Nicotinamide promotes pancreatic differentiation through the dual inhibition of CK1 and ROCK kinases in human embryonic stem cells

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Abstract

Background: Vitamin B3 (nicotinamide) plays important roles in metabolism as well as in SIRT and PARP pathways. It is also recently reported as a novel kinase inhibitor with multiple targets. Nicotinamide promotes pancreatic cell differentiation from human embryonic stem cells (hESCs). However, its molecular mechanism is still unclear. In order to understand the molecular mechanism involved in pancreatic cell fate determination, we analyzed the downstream pathways of nicotinamide in the derivation of NKX6.1\textsuperscript{+} pancreatic progenitors from hESCs.

Methods: We applied downstream modulators of nicotinamide during the induction from posterior foregut to pancreatic progenitors, including niacin, PARP inhibitor, SIRT inhibitor, CK1 inhibitor and ROCK inhibitor. The impact of those treatments was evaluated by quantitative real-time PCR, flow cytometry and immunostaining of pancreatic markers. Furthermore, CK1 isoforms were knocked down to validate CK1 function in the induction of pancreatic progenitors. Finally, RNA-seq was used to demonstrate pancreatic induction on the transcriptomic level.

Results: First, we demonstrated that nicotinamide promoted pancreatic progenitor differentiation in chemically defined conditions, but it did not act through either niacin-associated metabolism or the inhibition of PARP and SIRT pathways. In contrast, nicotinamide modulated differentiation through CK1 and ROCK inhibition. We demonstrated that CK1 inhibitors promoted the generation of PDX1/NKX6.1 double-positive pancreatic progenitor cells. shRNA knockdown revealed that the inhibition of CK1\textsubscript{α} and CK1\textsubscript{ε} promoted pancreatic progenitor differentiation. We then showed that nicotinamide also improved pancreatic progenitor differentiation through ROCK inhibition. Finally, RNA-seq data showed that CK1 and ROCK inhibition led to pancreatic gene expression, similar to nicotinamide treatment.

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Conclusions: In this report, we revealed that nicotinamide promotes generation of pancreatic progenitors from hESCs through CK1 and ROCK inhibition. Furthermore, we discovered the novel role of CK1 in pancreatic cell fate determination.

Keywords: Human embryonic stem cells, Pancreatic progenitors, Nicotinamide, Casein kinase 1 (CK1), Rho-associated protein kinase (ROCK), Kinase inhibitor

Background
Unexpected side effects of a specific drug often imply additional targets. These off-target phenomena are valuable resources to reveal novel molecular mechanisms and could help to identify new applications of clinically approved drugs. Human embryonic stem cells (hESCs) are pluripotent and can be differentiated into all cell types. hESC differentiation provides an excellent platform for people to examine key biological processes [1–4]. By delineating the molecular mechanisms of off-target effects in stem cell differentiation, new stem cell applications could be developed from known modulators.

Nicotinamide (NAM) is a multi-target drug that is widely used as a topical treatment for acne, eczema and other skin conditions. Nicotinamide belongs to the vitamin B3 family that also includes niacin. Vitamin B3 are converted to the coenzyme nicotinamide adenine dinucleotide (NAD) that is essential for energy metabolism [5]. Micromolar-range nicotinamide is sufficient to carry out metabolic functions. When its concentration is elevated to millimolar level, nicotinamide becomes a multi-target compound that is widely utilized in disease treatments and cell culture [6]. Nicotinamide can regulate DNA repair and apoptosis by inhibiting NAD*− dependent enzymes such as poly (ADP-ribose) polymersases (PARP) [7]. Nicotinamide can also inhibit Sirtuins (SIRT) to influence epigenetic modification and metabolism [8]. Because of the complexity of kinase cascades and their crosstalk with other pathways, much is unknown about nicotinamide’s function as a kinase inhibitor in disease treatments and cell culture.

