artemisinins treatment increased endocrine

specific genes' expression but not exocrine specific genes. With the single cell RNA-seq assay I confirmed that artemisinins induced loss of alpha cell specific transcripts in human alpha cells. Identification of a chemical which can induce alpha to beta reprogramming is a good starting point for deeply understanding the mechanism of transdifferentiation. Due to the fact that artemesinins' effects on mammalian cells have never been studied previously, a chemical pull-down was performed in alpha cell with artesunate as the bait. A GABA receptor cofactor, gephyrin was found to be a specific interactor of artemisinins. With further experiments I proved artemisinins induced alpha to beta reprogramming via enhancement of GABA signaling.

GABA signaling is not only an inhibitory signaling in synaptic transmission but also plays important role in the development of neurons[153,154]. The presence of GABA is not limited to the brain but also in serum[155]. It has been shown that GABA treatment can rescue mouse from insulin deficiency, which suggests the positive effects of GABA on pancreatic islets[156,157]. Considering the physiological function of GABA in islets is to suppress glucagon secretion from alpha cells[158,159], I hypothesize that inhibition of glucagon secretion leads to the reprogramming event induced by artemisinins.

Loss of essential proteins for glucagon secretion, Scg5, led to an increase of beta cells mass and beta to alpha cell ratio *in vivo*[160]. This serves as a strong evidence for the hypothesis that suppression of glucagon secretion induces alpha cell reprogramming. This hypothesis was validated by two experiments: 1. Application of recombinant glucagon together with artmisinins abolished the alpha-to-beta transdifferentiation; 2. Chemical probes which could inhibit glucagon secretion induced alpha-to-beta reprogramming. In summary I discovered artemisinins enhanced GABA signaling, suppressed glucagon secretion and induced alpha to beta transdifferentiation. This study provides the first compound to induce alpha-to-beta transdifferentiation and the first mechanistic understanding of it.

Similar as the alpha-to-beta cell transdifferentiation, I established a cell line model for beta cell dedifferentiation. After exploring a few different methods, I found FoxO inhibitor (FoxOi) treated Min6 cells (a mouse beta cell line) exhibited all the dedifferentiation-related phenotypes. Comprehensive analysis of the transcriptional changes in Min6 cells with FoxO inhibition revealed the enhancements of lysosomal acidification as critical factors of beta cell dedifferentiation. This discovery is in the same line as a recent publication showing dedifferentiated beta cells exerted metabolic inflexibility compared to normal beta cells[141]. Furthermore, chloroquine was used to neutralize the acidification and it indeed abolished the misexpression of glucagon in dedifferentiated beta cell.

One great advantage of cell line model is it is feasible to perform phenotype based chemical screenings. By such a screening loperamide was discovered to strongly suppress the misexpression of glucagon in dedifferentiated beta cells and rescue the transcription of beta cell essential transcription factors Mafa, Mnx1 and Pax4. Importantly, loperamide specifically inhibited glucagon transcription in human islet without dramatic effects on insulin transcription. Literature shows that loperamide exerts protective effects on diabetic rats[161,162]. All of these evidences suggest loperamide has the possibility to prevent beta cell dedifferentiation in type II diabetes.

Loperamide is annotated as a mu-opioid receptor agonist. All the experiments I did pointed out the existence of additional targets contributing to the effects of loperamide on beta cell dedifferentiation. Further literature studies and experimental works brought a hypothesis that loperamide inhibited beta cell dedifferentiation by increasing mobilization of intracellular calcium ions and enhancement of FoxO1. Loperamide's enhancing effects on FoxO1 was validated by an independent phenotypic change to beta cell dedifferentiation--Dauer formation of C.elegans. From the biological perspective, this beta cell dedifferentiation model unveils that

lysosomal acidification as the essential factor of beta cell dedifferentiation. From the translational medicine perspective, the discoveries of chloroquine and loperamide from this project show the possibility of introducing new therapies to type II diabetes.

