Nicotinamide Promotes Cell Survival and Differentiation as Kinase Inhibitor in Human Pluripotent Stem Cells

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SUMMARY

Nicotinamide, the amide form of vitamin B3, is widely used in disease treatments and stem cell applications. However, nicotinamide's impact often cannot be attributed to its nutritional functions. In a vitamin screen, we find that nicotinamide promotes cell survival and differentiation in human pluripotent stem cells. Nicotinamide inhibits the phosphorylation of myosin light chain, suppresses actomyosin contraction, and leads to improved cell survival after individualization. Further analysis demonstrates that nicotinamide is an inhibitor of multiple kinases, including ROCK and casein kinase 1. We demonstrate that nicotinamide affects human embryonic stem cell pluripotency and differentiation as a selective kinase inhibitor. The findings in this report may help researchers design better strategies to develop nicotinamide-related stem cell applications and disease treatments.

INTRODUCTION

Balanced cellular metabolism and signaling regulation are both essential for mammalian cells to survive, proliferate, and function. Nutrients are conventionally thought to act only as enzyme cofactors or energy sources. However, recent advances demonstrate that specific nutrients could also be involved in functions beyond nutritional support, such as epigenetic regulations and kinase cascades (Blaschke et al., 2013; Chen et al., 2013; Gao et al., 2016; Liu et al., 2017; Lu and Thompson, 2012; Sciacovelli et al., 2016; Tzatsos and Kandror, 2006; Yuan et al., 2013). In this report, we explored nicotinamide's role in stem cell regulation, and showed its regulatory roles as a kinase inhibitor.

Nicotinamide is the amide form of niacin, and both of them belong to the vitamin B3 family. They are the precursors of nicotinamide adenine dinucleotide (NAD), which acts as coenzyme in multiple cellular processes, including energy metabolism and DNA repair. Nicotinamide can be converted into nicotinamide mononucleotide (NMN) by nicotinamide phosphoribosyltransferase (NAMPT), which is then turned into NAD+ by nicotinamide mononucleotide adenylyltransferase (NMNAT) (Maiese et al., 2009). The normal plasma concentration of nicotinamide and niacin is around 5 μM (Odum and Wakwe, 2012). Deficiencies in nicotinamide and niacin could lead to decreased NAD+ production and cause pellagra, which affects the skin, digestive system, and CNS (Prakash et al., 2008). Nicotinamide, but not niacin, is also an inhibitor of sirtuin and poly(ADP-ribose) polymerase (PARP), which regulate protein deacetylation and DNA repair (Avalos et al., 2005; Jackson et al., 2003; Kuchmerovska et al., 2004; Saldeen and Welsh, 1998).

Nicotinamide has been widely used to treat diseases such as diabetes, schizophrenia, Alzheimer’s disease, psoriasis, obesity, and cancer (Aisen et al., 2008; Bagcchi, 2015; Chase et al., 1992; Chen et al., 2015; Gale, 2004; Knip et al., 2000; Siadat et al., 2013; Smythies, 1973). High dosage of nicotinamide is often required in clinical treatment, and the concentration in serum could reach the millimolar range (Dragovic et al., 1995). At the same time, nicotinamide is also used in various cell culture practices. High dosage (more than 10 mM) of nicotinamide promotes cell survival in different cell types, including neural, liver, and heart cells (Ieraci and Herrera, 2006; Lin et al., 2000; Shen et al., 2004; Shi et al., 2012; Tong et al., 2012). Nicotinamide is extensively used in the in vitro culture of organoids, including cell types from colon, liver, pancreas, and fallopian tube (Huch et al., 2013b, 2015; Kessler et al., 2015; Sachs et al., 2018; Sato and Clevers, 2015; Sato et al., 2011; Yin et al., 2016). Nicotinamide also enhances expansion of adult stem cells from pancreas, colon, bone marrow, and umbilical cord (Horwitz et al., 2014; Huch et al., 2013a; Jung et al., 2011; Peled et al., 2012; Sugiyama et al., 2013). In pluripotent stem cells, nicotinamide promotes reprogramming, improves maintenance (Son et al., 2013), and facilitates cell differentiation.
to various lineages, including neural, pancreatic, and cardiac lineages (Buchholz et al., 2013; Griffin et al., 2017; Idelson et al., 2009; Nostro et al., 2015; Parsons et al., 2011; Vaca et al., 2008). Despite its numerous applications, the molecular mechanisms of nicotinamide are still unclear in many circumstances.

In this study, we set to explore the roles of common vitamins in human pluripotent stem cells (hPSCs), and identified nicotinamide as a regulator of hPSC pluripotency, survival, and differentiation. Nicotinamide promoted hPSC cell survival and differentiation. Further analysis showed that nicotinamide promoted cell survival as a Rho-associated protein kinase (ROCK) inhibitor, while it also inhibited other kinases including casein kinase 1 (CK1) and a few others. Finally, we demonstrated that nicotinamide also initiated differentiation as a kinase inhibitor. Our study revealed the mechanisms underlying nicotinamide’s key functions, and expanded our understanding of its application in cell culture practices.

RESULTS

Nicotinamide Promotes hPSC Survival after Individualization through the Regulation of ROCK Pathway

hPSCs are vulnerable to cell death after individualization (Chen et al., 2010; Ohgushi et al., 2010). To identify the function of vitamins in stem cell regulation, we tested a set of 12 vitamins at three doses (based on their concentration in DMEM/F12) on cell survival after dissociation in H1 human embryonic stem cells (hESCs) (Figure S1A). Nicotinamide was the only vitamin that promoted hESCs survival after individualization, while high concentrations of retinol and cholecalciferol inhibited cell survival (Figure S1A). The effect of nicotinamide was dose dependent. Nicotinamide promoted survival of individualized cells at 5 and 10 mM, but at 25 mM showed significant toxicity to hESCs (Figure 1A). We then examined cell apoptosis during passage, and found that 10 mM nicotinamide significantly reduced the Annexin V-positive and propidium iodide-negative cells (Figures S1B and S1C). It suggested that nicotinamide suppressed apoptosis, and the observation was consistent with the improved cell survival by nicotinamide. Microscopy images showed that nicotinamide also suppressed the cell blebbing phenotype after dissociation in a dose-dependent manner (Figures 1B and 1C). The beneficial effect was also observed in other pluripotent stem cells (Figures S1D–S1F) as well as on different coating surfaces (Figures S1G and S1H).