Nicotinamide has been widely applied in various aspects of stem cell culture for hESC and human-induced pluripotent stem cells (hiPSC). Nicotinamide suppresses apoptosis and improves reprogramming efficiency [9, 10]. Nicotinamide is also beneficial for the differentiation toward different somatic cell types, such as CD34+ hematopoietic progenitors [11], retinal pigment epithelium (RPE) [12], and cardiomyocytes from hESC or hiPSC [13]. hESCs have been induced to pancreatic progenitors, putative β cells, or bona fide β cells by nicotinamide [14–19]. Nicotinamide promotes the development and self-renewal of murine pancreatic progenitors [20], and it also sustains the expression of pan-pancreas marker PDX1 [21] and endocrine marker NKX6.1 in the development of human pancreatic progenitors in serum-containing conditions [22]. However, the mechanism of nicotinamide in pancreatic lineage differentiation remains unclear. Although PARP and SIRT pathways are involved in nicotinamide-associated differentiation, little data is available to validate the mechanism of nicotinamide in cell fate determination processes. We recently show that nicotinamide promotes cell survival as a ROCK inhibitor and induces RPE through CK1 inhibition [6].

Nicotinamide is beneficial for pancreatic progenitor induction, but its downstream effector is unclear in this process. In order to understand the mechanism of nicotinamide in pancreatic in vitro development, we established a serum-free, chemically defined platform to analyze its function in pancreatic differentiation. We hope to reveal critical signaling pathways in pancreatic progenitor differentiation and develop novel methods to generate pancreatic cells from human pluripotent stem cells.

Materials and methods
hESC culture
Human ESCs (H1 and H9 lines from WiCell Research Institute, Inc., Madison, WI, http://www.wicell.org) were cultured in Matrigel-coated 6-well plates in E8 medium [23, 24]. Medium was changed daily, and cells were passaged every 3–4 days before they reach 60–70% confluence.

Pancreatic progenitor differentiation
The differentiation was carried out in serum-free conditions as illustrated in Fig. 1a. Sixty to 70% confluent hESCs were dissociated with EDTA, passaged onto Matrigel-coated 24-well plate (passaging ratio 1:24), and cultured until 40–50% confluence. Cells were then treated with 5 μM CHIR99021 and 100 ng/ml Activin A in differentiation medium (DMEM/F12, transferrin, chemically defined lipid concentrate, ascorbic acid, and sodium selenite) for 24 h, followed by 100 ng/ml Activin A, 100 nM LDN193189, and 2 μM IWP2 treatment in differentiation medium for another 48 h to induce definitive endoderm formation (Stage 1). Subsequently, primitive gut tube cells were induced under 50 ng/ml KGF in DMEM/F12 with 0.2% NaHCO3 and 1x Glutamax (Stage 2). Posterior foregut cells were induced
under 100 ng/ml Noggin, 1 mM cyclopamine-KAAD, 10 μM TTNPB treatment in DMEM HG with 1% B27 (without insulin), and 1× GlutaMAX (Stage 3). Subsequently, pancreatic progenitors were generated under 100 ng/ml Noggin and 500 nM TPB treatment in DMEM HG with 1% B27 (without insulin), 1× GlutaMAX, and factors involved in nicotinamide-associated pathways as specified (Stage 4). Chemicals used include nicotinamide (10 mM), niacin (1, 5, 10 mM), PARP inhibitor ABT888 (10, 50, 500 nM), SIRT inhibitor EX527 (1, 10, 20 μM), ROCK inhibitor Y27632 (10 μM), CK1 inhibitor D4476 (5 μM), PF4800567 (10 μM) and LH846 (10 μM).

**Real-time PCR**

Total mRNA was extracted with RNAiso-plus (TAKARA, cat. no.108-95-2), and reverse transcription from mRNA to cDNA was performed with High Capacity cDNA Reverse Transcript kit (Applied Biosystems, cat. no. 4368813) following the manufacturer’s instructions. Real-time quantitative PCR was conducted with SYBR Premix Ex Taq (TAKARA, cat. no. RR420) and the Quantstudio-7 system (Applied Biosystems). The relative amounts of the amplified nucleotide fragment were calculated by the $2^{\Delta\Delta C_{t}}$ method. Expression levels were normalized to the housekeeping gene TBP and compared with undifferentiated hESCs.