Future plans

It is almost 100 years since the first medical usage of insulin on type I diabetes patients. Although quite a lot of non-insulin drugs have been approved as treatments to diabetes, none of them showed as dramatic effects as the initial insulin therapy done by Banting, Best, and Collip in 1922 in Toronto, Canada. In the past century scientists tried to dissect the developmental process of islet with different knock out rodent models and zebrafish models. In this way the major transcription factors which are essential for beta cell development have been identified. In general these transcription factors are also essential for human beta cell development and functions based on MODY genetics. But this knowledge cannot be directly translated into any possible therapies for diabetes. Based on what have been achieved by my PhD study, I would like to propose two possible directions to identify new targets for diabetes therapy.

The first is a hypothesis-free approach based on transcriptional analysis. One shortage of previous transcriptional studies in diabetes context is they all focused on one transcriptional profile from their own research and did not link it to any other public resources. Actually, investigating different data bases derived from different research projects has led to the discovery of CD44 in adipocytes as a therapeutic target of diabetes[163-165]. I suggest that all the data sets got from my projects should be systemically studied together with public data sets. With this analysis the most robustly changed gene in all the reprogramming events could be picked up and the ones encoding druggable proteins could be followed by drug discovery in future.

As a show case, I focused on the expression changes of all the solute ligand carriers (SLCs). This is a big family containing multiple membrane proteins which are responsible for transportation of ions, amino acid and other factors for cells. With preliminary study *Slc7a2* was found to be robustly regulated in different reprogramming process. Under physiological conditions *Slc7a2* is

highly expressed in alpha cells comparing beta cells in both human[143] (from the first project of my PhD study) and mouse islets[134] (from public data base). It was always upregulated when beta cells were reprogrammed into alpha cells or dedifferentiated cells, and downregulated when alpha cells were reprogrammed into beta cells (**Fig. 1**). Slc7a2 is responsible for transportation of cationic amino acids including arginine, lysine and ornithine. The presence of this protein in pancreas is confirmed by human proteome map[166] (http://www.humanproteomemap.org) (**Fig. 2**). It is known that uptake limitation of certain group of amino acids could affect the status of cells in multiple aspects[167]. Although it is not proved yet if gain or loss of Slc7A2 can induce any reprogramming events in the islets, this show case suggests the possibility of integrating all the data sets in my study with public available data sets to identify new drug targets for beta cell reprogramming.

The second is a hypothesis-based approach derived from the artemisinins and FoxOi projects. In both projects, I observed the importance of extracellular glucagon concentration for keeping alpha cell identity. Recombinant glucagon supplements did abolish the increase of insulin content induced by artemether and decrease of glucagon transcription induced by chloroquine. These phenotypic changes highlight the essentiality of proper extracellular glucagon concentration for keeping stable alpha cell identity. Taking the similarity between alpha cell and beta cell into account, it is natural to hypothesize that hormones secreted by islets could regulate islet cell identities. Actually it has been reported that defects of insulin transcription or secretion lead to beta cell dedifferentiation[168,169] while lack of glucagon due to protein processing defects lead to loss of alpha cells[160]. One remaining question is which receptors on plasma membrane are responsible for the effects of insulin or glucagon.

Before answering this question we need to answer a basic question: which receptors are present in alpha or beta cell plasma membrane? With the recent progress of membrane proteomics

technology it is possible to address this question[170]. After identifying the receptors with cell-type specific expression pattern a few functional assay could be applied to test their roles on alpha/beta cell identity. For example we can knock out the alpha cell specific plasma membrane receptors with Crispr-Cas9 technology to see if it affects the transcription of alpha or beta cell specific genes. Any interesting receptors identified from this project could be targets suitable for drug discovery.