To understand nicotinamide’s role in cell survival, we tested modulators of a few known nicotinamide targets, including sirtuin inhibitors (EX527 and SirReal2) and PARP inhibitor (ABT888). However, neither single inhibitor nor their combination demonstrated the ability to improve cell survival (Figure S1I). It indicates that nicotinamide could function through some other pathways to promote cell survival. It is well known that individualized hESCs were killed through ROCK/actomyosin activation (Chen et al., 2010; Ohgushi et al., 2010). We compared the impact of nicotinamide on cell survival with ROCK inhibitor Y27632. After cell individualization and passaging, nicotinamide improved cell survival with similar efficiency as ROCK inhibitor. However, no additive beneficial effect was observed when they were applied together (Figure 1D), which suggested that nicotinamide and ROCK inhibitor possibly functioned through the same pathway.

We then analyzed the impact of nicotinamide on the ROCK pathway. ROCK directly phosphorylates myosin phosphatase-targeting protein (MYPT) at Thr696, and also regulates the phosphorylation of myosin light chain (MLC) directly or indirectly through MYPT (Totsukawa et al., 2000). After dissociation, the phosphorylation of MLC and MYPT increased, and Y27632 and nicotinamide suppressed the phosphorylation of both MLC and MYPT significantly (Figure 1E). This impact of nicotinamide was dose dependent (Figure 1F). Immunostaining results showed that both nicotinamide and Y27632 decreased the colocalization between p-MLC (Ser19) and actin filament after hESC dissociation (Figure 1G). These data indicated that nicotinamide was a modulator of the ROCK pathway.

Nicotinamide Is a Direct ROCK Inhibitor Independent of NAD Pathway

Nicotinamide is the precursor of NAD+ and NADH, so we tested whether nicotinamide improved cell survival through NAD metabolites. Niacin, NMN, NAD+, and NADH were added to individualized cells, but none of them had significant effect on cell survival (Figure 2A), and these molecules did not block the cell blebbing after individualization (Figure S2A). NAMPT converts nicotinamide into NMN, but NAMPT inhibitors did not alter nicotinamide impact on cell survival (Figures 2B and S2B). Niacin also had no impact on the phosphorylation of MLC and MYPT (Figure 2C). These results suggested that the effects of nicotinamide on cell survival and ROCK pathway regulation were possibly independent of the NAD pathway, and nicotinamide itself might be the direct effector.

To study how nicotinamide inhibited ROCK, we tested the protein level of ROCK1 and ROCK2 during dissociation, and found that nicotinamide had no impact (Figure S2C). Then we evaluated the activity of ROCK1 and ROCK2 in vitro with different doses of nicotinamide.
and niacin (Figures 2D and 2E). Surprisingly, the addition of niacinamide significantly suppressed ROCK1 and ROCK2 activity in a dose-dependent manner, but niacin had almost no effect (Figures 2D and 2E). Computational simulation demonstrated that niacinamide could potentially interact with key amino acid functional groups in the active site of ROCK2 (Figure S2D). The binding constant assay also confirmed the inhibition of ROCK1 and ROCK2 by niacinamide (Figures 2F and 2G).

**Figure 1. Nicotinamide Promotes hESC Survival through the Inhibition of the ROCK-Actomyosin Axis**

(A) Dose-dependent effect of nicotinamide on cell survival after dissociation. hESCs (H1 cells unless otherwise stated) were counted 24 hr after individualization. The cell survival index represents the number of surviving cells divided by the input cell number (n = 3). Nam, Nicotinamide.

(B) Phase contrast images after individualization. hESCs were dissociated by TrypLE, neutralized by 0.5% BSA, and then treated with the indicated concentration of nicotinamide for 30 min. Scale bar, 20 μm.

(C) The percentage of blebbing cells under nicotinamide treatments at different concentrations. The percentage of blebbing cells was normalized by the total cell number (n ≥ 5 images).

(D) The comparison of nicotinamide and ROCK inhibitor Y27632 on cell survival after individualization (n = 3). Nam, nicotinamide 10 mM; ROCKi, Y27632 10 μM.

(E) The phosphorylation of MYPT1 (Thr 696) and MLC (Ser 19) in individualized hESCs under nicotinamide treatment. 10 μM ROCK inhibitor (Y27632) was used as positive control. Top, western blot image. Bottom, quantification of the western blot results (n = 3).

(F) Dose-dependent effect of nicotinamide on the phosphorylation of MYPT1 (Thr 696) and MLC (Ser 19). Individualized hESCs were treated with nicotinamide at indicated concentrations for 1 hr. Top, western blot image. Bottom, quantification of the western blot results (n = 3).

(G) Confocal images of individualized hESCs treated with 10 mM nicotinamide (Nam) or 10 μM ROCK inhibitor Y27632 (ROCKi). Red, phalloidin 594; green, p-MLC (Ser19). Scale bar, 10 μm.

Data are shown as means ± SEM. *p < 0.05 compared with control.

**Nicotinamide Regulates More Than the ROCK Pathway**

ROCK inhibitor Y27632 increases the cloning efficiency of hPSCs (Chen et al., 2010), so we examined the impact of nicotinamide on cloning efficiency. Compared with Y27632, nicotinamide-treated cells showed much smaller improvement in cloning efficiency (Figure 3A), even though both reagents had similar impact on 24-hr cell survival (Figure 1D). High concentrations of nicotinamide decreased the cell growth rate of hESCs (Figure 3B) and
reduced the mRNA level of NANO and POUF1 (Figures 3C and 3D), which indicated that nicotinamide possibly induced hESC differentiation. Among the set of 12 vitamins examined in the differentiation of hESCs, nicotinamide was the only one that affected the pluripotency of hESCs (Figures S3A and S3B). Taken together, this evidence suggests that nicotinamide may have additional functions on pluripotency other than regulating the ROCK pathway.