**Immunostaining**

Putative pancreatic progenitors were fixed with 4% paraformaldehyde at room temperature for 20 min, rinsed with 1× PBS 3 times, permeabilized with 0.5% Triton X-100 for 20 min, and then stained with primary and secondary antibodies following standard protocols. Primary antibodies include goat anti-PDX1 (Cell Signaling, cat. no. 5679 at 1:2000 dilution) and mouse anti-NKX6.1 (BD biosciences, cat. no. 563338 at 1:200 dilution). The nuclei were stained with Hoechst 33342 (Abnova, cat. no. U0334 at 1:10,000 dilution). Stained cells were
visualized using Zeiss Axio Observer fluorescence microscope with ApoTome.

Flow cytometry
Putative pancreatic progenitors were harvested by TrypLE (37 °C, 5 min) and neutralized with 5% BSA. After washing with DPBS, cells were permeabilized with 0.2% Triton X-100, 5% BSA in DPBS for 1 h on ice. Primary antibodies goat anti-human PDX1 (Cell Signaling, cat. no. 5679 at 1:1000 dilution) and mouse anti-human NKX6.1 conjugated by Alexa Fluor® 647 (BD Biosciences, cat. no. 563338 at 1:20 dilution) were incubated with cells in 1% BSA in DPBS for 1 h at room temperature in the dark. After washing, Alexa Fluor® 488-conjugated anti-goat secondary antibody was used at 1:1000 dilution in 1% BSA in DPBS for 1 h at room temperature in the dark for PDX1 staining. After washing, cells were resuspended in DPBS for analysis using BD ACCURI C6. Undifferentiated hESCs were stained with PDX1 or NKX6.1 as a negative control for gating.

Western blot
Cells were lysed with RIPA buffer supplemented with phosphatase inhibitor cocktail (Sigma-Aldrich, cat. No. P5726) and proteinase inhibitors. The lysate was sonicated and heated at 95 °C before loading onto 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), and the separated proteins were transferred to PVDF membrane. The membrane was blocked with 5% BSA in TBST at room temperature for 30 min, washed with TBST (5 min each time, 3 times), incubated with primary antibodies at 4 °C overnight, washed with TBST (5 min each time, 3 times), and incubated with peroxidase-conjugated secondary antibodies. Signal strength was semi-quantitatively determined by optical densitometry using ImageJ Lab. Primary antibodies include phospho-β-catenin (Ser45) (Cell Signaling, cat. no. 9561 s at 1:1000 dilution), β-catenin (Upstate, cat. no. 06-734 at 1:1000 dilution), phospho-MLC (Ser19) (Cell Signaling, cat. no. 3675 s at 1:2000 dilution), MLC (Sigma-Aldrich, cat. no. SAB1403431 at 1:2000 dilution) and GAPDH (Santa Cruz, cat. no. sc-25778 at 1:2000 dilution).

Cloning and generation of shRNA in hESCs lines
The psi-LVRU6P plasmid was utilized to create shRNA-expressing constructs. Target sites of shCK1α, shCK1β and shCK1ε are GGAAAGTGGCAGTGAAGCTAGA, GGTCCCTGGAGATATCACC and GGTGTGCCATC AACGTGGAATG. The lentivirus particles were produced in 293FT cells transfected with psPAX2, pMD2.G and psi-LVRU6P-shRNA-containing plasmids. H1 cells were transduced with the lentivirus and stable cell lines were established by puromycin selection.