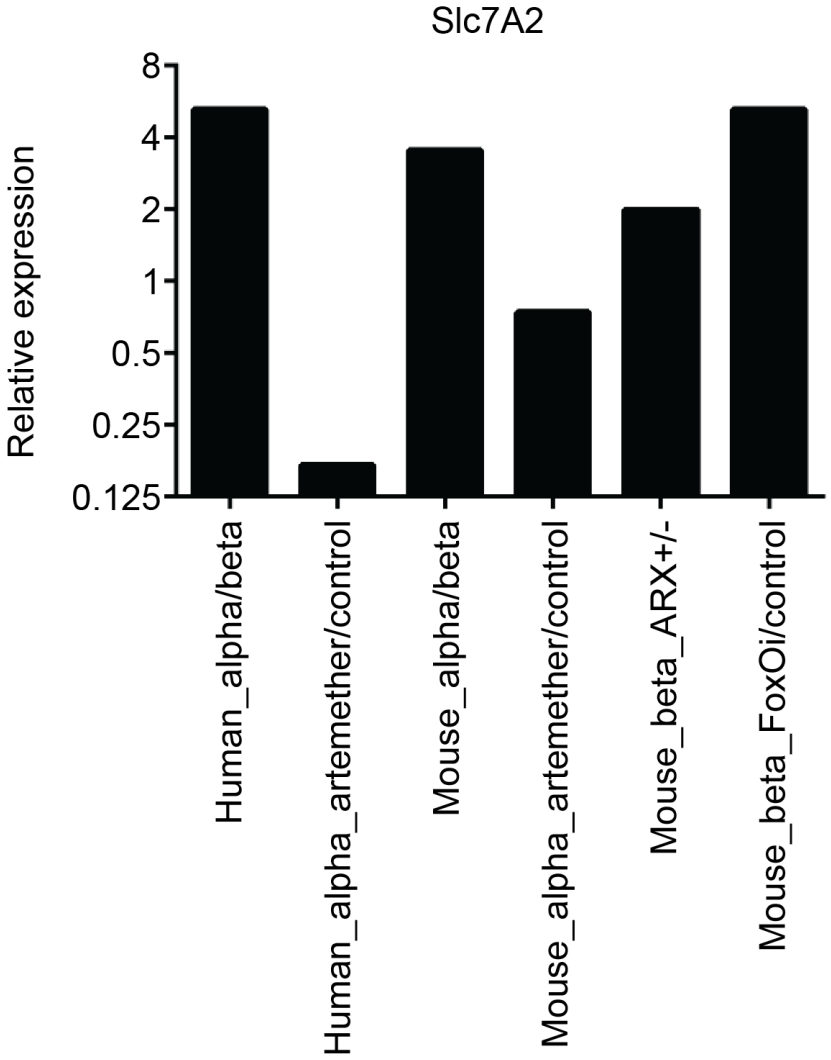


Fig 1. Relative expression of Slc7A2 in multiple reprogramming events.