To study the other functions of nicotinamide in hESCs beyond ROCK inhibition, we analyzed the global gene expression profile after 24 hr of nicotinamide and ROCK inhibitor treatment (Table S1). Hierarchical clustering analysis showed that nicotinamide treatment was not clustered with the ROCK inhibitor group (Figure 3E). Compared with control, nicotinamide increased the expression of 371 genes, and decreased the expression of 640 genes after a 24-hr treatment. However, only a small portion of these genes were shared by the cells treated with ROCK inhibitor (Figure S3C). The KEGG analysis showed that the genes downregulated by nicotinamide were enriched in pathways associated with pluripotency of stem cells, phosphatidylinositol 3-kinase, metabolism, transcription, and cancer (Figure 3F), and the gene expression patterns were different compared with the genes downregulated by the ROCK inhibitor (Figure S3D). The genes upregulated by nicotinamide were also enriched in different pathways from those upregulated by the ROCK inhibitor (Figures S3E and S3F). These data indicated that nicotinamide had multiple functions in hESC regulation.

Nicotinamide Affects hESC Differentiation in Multifaceted Manner

Because nicotinamide was a direct ROCK inhibitor at high concentration, we hypothesized that nicotinamide might be able to inhibit other kinases. Considering that most nicotinamide effects appeared at 10 mM in cell culture, we measured cellular nicotinamide amounts when 10 mM of nicotinamide was added in the medium. Liquid chromatography-mass spectrometry (LC-MS) results demonstrated that cellular nicotinamide concentration was around
1.5 mM after 1 hr of incubation (Figure 4A). Based on the above information, the KINOMEscan assay in a competition-binding method was used to screen the interaction between nicotinamide and the active sites of 97 kinases (Davis et al., 2011; Egan et al., 2015; Fabian et al., 2005; Somoza et al., 2015), and the screening was performed at 1 and 3 mM. We found that multiple kinases were significantly inhibited by nicotinamide (Figure 4B; Table S2). Nicotinamide inhibited 96.7% of kinase-ligand interaction of ROCK2 at 3 mM, which is consistent with the in vitro kinase assay (Figure 2D). It also inhibited 92.3% of kinase-ligand interaction of CK1δ (Figure 4B; Table S2). The kinase

Figure 3. Nicotinamide Has More Functions Than the ROCK Inhibitor
(A) Comparison of ROCK inhibitor Y27632 (ROCKi) and nicotinamide (Nam) on cloning efficiency (n = 3).
(B) Dose-dependent effect of nicotinamide on cell growth. hESCs were treated with different doses of nicotinamide, and cell number was counted every day. The fold change was calculated by dividing the cell number by day 0 cell count (n = 3 technical replicates). The results were repeated three times.
(C and D) Dose-dependent effect of nicotinamide on pluripotency. NANOG (C) and POU5F1 (D) expression (normalized to control without treatment) were analyzed by qPCR after 3 days of differentiation (n = 3).
(E) Hierarchical clustering of samples with indicated treatments in microarray analysis. The phylogenetic relationships of genes are shown on the left, and the cluster relationship of samples is indicated on the top. hESC samples were collected after 24 hr of treatment and compared with untreated control and differentiated mesoderm cells. ROCKi, Y27632 (10 μM for 24 hr). Nam, nicotinamide (10 mM for 24 hr). Mesoderm, hESC-derived mesoderm cells.
(F) Bubble plot of enriched KEGG pathways from nicotinamide downregulated genes. Data are shown as means ± SEM. *p < 0.05 compared with control.
Figure 4. Nicotinamide Inhibits CK1 and Promotes hESC Differentiation

(A) The cellular concentration of nicotinamide determined by LC-MS after 1 hr of treatment. hESCs were cultured in normal E8 medium without additional nicotinamide (Control), or E8 medium with additional 10 mM nicotinamide (Nam) (n = 3).

(B) The kinase screening profile for nicotinamide, obtained using the DiscoverRx KINOMEscan service. Nicotinamide was screened at 1 and 3 mM for its ability to inhibit the binding of 97 kinases to substrates in the assay. % Ctrl represents the results of primary screen on binding interactions, and lower numbers indicate stronger hits (see also Table S2).

(C–E) Binding constant measurements for the interactions of nicotinamide with CK1δ, CK1α (D), and CK1c (E). The x axis indicates the nicotinamide concentration (µM) in log10 scale (n = 2 technical replicates).

(legend continued on next page)
binding constants of nicotinamide with CK1δ, CK1ζ, and CK1ε were 352.512, 546.580, and 612.076 μM, respectively (Figures 4C–4E). β-Catenin is the substrate of CK1ζ, and is specifically phosphorylated at Ser45 (Amit et al., 2002; Liu et al., 2002). We examined β-catenin phosphorylation (Ser45) in hESC, and found that nicotinamide and CK1 inhibitor significantly suppressed Ser45 phosphorylation (Figures 4F and 4G). We also evaluated the impact of nicotinamide on the CK1α activity in vitro using the bioluminescent kinase assay, and the result showed that nicotinamide inhibited CK1α in a dose-dependent manner (Figure 4H).

Based on the findings in the kinase screen, we examined whether any of the nicotinamide-inhibited kinases or other targets could affect differentiation in hESCs. hESCs were treated with a set of small molecules that modulated the activities of nicotinamide targets, and allowed to spontaneously differentiate for 3 days. Similar to nicotinamide, CK1 inhibitor D4476 significantly reduced the mRNA level of pluripotency markers (NANOG and POU5F1); in contrast, other inhibitors in ROCK, PARP, and sirtuin pathways did not have a significant impact (Figures 4I and 4J). In embryoid body differentiation, nicotinamide inhibited the expression of meso-endoderm marker genes (MIXL1, TBX3, EOMES, and SOX17), and induced the expression of ectoderm marker genes (PAX6 and NEUROD1). CK1 inhibitor D4476 demonstrated a similar effect (Figure S3G). We also confirmed that both nicotinamide and CK1 inhibitor D4476 blocked meso-endoderm differentiation in BMP4-induced differentiation (Figures S3H–S3J). These results suggest that nicotinamide could lead to hPSC differentiation through the inhibition of CK1.