RNA sequencing
Total mRNA was extracted on day 13 (stage 4) using RNAiso-plus (TAKARA, cat. no.108-95-2). The RNA libraries were generated using TruSeq RNA Sample Preparation kit (Illumina), and cDNA fragments were enriched by PCR using Illumina TruSeq PCR primers. Each library was sequenced as paired-end reads in HiSeq 2000/1000 (Illumina). The sequencing data of this report have been uploaded in NCBI’s BioProject and Sequence Read Archive (SRA), and these data are accessible through BioProject accession number PRJNA701836 or SRA accession number SRP306377.

Bioinformatics analysis
Transcripts Per Million (TPM) were used to normalize gene read counts in all samples. Values for the heat map were calculated by log2 (TPM of each gene in each sample/mean TPM of each gene in all samples). R package ggplot2 was used to generate heatmaps. R package EdgeR was used to pick differentially expressed genes (DEG) with p value < 0.01, log2 (fold change) > 2 or < −2. The Euclidean distances between selected genes in each sample were used as the clustering method for the heatmap cluster. Cell type enrichment analysis was performed using the section of “GTEx Tissue Sample Gene Expression Profiles up” in Enrichr (https://maayanlab.cloud/Enrichr/).

Statistical analysis
Data represent mean ± SEM of ≥3 independent experiments unless specified. Statistical significance was tested by either paired t test or one-way ANOVA analysis. p < 0.05 represents statistical significance.

Result
Enhancement of pancreatic progenitor induction by nicotinamide depends on kinase inhibition, but not on PARP and SIRT regulation
Nicotinamide has been used to induce pancreatic progenitors from hESCs [21, 22], but its molecular mechanism is unclear. In order to examine the function of nicotinamide, a serum-free platform was established to stepwisely induce pancreatic progenitors (Fig. 1a). After H1 hESCs differentiated to posterior foregut (Stage 3), noggin and TPB were applied to induce pancreatic progenitors (Stage 4). We showed that the addition of nicotinamide in stage 4 significantly enhanced the expression of pancreatic progenitor markers NKX6.1 and PDX1 (Fig. 1b). This system was utilized to study nicotinamide regulation of pancreatic specification in this report.

Nicotinamide is best known to serve as the substrate to produce NAD as well as an inhibitor for PARP and SIRT pathways (Fig. 1c). Niacin was tested in Stage 4, but it failed to enhance pancreatic differentiation as
shown by the mRNA levels of PDX1 and NKX6.1 and percentage of PDX1+/NKX6.1+ cells (Fig. 1d, Fig. S1c, d). It suggested that nicotinamide did not modulate differentiation through NAD metabolism. When PARP inhibitor (ABT888) and SIRT inhibitor (EX527) were applied during Stage 4, they did not improve NKX6.1 and PDX1 mRNA levels and percentage of PDX1+/NKX6.1+ cells (Fig. 1d, Fig. S1c, d). It indicated that nicotinamide did not modulate pancreatic differentiation through PARP and SIRT inhibition.

We recently found that nicotinamide was also a kinase inhibitor with multiple targets including ROCK and CK1 (Fig. S1a) [6], so we examined whether nicotinamide modulates pancreatic differentiation as a kinase inhibitor. When CK1 inhibitor D4476 and ROCK inhibitor Y27632 were individually applied in stage 4, each of them significantly increased the expression of PDX1 and NKX6.1, and this phenotype was similar to that of nicotinamide (Fig. 1d, Fig. S1e). Besides the H1 cell line, we showed that H9 hESCs can also be induced to pancreatic progenitors as shown by the significant increase of the mRNA levels of PDX1 and NKX6.1 upon CK1 or ROCK inhibition (Fig. S1b). These results indicated that nicotinamide influenced pancreatic differentiation as a kinase inhibitor. The cell fate determination could be carried out through CK1 and ROCK in parallel.