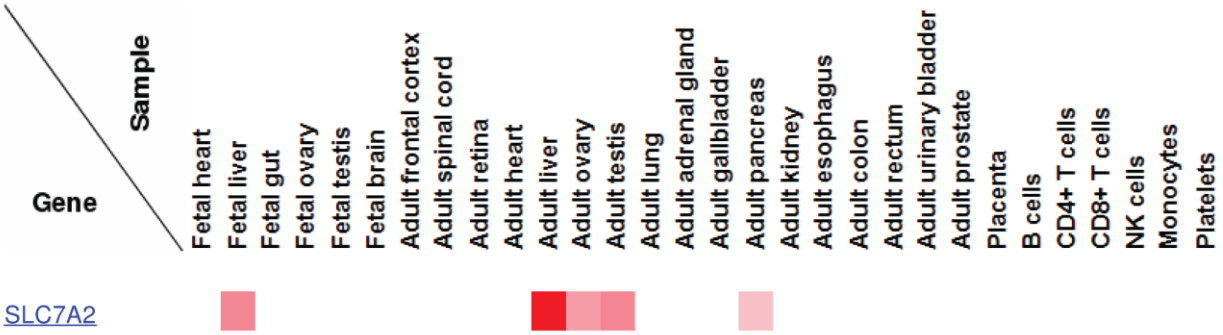
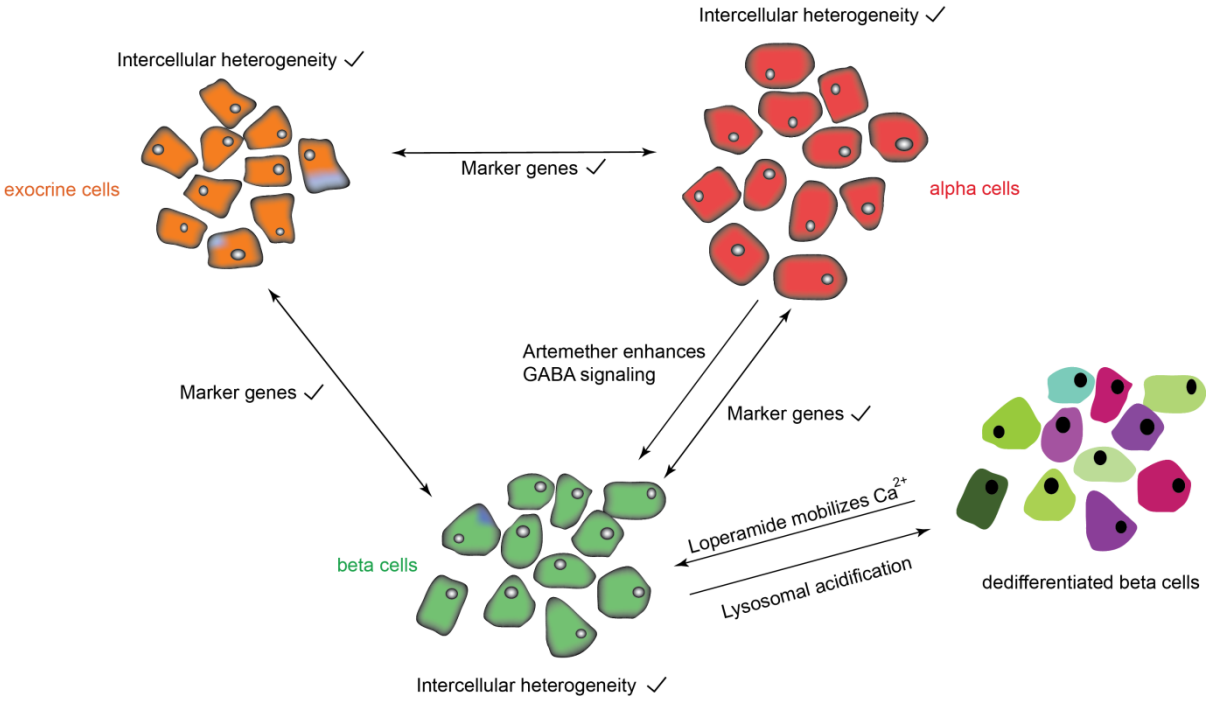


Fig 2. Relative expression of Slc7A2 in multiple organs. Data derived from Human Proteome Map.

Conclusion

In conclusion, with my PhD study I established one reference data set of human islet cells in single cell resolution and two cell line models for islet reprogramming studies, discovered three drugs- artemether, chloroquine and loperamide-which could be translated into clinical applications and revealed three mechanisms for islet reprogramming-GABA signaling for alpha-to-beta transdifferentiation, lysosomal acidification for beta cell dedifferentiation and calcium mobilization for glucagon transcription. For the follow-up projects, I proposed one project about systemically analyzing transcriptional profiles-with Slc7A2 as a show case and one project based on membrane proteomoics to help us better understand insulin and glucagon's effects on islet reprogramming. I hope my work could finally be transformed to clinical applications to help diabetic patients in future.



Summary of conclusion for this thesis.

Curriculum Vitae

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2008.9-2009.9 Fudan University OB&GYN Hospital

2007.6-2008.6 Shandong University Qilu Hospital

2006.9-2007.6 Jinan City Hospital

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Research Project

Molecular mechanism and novel therapies for diabetes

Conferences

2013 Keystone symposia: Diabetes — New Insights into Mechanism of Disease and its Treatment (poster)

2014 EMBO|EMBL Symposium: Translating Diabetes (poster)

2015 the 12th German Pancreatic Islet Workshop (short talk)

2015 Gordon research conference Pancreatic Diseases (short talk)

Publications

Li Bin, Li Jin, et al. Suppression of esophageal tumor growth and chemoresistance by directly targeting the PI3K/AKT pathway. Oncotarget *5*, 11576-11587.

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