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Research article

Yokukansan, a Kampo medicine, enhances the level of neuronal lineage markers in differentiated P19 embryonic carcinoma cells

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ABSTRACT

Yokukansan (YKS), a traditional Japanese Kampo medicine, affects neurological and psychiatric disorders. It ameliorates hippocampal neurogenesis in animals. However, its effect on neuronal cell differentiation remains unclear. Therefore, we investigated the effects of YKS on pluripotent P19 embryonic carcinoma cells as neuronal differentiation model cells.

Western blotting and immunocytochemistry revealed that 10 μg/mL YKS treatment during embryoid body formation or neuronal differentiation increased the expression of the neuronal stem cell marker, Nestin, by 1.9-fold and 1.7-fold, respectively, and of the mature neuron marker, NeuN, by 1.5-fold and 1.4-fold, respectively.

We examined the effect of YKS on intracellular signaling pathways in P19 cells and found significant elevation in phospho-PDK1 and phospho-mTOR expression (1.1-fold and 1.2-fold, respectively). Therefore, we investigated the effect of PDK1 and mTOR inhibitors on the level of neuronal lineage markers. We found that the mTOR inhibitor significantly abolished the YKS effect on the level of neuronal lineage markers.

Moreover, to identify the target(s) of YKS, antibody array analysis that simultaneously detects 16 phosphorylated proteins was performed. YKS significantly upregulated 10 phosphorylated proteins including PDK1, Akt, AMPK, PRAS40, mTOR, p70 S6 kinase, GSK-3α, Bad and ERK1/2 under cell proliferation conditions. These results suggest that YKS simultaneously activates multiple signaling pathways.

Thus, we concluded that YKS enhances the level of neuronal lineage markers in differentiated P19 cells, however it does not induce neuronal differentiation. Furthermore, mTOR is the predominant mediator of the YKS effect on these cells.

1. Introduction

Yokukansan (YKS) is a Japanese traditional medicine called “Kampo medicine” that originated from the Chinese herbal medicine “Yi-Gan San”. YKS consists of seven kinds of crude drugs: Atractylodes lancea rhizome, poria sclerotium, Cnidium rhizome, Uncaria hook, Japanese Angelica root, Bupleurum root and Glycyrrhiza. YKS has been used in Japan to treat patients who have symptoms such as nervousness, short temper, irritability, sleeplessness, night crying in infants and young children and it has no severe adverse effects. In a previous study, YKS improved behavioral and psychological symptoms of dementia, namely hallucinations, agitation and aggressiveness in patients with Alzheimer's disease [1]. Therefore, YKS is effective for treating neurological and psychiatric disorders.

Using animal models, neuropharmacological studies have improved our understanding of the therapeutic effects of YKS [2]. For instance, YKS ameliorates hippocampal neurogenesis in rats and mice [3, 4]. YKS maintains neuronal survival and function by inducing multiple beneficial effects, including anti-apoptosis, neurite outgrowth and neurogenesis [2]. However, to date, the effect of YKS on neurogenesis at the cellular level and its mechanism have not been fully investigated.

P19 cells can be differentiated into neurons by combining retinoic acid (RA) stimulation and cell aggregation in suspension culture [5, 6], and can differentiate into neural stem cells and subsequently into neurons [7]. Therefore, these cells are widely used as neuronal differentiation model cells. In this study, we investigated the effects of YKS on neurogenesis and neuronal differentiation at the molecular level using

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retinoic acid-treated P19 cells.

2. Materials and methods

2.1. Materials

Antibodies against NeuN, ERK1/2, p-ERK1/2 (Thr-202/Tyr-204), GSK-3β, p-GSK-3β (Ser-9), mTOR, p-mTOR(Ser-2448), p-mTOR(Ser-2481), PDK1, p-PDK1(Ser-241) and secondary horseradish peroxidase (HRP)-conjugated antibodies (anti-rabbit and mouse IgG) were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). Anti-β-tubulin antibody was obtained from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Anti-Nestin antibody was from Sigma-Aldrich (St Louis, MO, USA). PDK1 inhibitor OSU-03012 was obtained from Selleck (Houston, TX, USA) and mTOR inhibitor Torin 1 was from Cayman Chemical (Ann Arbor, MI, USA).