**Generic chemical inhibition of CK1 promotes pancreatic progenitor induction**

We further examined how nicotinamide and CK1 affected pancreatic progenitor differentiation in Stage 4 (Fig. 1a). Similar to nicotinamide, CK1 inhibitor D4476 not only significantly increased the expression of NKX6.1 and PDX1, but also promoted the expression of additional pancreatic progenitor marker genes PTF1A and SOX9 as well as endocrine precursor marker NGN3 (Fig. 2a, Fig. S2a). Immunostaining results demonstrated that nicotinamide and D4476 both increased the expression of PDX1 and NKX6.1 (Fig. 2b), which were consistent with flow cytometry results (Fig. 2c). These results supported our hypothesis that nicotinamide induced pancreatic progenitors as a CK1 inhibitor.

We then inspected whether downstream factors of CK1 were responsible for the cell fate induction. Previous study revealed that GSK-3 dependent phosphorylation of β-catenin relies on prior priming phosphorylation of β-catenin at Ser45 by CK1 in canonical Wnt pathway [25]. We showed that both nicotinamide and D4476 suppressed the phosphorylation of β-Catenin (Ser45) in putative pancreatic progenitors (Fig. 2d, d’). This result indicated that nicotinamide and D4476 might promote pancreatic progenitor induction through Wnt pathway.

**Inhibition of CK1α and CK1ε promotes pancreatic progenitor induction**

The human genome contains multiple CK1 isoforms with distinct functions. D4476 is a pan CK1 inhibitor that targets CK1α, CK1δ and CK1ε. We tried to determine which CK1 isoform was involved in pancreatic differentiation. CK1 isoform-specific inhibitors were applied in Stage 4 (Fig. 1a), and we showed that CK1ε inhibitor PF4800567 hydrochloride significantly improved the expression of pancreatic progenitor-specific marker genes as well as endocrine precursor marker NGN3 (Fig. 3a), and it also increased the percentage of PDX1+/NKX6.1+ cells (Fig. 3b). In contrast, CK1δ inhibitor LH 846 did not significantly improve pancreatic differentiation.

We then used shRNA to knockdown the gene expression of CK1α, CK1δ and CK1ε, respectively (Fig. S3a, b). The knockdown of CK1α and CK1ε significantly upregulated the mRNA levels of typical pancreatic progenitor markers (NKX6.1, PDX1, PTF1A and SOX9) (Fig. 3c), and the percentage of PDX1+/NKX6.1+ cells was also increased in these two knockdown cell lines (Fig. 3d, Fig. S3c). In contrast, CK1δ knockdown did not improve pancreatic differentiation (Fig. 3c, d, Fig. S3c). These data indicated that CK1α and CK1ε suppressed pancreatic differentiation, which was enhanced by CK1 inhibition.

**Nicotinamide also promotes pancreatic differentiation through ROCK inhibition**

Consistent with previous report on the targets of nicotinamide [6], we found that both CK1 and ROCK were involved in pancreatic progenitor differentiation under nicotinamide treatment (Fig. 1c). We compared the impact of nicotinamide and ROCK inhibitor Y27632 in Stage 4 of pancreatic differentiation. Both nicotinamide and Y27632 significantly improved the expression of pancreatic progenitor genes, including NKX6.1, PDX1, PTF1A and SOX9 (Fig. 4a, Fig. 4a), while NGN3 was only significantly upregulated under nicotinamide treatment. Nicotinamide or Y27632 had no significant impact on the mRNA level of endocrine marker NEUROD1 (Fig. S4a). Immunostaining showed that the expression of PDX1 and NKX6.1 was upregulated by nicotinamide and Y27632 treatments (Fig. 4b). Flow cytometry results also showed that both nicotinamide and Y27632 upregulated the percentage of PDX1+/NKX6.1+ double-positive cells (Fig. 4c).

Myosin Light Chain 2 (MLC2) phosphorylation is downstream of ROCK kinase [26], so we evaluated MLC2 phosphorylation at Ser19 during Stage 4 (from day 10 to day 13). Under Noggin/TPB treatment, MLC2 phosphorylation gradually increased from day 10 to day 13 (Fig. 4d, d’). Both nicotinamide and Y27632
significantly decreased MLC2 phosphorylation. However, D4476 did not significantly suppress MLC2 phosphorylation (Fig. 4e, e`). These data suggest that CK1 and ROCK regulate pancreatic differentiation through distinct pathways.