Nicotinamide was reported as an inducer of retinal pigment epithelium (RPE) differentiation (Buchholz et al., 2013), so we explored whether nicotinamide affects RPE differentiation through CK1 inhibition (Figure 4K). Consistent with previous reports, nicotinamide increased the expression of early eye field markers LHX2, PAX6, and RAX on day 6 of RPE differentiation. ROCK inhibitor, SIRT2 inhibitor, PARP inhibitor, and niacin alone had little effect, while joint treatment with ROCK inhibitor and SIRT1 inhibitor improved the mRNA level of LHX2, PAX6, and RAX, even though the level was much lower than with nicotinamide (Figures S4A–S4C). At the same time, CK1 inhibitor D4476 significantly induced the expression of early eye field markers LHX2, PAX6, and RAX (Figures 4L–4N). The positive impact of nicotinamide, CK1 inhibitor, and CK1/ROCK dual inhibition on RPE differentiation was further confirmed by LHX2 immunostaining (Figure 4O) and flow cytometry analysis (Figure S4D). Similar results were obtained with H9 (Figures S4E–4G) and human induced pluripotent stem cell lines NL1 (Figures S4H–S4J) and NL4 (Figures S4K–S4M). These results indicate that the effect of nicotinamide on RPE differentiation potentially relies on its inhibition on ROCK and CK1 pathways.

**DISCUSSION**

Nicotinamide is widely used in disease treatments and stem cell applications, but many of its effects cannot be explained by its role in nutritional regulation. We demonstrated that nicotinamide regulates stem cell survival and differentiation through the inhibition of specific kinases. Besides its complicated role in metabolism, DNA repair, and epigenetic modification, nicotinamide can modulate various cellular functions through kinase cascades. This is consistent with the diverse applications related to nicotinamide.

Nicotinamide has long been used in stem cell culture to improve stem cell performance. Nicotinamide enhanced cell survival and reprogramming, but its function was attributed to its role in the sirtuin pathway and nutritional regulation (Avalos et al., 2005; Son et al., 2013). Our study showed that nicotinamide was a ROCK inhibitor. ROCK inhibitors are known to suppress actomyosin contraction, improve cell survival, and enhance reprogramming.
efficiency (Chen et al., 2010; Ohgushi et al., 2010; Watanabe et al., 2007). It is possible that nicotinamide benefits the stem cell culture through its role as a ROCK inhibitor. It is noteworthy that nicotinamide is used in many organoid culture systems, which are also benefited by ROCK inhibition in organoid formation (Miyoshi and Stappenbeck, 2013). The positive effect of nicotinamide on organoid culture may also be related to its role as a ROCK inhibitor.

Nicotinamide is used in many different stem cell differentiation platforms, and our data show that this function is likely not based on its inhibition of ROCK. The kinase screen data in this report showed that nicotinamide also inhibited CK1 and other kinases that are associated with pluripotency. In the limited tests, we showed that some of nicotinamide’s impacts on differentiation could potentially be explained by its ability to inhibit CK1 pathways. In RPE differentiation, combination of ROCK and CK1 inhibitors achieved similar effects as nicotinamide alone, which supports our argument that nicotinamide might drive differentiation through CK1 modulation.

Unlike nicotinamide, niacin does not inhibit either ROCK or CK1, even though both belong to the vitamin B3 family. It is important to consider their differential impact on cellular functions when people plan to use vitamin B3 for specific treatments. The kinase pathways affected by nicotinamide could provide valuable references for relevant clinical applications.

We noticed that nicotinamide was effective in kinase inhibition only at high concentrations, and there is an obvious dose-dependent effect. The high concentration of nicotinamide is often used in disease treatment and cell culture. However, the level of nicotinamide in the serum is much lower. This suggests a dual role of nicotinamide controlled by its cellular concentration. Low-level nicotinamide is sufficient to meet cellular needs as a nutrient, but high concentrations lead to kinase inhibition and subsequently affect survival and differentiation. This partially explains why nicotinamide’s effect on kinase activity was not previously revealed.

In summary, this report revealed nicotinamide as a kinase inhibitor regulating stem cell survival and differentiation. These results have practical implications for nicotinamide-related treatments, and provide another angle to further improve its applications.

**EXPERIMENTAL PROCEDURES**

Experimental procedures are also provided in Supplemental Information.

**hPSC Culture and Survival Assays**

The use of hESCs and hiPSCs was approved by the Institutional Review Board at the University of Macau. hESC culture and survival assays were carried out as described previously (Chen et al., 2010). See Supplemental Information for more details.

**hESC Differentiation to Early RPE Lineage**

hPSCs were passaged 1:6 on Matrigel (Corning Life Sciences) in E8 medium with 10 μM Y27632 and changed to fresh E8 after cell attachment for 24 hr. Then the differentiation was induced following the methods reported previously with slight modifications (Buchholz et al., 2013). The detailed method is in Supplemental Information.

**Statistical Analysis**

Data are shown as means ± SEM of at least three independent experiments unless otherwise specified, and Student’s t test was used for statistical analysis. p values < 0.05 were considered significant.

**ACCESSION NUMBERS**

The accession number for the microarray data reported in this paper is GEO: GSE121230.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.10.023.

**AUTHOR CONTRIBUTIONS**

G.C., Y.M., and J.A.T. conceived and designed the study. Y.M., W.L., and G.C. performed the cell survival assays and ROCK-related experiments. F.X. measured intracellular nicotinamide concentration by LC-tandem MS. Y.M. and X.Z. performed hPSC differentiation experiments. Z.R., C.S., and Y.M. prepared samples for microarray and analyzed the data. V.Y.-F.W. contributed to the structure modeling. L.L and Y.M. performed the Annexin V and propidium iodide staining. Y.M., W. L., and G.C. wrote the paper. Most authors contributed to the editing and proofreading of the manuscript.

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Supplemental Information

Nicotinamide Promotes Cell Survival and Differentiation as Kinase Inhibitor in Human Pluripotent Stem Cells

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Figure S1, Related to Figure 1. Nicotinamide improves cell survival in hESCs and iPSCs.

(A) The effect of vitamins on hESC (H1 cells unless otherwise stated) survival 24 hours after dissociation. The cell survival index represents the number of surviving cells divided by the input cell number (n = 3 technical replicates). The result was repeated twice.

(B) Nicotinamide decreased the apoptotic hESCs after 8 hours dissociation.

(C) The quantification of Annexin-V positive propidium iodide negative hESCs after 8 hours dissociation (n = 3 independent experiments). Nam, nicotinamide 10 mM.

(D-F) Dose-dependent effect of nicotinamide on individualized H9 (D), NL1 (E) and NL4 (F) cell survival (n = 3 independent experiments).