We then modulated pancreatic differentiation through different combinations of ROCK and CK1 inhibition. We showed that the combination of ROCK and CK1 inhibition led to a more significant increase of PDX1 and PTF1A expression, in comparison with ROCK inhibitor alone (Fig. S4b). When Y27632 or D4476 was applied to cells in the presence of nicotinamide, neither of them had an additive effect in the differentiation of PDX1+/NKX6.1+ cells (Fig. S4c). These results suggest that nicotinamide may induce pancreatic differentiation through a synthetic effect of CK1 and ROCK inhibition.
Fig. 3 Inhibition of CK1α and CK1ε promotes pancreatic progenitor induction. 

a RT-qPCR analysis of pancreatic progenitors on day 13 treated with D4476, PF4800567 and LH846 to measure mRNA levels of NKX6.1, PDX1, PTF1A and NGN3 (n > 3), *p < 0.05; b Flow cytometry analysis of pancreatic progenitors on day 13 for PDX1+ and NKX6.1+ percentage under Mock, D4476, PF4800567 and LH846 conditions (n = 3); c RT-qPCR analysis of pancreatic progenitors on day 13 induced from shCK1α, shCK1δ and shCK1ε cell lines to measure mRNA level of NKX6.1 (n = 3), *p < 0.05; d Flow cytometry analysis of pancreatic progenitors on day 13 induced from shCK1α, shCK1δ and shCK1ε cell lines for percentage of PDX1+ / NKX6.1+ (n = 3)
Fig. 4 (See legend on next page.)
Global gene expression of pancreatic progenitors under different treatments

In order to evaluate the pancreatic progenitors induced by different methods, RNA-seq profiles were obtained to examine global gene expression. In comparison to the DE condition, genes in cluster 1 (representing pancreas and stomach) were significantly upregulated by D4476, Y27632 and nicotinamide treatments, and the highly upregulated genes such as FOXA1, JAG1, ANKRD1, PFKFB3, PRSS23, CDH6, IRS1, TANC1, LAMC1, LAMC2 and CSRP1 were related to the pancreas. Meanwhile, genes in cluster 2 (representing stomach) were upregulated by D4476 or Y27632 treatment, but not by nicotinamide treatment. Genes in cluster 3 (representing pancreas) were only upregulated by D4476 treatments (Fig. 5a), and the genes of pancreatic organogenesis such as PRDM16, GATA2 and MAOA were markedly upregulated. These data indicated that specific CK1 inhibition could be a useful approach to induce a subset of pancreatic marker genes in future applications. We further showed that most pancreatic progenitor markers are upregulated in nicotinamide, CK1 inhibition and ROCK inhibition conditions (Fig. 5b). We analyzed representative gene expression in pancreatic sub-cell types by analyzing published data of a single cell analysis of the human pancreas [27]. The top 30 endocrine-specific genes were generally upregulated in each treatment. However, D4476 led to enhanced expression of more genes representing Beta cells than Y27632 and nicotinamide, indicating that the putative pancreatic progenitors generated via CK1 inhibition may be more likely to go into endocrine cell fates than ROCK inhibition and nicotinamide (Fig. 5c, Fig S5a). To further evaluate the potential of the pancreatic progenitor cells, we further induced hESCs into β-like cells (Fig. S6a), and immunostaining showed that more insulin-producing cells were generated from nicotinamide, D4476 and Y27632 treated pancreatic progenitors (Fig. S6b).

Discussion

Nicotinamide is involved in diverse biological processes, but its distinct molecular mechanisms in specific applications are not fully defined. Besides its role in metabolism and epigenetic regulation, nicotinamide is emerging as a potent modulator of kinase cascades. By examining nicotinamide function in hESC differentiation, we revealed that nicotinamide inhibits both CK1 and ROCK pathways to promote pancreatic progenitor cell fate.