(G-H) Nicotinamide improved hESC survival after dissociation on surfaces coated with vitronectin (G) and E-cadherin (H) (n = 3 independent experiments). ROCKi, Y27632 10 μM. Nam, nicotinamide 10 mM.

(I) The comparison of nicotinamide, Sirtuin inhibitors and PARP inhibitor on cell survival after individualization. Nam, nicotinamide 10 mM; SIRT1i, Ex527 10 μM; SIRT2i, SirReal2 500 nM; PARPi, ABT888 50 nM; 3i, Ex527 10 μM + SirReal2 500 nM + ABT888 50 nM (n = 3 independent experiments).

Data are shown as mean ± SEM. *, p < 0.05 compared with control.
Figure S2. Related to Figure 2. hESC survival is not improved through SIRT, PARP or NAD pathway.
(A) Nicotinamide decreased the percentage of blebbing cells, but niacin, NMN, NAD+ and NADH had no effect. hESCs were dissociated by TrypLE for 5 minutes, neutralized by 0.5% BSA, and then treated with 10 mM nicotinamide (Nam), 5 mM Niacin, 5 mM NMN, 5 mM NAD+, or 5 mM NADH for 30 minutes. The percentage of blebbing cells was normalized by the total cell number (n = 5 images). Data are representative of three independent experiments.
(B) Nicotinamide phosphoribosyltransferase (NAMPT) inhibitor STF118804 did not block the effect of nicotinamide on cell survival (n = 3 independent experiments). White, STF118804 alone; red, STF118804 together with nicotinamide; blue, STF118804 together with ROCK inhibitor. Nam, nicotinamide 10 mM; ROCKi, Y27632 10 μM.
(C) Nicotinamide and ROCK inhibitor did not change the protein level of ROCK1 and ROCK2 after the indicated time of individualization (n = 3 independent experiments). Nam, nicotinamide 10 mM, ROCKi, Y27632 10 μM.
(D) Summary of the binding of nicotinamide to ROCK2 and CK1δ predicted by structure modeling. Data are shown as mean ± SEM. *, p < 0.05 compared with control.
Figure S3

A

B

C

D

E

F

G

H

I

J

Legend:
- Ct
- Retinol
- Thiamine
- Nicotinamide
- D-Pantothenic acid
- Pyridoxal hydrochloride
- Biotin
- Folic acid
- Vitamin B12
- L-Ascorbic acid
- Cholecalciferol
- α-Tocopherol
- Vitamin K

Concentration (μM)

Fold change of POU5F1

Fold change of NANOG

Summary of signaling pathways:
- Wnt signaling pathway
- Transcriptional misregulation in cancer
- TNF signaling pathway
- Signaling pathways regulating pluripotency of stem cells
- Pathways in cancer
- HTLV-I infection
- Basal cell carcinoma
- Amoebiasis
- African trypanosomiasis

Figures showing gene expression changes under different conditions:

- ROCKi
- Nam
- CK1i

Gene expression heatmaps and bar graphs for various genes in different conditions.
Figure S3, Related to Figure 3. Nicotinamide and ROCK inhibitor demonstrate different impacts on gene expression and differentiation potential.

(A-B) The effect of vitamins on hESC pluripotency during differentiation. *NANOG* and *POU5F1* expression were detected by Q-PCR after three days of differentiation. Data shown as mean ± SEM of duplicate wells and are representative of two independent experiments.

(C) Venn diagram representing the number of differentially expressed genes in microarray analysis. hESCs were treated with Nicotinamide (10 mM) and ROCK inhibitor (Y27632 10 μM) in E8 medium for 24 hours. Green, genes up-regulated by ROCK inhibitor treatment; blue, genes up-regulated by nicotinamide treatment; pink, genes down-regulated by nicotinamide treatment; yellow, genes down-regulated by ROCK inhibitor.

(D-F) Bubble plot of enriched KEGG pathways from ROCK inhibitor down-regulated genes (D), nicotinamide up-regulated genes (E) and ROCK inhibitor up-regulated genes (F). Rich factor is the ratio of the treatment-regulated gene number to the total gene number of a certain pathway. A Q value is the corrected p value ranging from 0 to 1. The color and size of the dots indicated the range of the Q-value and the number of genes mapped to the specific pathways.

(G) Heatmap comparing the gene expression profiles of hESCs, and day 14 embryoid bodies were treated with DMSO (Control), ROCK inhibitor (ROCKi, Y27632 10 μM), nicotinamide (Nam, 10 mM) or CK1 inhibitor (CK1i, D4476 5 μM) (n = 3 independent experiments).

(H-J) Nicotinamide and CK1 inhibitor decreased the gene expression of *TBXT* (H), *MIXL1* (I) and *SOX17* (J) in BMP4 induced meso-endoderm differentiation. hESCs were treated with 20 ng/ml BMP4 in E8 medium for 2 days, and then the expression level of meso-endoderm marker genes were analyzed by Q-PCR, normalized to GAPDH and to BMP4 control (n = 3 independent experiments).

Data shown as mean ± SEM. *, p < 0.05, ***, p < 0.001 compared with control.
Figure S4, Related to Figure 4. Nicotinamide promotes early RPE differentiation in hESCs and iPSCs.

(A-C) Effect of indicated inhibitors on the expression of early eye field markers LHX2 (A), RAX (B) and PAX6 (C) on day 6 of RPE differentiation. ROCKi, Y27632 10 μM; SIRT1i, EX527 10 μM; SIRT2i, SirReal2 500 nM; PARPi, ABT888 50 nM (n = 3 independent experiments).

(D) Nicotinamide and CK1 inhibition increased the population of LHX2 positive cells on day 12 of early RPE differentiation. ROCKi, Y27632 10 μM; Nam, nicotinamide 10 mM; CK1i, D4476 5 μM. The grey line showed the LHX2 level of hESCs, and the red line showed the LHX2 level of differentiated cells with indicated treatment (n = 3 independent experiments).

(E-M) Effect of nicotinamide and CK1 inhibition on the expression of early eye field markers in the early RPE differentiation of hESC line H9 (E-G) and iPSC line NL1 (H-J) and NL4 (K-M), analyzed by real-time PCR on day 6 of differentiation (n = 3 independent experiments). ROCKi, Y27632 10 μM; Nam, nicotinamide 10 mM; CK1i, D4476 5 μM.

Data shown as mean ± SEM. *, p < 0.05 compared with control.
## Supplemental Experimental Procedures

### Reagents

| Antibodies                                                                 | Source          | Identifier                  |
|----------------------------------------------------------------------------|-----------------|-----------------------------|
| Anti-phospho-Myosin Light Chain 2 (Ser19) (used at 1:500 for WB, 1:100 for IF) | Cell signalling | Cat.# 3671; RRID: AB_330248 |
| Anti-Myosin (Light Chains) (used at 1:1000)                                | Sigma           | Cat.# M4401; RRID: AB_477192 |
| Anti-phospho-MYPT1 (Thr696) Antibody (used at 1:500)                      | Millipore       | Cat.# ABS45; RRID:AB_11212365|
| Anti-MYPT1 Antibody (used at 1:1000)                                       | Millipore       | Cat.# 07-672; RRID:AB_417394|
| Anti-LHX2 Antibody (used at 1:100)                                        | DSHB            | Cat.# PCRPLHX2-2E3          |
| Anti-ROCK1 (used at 1:1000)                                                | Beyotime        | Cat.# AF0276                 |
| Anti-ROCK2 (used at 1:1000)                                                | Millipore       | Cat.# 04-841                 |
| Anti-β-Catenin (12F7) (used at 1:500)                                      | Santa Cruz      | Cat.# 59737                  |
| Anti-phospho-β-Catenin (Ser45) (used at 1:500)                             | Cell signalling | Cat.# 9564S                 |
| Alexa Fluor® 488 AffiniPure Goat Anti-Mouse IgG                             | Jackson         | Cat.# 115-545-071; RRID:AB_2338847 |
| Alexa Fluor® 488 AffiniPure Goat Anti-Rabbit IgG                           | Jackson         | Cat.# 111-545-046; RRID:AB_2338050 |

### Chemicals and recombinant proteins

| Chemicals and recombinant proteins                                                                 |
|-----------------------------------------------------------------------------------------------------|
| Penicillin/streptomycin                                                                             |
| Transferrin                                                                                         |
| Ascorbic acid                                                                                       |
| Insulin                                                                                             |
| Recombinant human TGFβ                                                                             |
| Recombinant human Activin A                                                                        |
| Recombinant Human Noggin                                                                            |
| Recombinant Human BMP4                                                                             |
| Y-27632                                                                                             |
| Nicotinamide                                                                                        |
| Nicotinic acid                                                                                      |
| Retinol                                                                                             |
| Thiamine                                                                                           |
| D-pantothenic acid hemicalcium salt                                                                 |
| Pyridoxine hydrochloride                                                                           |
| Biotin                                                                                             |
| Folic acid                                                                                          |
| Vitamin B12                                                                                        |
| Cholecalciferol                                                                                    |
| (+)alpha-tocopherol                                                                                |
| Vitamin K1                                                                                          |
| SirReal2                                                                                           |
| EX 527 (Selisistat)                                                                                |
| ABT-888                                                                                            |
| β-nicotinamide adenine dinucleotide                                                                  |
**Other reagents**

| Item                                                                 | Brand          | Catalog Number |
|----------------------------------------------------------------------|----------------|----------------|
| β-nicotinamide adenine dinucleotide reduced disodium salt hydrate     | Sigma          | Cat.# N8129    |
| β-Nicotinamide mononucleotide                                         | Sigma          | Cat.# N3501    |
| D4476                                                                | Selleck        | Cat.# S7642    |
| BSA                                                                  | Sigma          | Cat.# A7030    |
| Matrigel                                                             | Corning        | Cat.# 354230   |
| TrypLE                                                               | ThermoFisher   | Cat.# 12563-029|
| DMEM/F12                                                             | ThermoFisher   | Cat.# 11330057 |
| RNAiso Plus                                                          | Takara         | Cat.# 9109     |
| Applied Biosystems™ High-Capacity cDNA                                | Applied        | Cat.# 4368813  |
| Reverse Transcription Kit                                            | Biosystems     |                |
| SuperSignal™ West Pico PLUS                                           | ThermoFisher   | Cat.# 34078    |
| Chemiluminescent Substrate                                           |                |                |
| Rho-associated kinase (ROCK) activity assay                          | Millipore      | Cat.# CS001    |
| SYBR® Premix Ex Taq™ (Tli RNaseH Plus)                               | Takara         | Cat.# RR420A   |
| Alexa fluor 594 phalloidin                                           | Invitrogen     | Cat.# A12381   |
| Dead Cell Apoptosis Kit with Annexin V FITC and PI                   | ThermoFisher   | Cat.# V13242   |
| Vectashield Antifade Mounting Medium                                  | Vector         | Cat.# H-1000   |
| CK1α1 Kinase Enzyme System                                           | Promega        | Cat.# V4484    |
| ADP-Glo™ Kinase Assay                                                | Promega        | Cat.# V9101    |

**Human PSC Culture**

Human ESCs (H1 and H9) and human iPSCs (NL1 and NL4) from NIH were cultured in E8 medium on Matrigel-coated plates for 3-4 days, and then passaged with DPBS/EDTA. The details of cell maintenance were described previously (Chen et al., 2010). In this study, most experiments were conducted on H1 hESC line unless otherwise stated, and some key experiments were also confirmed in H9 and iPSCs.

**Survival Assays**

The assay was performed as previously described with some modifications (Chen et al., 2010). Briefly, human ESCs and iPSCs were cultured for 3 days, dissociated with TrypLE, and neutralized with the medium containing 0.5% BSA. Cells were then harvested and counted, and 20,000-40,000 cells were plated into each well of 24-well plates containing 500ul medium and different reagents. After 24 hours, cells were dissociated with TrypLE, neutralized with 10% FBS, and counted by flow cytometry.

**Cell growth and Cloning assay**

The assay was performed as previously described with some modifications (Chen et al., 2010). Briefly, human embryonic stem cells (hESCs) (H1 cells were used unless otherwise stated) were cultured for 3 days, dissociated with TrypLE, and neutralized with medium containing 0.5% BSA. Cells were then harvested, counted, and seeded at the density of 500 per well in 12-well plates. The cloning efficiency was measured after 7 days.