Casein kinase 1 (CK1) is a family of serine/threonine kinases that are constitutively active in cells. CK1 isoforms are highly involved in circadian rhythms, nucleocytoplasmic shuttling of transcription factors, DNA transcription, and DNA repair. CK1 has been implicated in apoptosis and cell proliferation of pancreatic ductal adenocarcinoma cells, but its role in pancreatic differentiation was unknown [28]. In this study, we showed that the suppression of CK1α and CK1ε promoted pancreatic progenitor differentiation, implying that active CK1 pathway is inhibitory to pancreatic differentiation. Nicotinamide and CK1 inhibition probably acted through WNT signaling [29] to modulate pancreatic cell fate [30]. Recent studies showed that pancreatic progenitors can be categorized into three subgroups, including PDX1+/NKX6.1+, PDX1+/NKX6.1−, and PDX1−/NKX6.1+ cells [31−33]. PDX1+/NKX6.1+ cells are considered as bona fide beta cell precursors, which are significantly induced by CK1 inhibitor and nicotinamide. More work is necessary to explore the differentiation of the other two subtypes in vitro.

This study also showed that nicotinamide induced pancreatic cell fate as a ROCK inhibitor. ROCK pathway is involved in glucose-stimulated insulin secretion [34] and the disassembly of glucotoxicity-induced stress fibers [35]. Recent reports showed that ROCK inhibition promoted the differentiation of PDX1+ posterior foregut cells into pancreatic endoderm fate at low cell density [36]. We previously showed that nicotinamide enhances hESC survival by inhibiting MLCL2 phosphorylation as a ROCK inhibitor [6]. In pancreatic differentiation, nicotinamide likely promotes pancreatic progenitor fate through a similar mechanism as a ROCK inhibitor.

Although nicotinamide is best known as vitamin B3 for metabolism and inhibitors in SIRT and PARP pathways, we show that nicotinamide modulates pancreatic differentiation through its “off-target” effect as a kinase inhibitor. By exploring nicotinamide’s regulation of kinase targets,
Cluster 1: Pancreas, stomach
Cluster 2: Stomach
Cluster 3: Pancreas
Cluster 4: Blood vessel
Cluster 5: Blood vessel
Cluster 6: Brain
Cluster 7: Adipose tissue
Cluster 8: Definitive endoderm

Pancreatic progenitor marker genes

Top 30 specific genes of endocrine cells

Fig. 5 (See legend on next page.)
we will not only reveal the novel molecular mechanism, but also develop new methods in stem cell applications.

**Conclusion**

We demonstrated that the promotion of pancreatic progenitor differentiation by nicotinamide was through the dual inhibition of ROCK and CK1. These findings should be of broad interest to the stem cell community and regenerative medicine. Elucidating the mechanism of pancreatic progenitor and endocrine cell development is the foundation for in vitro induction of pancreatic progenitors and endocrine cell types.

**Abbreviations**

HESCs: Human embryonic stem cells; ROCK: Rho-associated protein kinase; CK1α: Casein kinase; MLCK2: Myosin light chain 2; NAM: Nicotinamide; NAD: Nicotinamide adenine dinucleotide; PARP: Poly (ADP-ribose) polymerases; SIRT: Sirtuins; PP: Pancreatic polypeptide cells.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13287-021-02426-2.