To test effect of reagents on growth, hESCs were cultured for 3 days, dissociated with TrypLE, and neutralized with medium containing 0.5% BSA. Cells were then harvested and split at a ratio of 1:12.
dilution in 24-well plates. Treatments were added to attached cells after 24 hours, and continued for indicated periods of time, and cell counts were determined using flow cytometer and compared to control before treatment.

**hESC differentiation**
For 3-day spontaneous differentiation, hESCs were dissociated by DPBS-EDTA and passaged at a 1:12 dilution into a 12-well plate coated with Matrigel. After 24 hours of cell attachment, the maintenance medium (E8) was replaced with 1ml of E6 medium (E8 minus TGFβ and FGF2), and the medium was changed every 1 or 2 days for 3 days. The embryoid body (EB) formation was performed as previously described with some modifications (Lin and Chen, 2008). Briefly, 70% confluent hESCs were dissociated with DPBS/EDTA, and passaged 1:2 into each well of AggreWell-800 (Corning) into E8 medium with 10 μM Y27632 for 24 hours. EBs were cultured in E8 medium for another 2 days with half-change of fresh medium every day. On the third day, EBs were removed from microwells by gently pipetting, and transferred into poly-HEMA coated 12-well plates in E6 medium to culture for 14 days. The medium was changed every 2 days. For meso-endoderm differentiation, hESCs were passaged at a 1:6 ratio by DPBS-EDTA into a 12-well plate coated with Matrigel. After 24 hours of cell attachment, medium was changed to E8 medium with 20 ng/mL BMP4 for 2 days. RNA was harvested for Q-PCR analysis.

**hPSC differentiation to early RPE lineage**
RPE differentiation was induced following the methods reported previously with slight modifications (Buchholz et al., 2013). hPSCs were passaged 1:6 onto Matrigel (Corning) in E8 medium with 10 μM Y27632 and changed to fresh E8 medium after cell attachment for 24 hours. From day 0 to 2, 50 ng/ml Noggin (R&D Systems), 3 μM IWP2 (Selleck), 10 ng/ml IGF1 (R&D Systems) and other chemicals were added to E6 medium. From day 2 to 4, 10 ng/ml Noggin, 3 μM IWP2, 10 ng/ml IGF1, 5 ng/ml FGF2 and other chemicals were added to E6 medium. From day 4 to 6, 3 μM IWP2, 10 ng/ml IGF1 and 20 ng/ml Activin A (R&D Systems) were added to E6 medium. Total RNA was harvested to analyze the gene expression. From day 6 to 12, the cells were cultured in E6 medium, and then harvested for flow cytometry analysis. At day 8, the differentiated cells were passaged by dispase II, cultured for another 4 days, and then harvested for immunostaining.

**Flow cytometry**
At day 12 of RPE differentiation, the differentiated cells were dissociated with TrypLE, and fixed with 1% paraformaldehyde in PBS at 37°C for 10 minutes. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes at room temperature. Primary antibody mouse anti-human LHX2 was incubated with cells at a 1:100 dilution in 1% BSA in PBS for 1 hour at room temperature. After washing, Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody was used at 1:1000 dilution in 1% BSA for 1 hour at room temperature. After washing, cells were re-suspended in PBS for flow cytometry analysis using BD Accuri C6. Undifferentiated hESCs were stained with LHX2 as negative control for gating.

**Annexin V and propidium iodide staining**
The annexin V and propidium iodide staining was performed following the protocol of the kit (ThermoFisher). Briefly, hESCs were dissociated with TrypLE to single cells, and seeded to Matrigel-coated 12-well plates with or without 10 mM nicotinamide. After 8 hours, cells were harvested, washed with PBS, and then re-suspended in 100 μL 1x annexin-binding buffer containing 5 μL annexin V and 100 μg/mL propidium iodide. After incubating for 15 minutes, 400 μL annexin-binding buffer was added, and samples were kept on ice for flow cytometry analysis using BD Accuri C6.

**ROCK activity measurement**

The assay was done following the instruction from the Rho-associated kinase (ROCK) activity assay kit (Millipore). Briefly, the reaction mixture was prepared which included 1 mU ROCK1 or ROCK2, 0.5 mM ATP, 75 mM MgCl₂ and assay dilution buffer; ROCK inhibitor or nicotinamide was added, and the reaction was incubated at 30°C for 30 minutes. After washing, anti-p-MYPT1 (Thr696) antibody was added, followed by incubation at room temperature for 1 hour. After washing, secondary antibody was added (room temperature for 1 hour). Then TMB/E substrate was added and allowed to develop for 1-5 minute before the reaction was stopped. The absorbance was measured at 450 nm.

**Kinase screen and Kd determination**

The kinase screen and Kd determination were performed by DiscoverX. Briefly, specific kinases were expressed in BL21 strain or HEK-293 cells, and then labeled with DNA tag for Q-PCR detection. Magnetic beads coated with streptavidin were incubated with biotinylated molecule ligands. The affinity resins were then blocked and washed before using for kinase assays. Binding reactions were performed by combining test compounds, affinity beads with ligand, and kinases. Kds were determined using 9-point 3-fold dilution series with DMSO control point. The reactions were performed in 384-well plate, and incubated at room temperature for 1 hour. After washing and elution, the kinase concentration in the eluates of beads was determined by q-PCR.

% Ctrl calculation:

\[
\frac{\text{test compound signal} - \text{background signal}}{\text{negative control signal} - \text{background signal}} \times 100
\]

Test compound: nicotinamide
Negative control: DMSO (100% Ctrl)
Positive control: control compound (0% Ctrl)

**CK1α activity measurement**

The assay was performed following instructions from the kit (Promega). Briefly, the reaction was prepared with 25 μL total volume containing 10 ng CK1α, 2.5 μg Casein, 10 μM ATP, CK1 inhibitor D4476 or different doses of nicotinamide in reaction buffer, and reacted at room temperature for 60 minutes. Then 25 μL ADP-Glo® reagent was added, and incubated at room temperature for 40 minutes. After that, 50 μL Kinase detection reagent was added and incubated at room temperature for 30 minutes. Luminescence was read by PerkinElmer Victor X3 Microplate Reader.

Percentage of CK1α activity calculation

\[
\frac{\text{test compound signal} - \text{background signal}}{\text{negative control signal} - \text{background signal}} \times 100
\]

Test compound: nicotinamide or D4476
Background signal: reaction without CK1α1
Negative control signal: reaction without any inhibitor.