**Additional file 1: Supplemental Figure 1.** Enhancement of pancreatic progenitor induction by nicotinamide depends on kinase inhibition, but not on PARP and SIRT regulation. A Nicotinamide’s molecular targets screened via KINOMEScan** confirmed that nicotinamide can directly bind and inhibit CK1α and ROCK, b RT-qPCR analysis for mRNA levels of NK06.1 and PDX1 in pancreatic progenitors on day 13 of differentiation from hESCs, treated with nicotinamide, D4476 and Y27632 (n = 3), *p < 0.05; c RT-qPCR analysis of pancreatic progenitors on day 13 for mRNA levels of NK06.1 and PDX1 under Mock, nicotinamide (10 mM), niacin (5 mM), PARP inhibitor ABT888 (10, 50, 100 mM) and SIRT inhibitor EX527 (1, 10, 20 μM) conditions (n = 3); d Flow cytometry analysis to test the effect of nicotinamide (10 mM), niacin (5 mM), ABT888 (10, 50, 100 mM) and EX527 (10 μM) on generation of PDX1+ NK6.1+ cells (n = 3); e Flow cytometry analysis to test the effect of nicotinamide (10 mM), D4476 (5 μM) and Y27632 (10 μM) on the generation of PDX1+ NK6.1+ cells (n = 3).

**Supplemental Figure 2.** Generic chemical inhibition of CK1α promotes pancreatic progenitor induction. a RT-qPCR analysis of pancreatic progenitors on day 13 treated with nicotinamide and D4476 to measure mRNA levels of NEUROD1 and NGN3 (n > 3), *p < 0.05. **Supplemental Figure 3.** Inhibition of CK1α and CK1ε promotes pancreatic progenitor induction; a RT-qPCR analysis to measure mRNA levels of CK1α and CK1ε in pancreatic progenitors on day 13 treated with Mock, nicotinamide, D4476 and Y27632 (n = 3), *p < 0.05; b Western blot analysis to assay protein level of CK1α, CK1ε and shCK1α, shCK1ε and shCK1α+CK1ε cell lines in pluripotency stage (n = 3), *p < 0.05; c Flow cytometry analysis of pancreatic progenitors on day 13 induced from CK1α, shCK1α and shCK1α+CK1ε cell lines for percentage of PDX1+ / NK6.1+ (n = 3). **Supplemental Figure 4.** Nicotinamide also promotes pancreatic differentiation through ROCK inhibition. a RT-qPCR analysis of pancreatic progenitors on day 13 treated with nicotinamide and Y27632 to measure mRNA levels of NEUROD1 and NGN3 (n > 3), *p < 0.05; b RT-qPCR analysis of pancreatic progenitors on day 13 treated with nicotinamide, Y27632, D4476 and Y27632+D4476 to measure mRNA levels of NK06.1, PDX1 and PTF1A (n > 3); *p < 0.05; c Flow cytometry analysis to test the effect of nicotinamide, nicotinamide+D4476 and nicotinamide+Y27632 on the generation of PDX1+/NK6.1+ cells (n = 3).

**Acknowledgements**

Dr. Ruyu Xie and Dr. Xinwei Wu of the University of Macau provided valuable suggestions to this project. We want to thank the council members of the Macau Society for Stem Cell Research (MSSCR) for the constructive discussions.

**Authors’ contributions**

YZ, YM and WL conducted stem cell maintenance and differentiation; YZ, YM and LL analyzed nicotinamide function; YZ characterized the differentiated cells; YZ, JX, ZR and ZZ conducted bioinformatics analysis; YZ and GC conducted stem cell maintenance and differentiation; YZ, YM and WL wrote the manuscript. All authors read and approved the final manuscript.

**Funding**

This project was funded by the University of Macau (File No. MYRG2018-00135-FHS and MYRG2019-00147-FHS), and also by the Science and Technology Development Fund, Macau SAR (File No. 131/2014/A3, 056/2015/A2, 0059/2019/A1, 0123/2019/A3 and FDCT/0011/2019/AFK).

**Availability of data and materials**

The data supporting the finding of this article are all online.

**Declarations**

**Ethics approval and consent to participate**

The use of hESCs was approved by the Institutional Review Board at the University of Macau.

**Competing interests**

The authors declare that they have no competing interests.

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Received: 1 March 2021 Accepted: 28 May 2021
Published online: 25 June 2021

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