**Immunostaining and Actin Staining**
Cells were fixed with 4% Paraformaldehyde in PBS for 10 minutes at room temperature. After washing, fixed cells were permeabilized with 0.3% Triton-X 100 in PBS for 20 minutes at room temperature. After washing with PBS, cells were blocked with 1% BSA in 0.1% Triton-X100 / PBS, and incubated with the primary antibodies (1:200 in PBS containing 1%BSA and 0.1% Triton-X100) overnight at 4 °C. The stained cells were washed with 0.1% Triton-X100 / PBS three times at room temperature, and then incubated with the secondary antibodies (1:1000) for 1 hour at room temperature. For actin staining, diluted phalloidin solution (1:100) was added to the cells, and incubated for 30 minutes at room temperature following the protocol of manufacturer (ThermoFisher). After washing, cells were stained with hoechst (1:10000). The samples were mounted with Vectashield (Vector Laboratories), and imaged with Carl Zeiss Confocal LSM710.

**Western blot**
Briefly, 30μg protein extracted from H1 cells was loaded into the lanes of a SDS-PAGE gel, and transferred to PVDF membranes after electrophoresis. The membranes were blocked with 5% non-fat milk in TBS-T for 1h at room temperature, and then incubated with primary antibodies overnight at 4°C. After washing, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase for 2 hours. The immuno-complexes were detected by the enhanced chemiluminescence method (ThermoFisher). The density of signals was quantified with Image J.

**Quantification of intracellular nicotinamide**
H1 cells were passaged and then cultured in 6-well plates with E8 medium (Ct) or E8 supplemented with 10 mM nicotinamide for 1h. After 1h, attached cells were dissociated by TrypLE, neutralized with 10% FBS/DMEM, and then counted by hemocytometer. Cells were also collected for nicotinamide concentration quantification by LC-MS/MS. Sample preparation is based on published procedures (Ying et al., 2012; Zhang et al., 2016). Briefly, spent media was removed, and cells were rinsed with 0.5 ml/well 0.9% (w/v) saline twice. Then 1 ml/well -80°C 80% methanol was added to quench metabolism, and cells were scrapped off. The metabolite-containing mixtures were put on ice and then centrifuged at 2000 x g for 15 min. The supernatant was collected and then evaporated by nitrogen-blowing. Samples were re-suspended using 50μl 50% acetonitrile. Waters Xevo TQD coupled with Waters Acquity UPLC system was used for quantification of nicotinamide.

**Microarray**
The experiment procedure of microarray was performed as previously described (Liu et al., 2018). RNAiso Plus was used to extract total RNA from the cells. Then RNA was converted to cRNA using SuperScript III kit and TargetAmp™-Nano Labeling Kit (Epibio) following the manufacturer’s instructions. The HumanHT-12 v4 Expression BeadChip Kit (Illumina) was used for sample hybridization.

**Bioinformatic analysis**
The bioinformatics analysis of the microarray result was based on the protocol reported previously (Liu et al., 2018). Briefly, the microarray data was analyzed using the arrayanalysis.org portal.
Data quality was inspected and assured through box plot and PCA plot. The raw data was processed through background correction, quantile normalization, and the variance stabilizing transformation (log2) (GEO accession number: GSE121230). Genes with a fold change ≥ 1.5 were considered significantly differentially expressed. Heatmap were created using the `pheatmap` package in R, and then hierarchical clustering was performed on both axes with euclidean metric for similarity and complete linkage clustering.

Functional enrichment on differentially expressed genes (DEGs) was analyzed with DAVID online tool (https://david.ncifcrf.gov/). DEGs were further classified by Gene ontology (GO) assignments. GO terms with a P-value < 0.05 were considered significantly enriched. Significantly enriched KEGG pathways were identified using Fisher’s exact test.

In bubble plot of enriched KEGG pathways, rich factor is the ratio of the nicotinamide down-regulated gene number to the total gene number of a certain pathway. A Q value is the corrected p value ranging from 0 to 1. The color and size of the dots indicated the range of the Q-value and the number of genes mapped to the specific pathway (Figure 3F).

**RNA extraction and Q-PCR**

Total RNA of hESCs and iPSCs was extracted using RNAiso Plus (Takara). About 500ng isolated RNA was reverse transcribed into cDNA using high-capacity cDNA reverse transcription kit (ThermoFisher). Q-PCR was performed on QuantStudio™ 7 Flex Real-Time PCR System using SYBR® Premix Ex Taq™ (Takara). All experiments were performed in duplicates, and relative gene expression was normalized by GAPDH. Primer sequences are listed in the following table.

| mRNA Transcript | Forward primer | Reverse primer |
|-----------------|----------------|----------------|
| POU5F1          | AAGCTGGAGTTTGTTGCAAGGTTT | TGAACCTACCTTCCTCCCTCAACCA |
| NANOG           | GATGGCTCAACACGGAGACTG | GCAGAAAGGTGGTTTGGCC |
| LHX2            | ATGCTGTCTCACAGTCTCGTG | GCATGGTCTCGCTCGTGTC |
| PAX6            | CCAGGCAATCGGTGGTAGT | ACGGGCACTCCCGCTTATAAC |
| RAX             | GAAATCTCAAAACTCAGCCC | CTTCACACTTTGCTCAGGAG |
| SOX2            | GGGAGAATGTGTTGCTG | CGCCGCGATGATTGTTATT |
| NEUROD1         | GCTGGCGAGATCCCCTACAGAC | AAATGGTGAAACTGGGCTG |
| MIXL1           | GGTACCACCGACATCCACTT | CGCCGTGTCTGGAAACCATA |
| TBX6            | DCCATTGGGATGACCCAGTT | ACCGCATTGTACACGTTG |
| EOMES           | GTGCCACGCTCTACCTTGTG | CCTGCCCTTTGCTGAATGAT |
| SOX17           | GCGACCGAATTTGGAACAGTA | GGATCAGGGACCTGTCACAC |
| CGB             | TCACCGTCAAACACCACATC | AGAGTGCAATGACAGCCTG |
| TACSTD2         | ACAACGATGGCCTCTACAGC | GTCCAGGTCTGAGTGTGGA |

**Supplemental References**

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