2.2. Reagents

YKS is composed of seven dried crude drugs: Atractylodes lancea rhizome (4.0 g, rhizome of *Atractylodes chinensis* Koidzumi collected in Shaanxi province), poria sclerotium (4.0 g, sclerotium of *Wolfiporia cocos* Ryvarden et Gilbertson, collected in Yanbian Korean autonomous region, China), Cnidium rhizome (3.0 g, rhizome of *Cnidium officinale* Makino, collected in Hokkaido prefecture), Uncaria hook (3.0 g, thorn of *Uncaria rhynchophylla* Miquel, collected in Jianxi province), Japanese Angelica root (3.0 g, root of *Angelica acutiloba* Kitagawa, collected in Kyoto prefecture), Bupleurum root (2.0 g, root of *Bupleurum falcatum* Linné, collected in Sichuan province) and Glycyrrhiza (1.5 g, root and stolon of *Glycyrrhiza uralensis* Fisher, collected in Inner Mongolia). These crude drugs are registered in the Pharmacopeia of Japan ver. 17. They were identified and authenticated by Dr Yutaka Yamamoto (Tochimoto Tenkaido Co. Ltd., Osaka, Japan), and were purchased from Tochimoto Tenkaido Co. Ltd. The mixture of the seven herbs was extracted with 10 times distilled hot water (220 mL) at 95 °C for 1 h. After cooling, the extract solution was filtered and lyophilized to produce a dry YKS powder. Next, the YKS powder was dissolved with distilled water. This solution was used in the experiments after a 0.22-μm filtration sterilization.

2.3. Cell culture and differentiation of P19 cells

P19 cells were cultured in Minimum Essential Medium Eagle, Alpha Modification (α-MEM; Wako) containing 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA). Cells were cultured at 37 °C in a 5% CO_2 atmosphere and passaged at least twice before differentiation. Cell differentiation was performed essentially as described previously [8]. Briefly, in condition 1, P19 cells (1×10^6) were plated on bacterial ϕ10 cm dishes (Iwaki, Shizuoka, Japan) containing medium with 0.5 μM all-trans RA (Wako) for 4 days to form embryoid bodies [EBs; 0–4 days *in vitro* (Div)]. Cells were harvested by centrifugation at 200 × *g* for 5 min and trypsinized. Next, 1.5×10^5 cells were seeded on poly-L-lysine-coated 6-well plates (Nippon Genetics, Tokyo, Japan) in the absence of RA, with or without YKS and kinase inhibitors at 1–2 days (4–6 Div). In condition 2, P19 cells (1×10^6) were plated on bacterial ϕ10 cm dishes containing medium with 0.5 μM RA (Wako), with or without YKS for 4 days to form EBs (0–4 Div). Cells were harvested by centrifugation at 200 × *g* for 5 min and trypsinized. Next, 1.5×10^5 cells were seeded on poly-L-lysine-coated 6-well plates in the absence of RA at 1–2 days (4–6 Div). Cells were harvested and analyzed by western blotting (WB).

2.4. Western blot analysis

Cultured cells were washed with phosphate-buffered saline (PBS) and collected in sodium dodecyl sulfate (SDS) sample buffer. The protein concentration of the cell lysates was determined by the bicinchoninic acid (BCA) protein assay kit (Takara, Shiga, Japan), using bovine serum albumin (BSA) as a standard. Protein samples were separated on SDS polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; EMD Millipore, Burlington, MA, USA). Membranes were blocked with 0.5% skim milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) for 1 h at room temperature (RT). Then, membranes were probed consecutively with primary antibodies and secondary antibodies. Protein bands were visualized by chemiluminescence (Chemi-Lumi One Super, Nacalai Tesque, Kyoto, Japan) and LAS-4000 mini (FUJIFILM, Tokyo, Japan).

2.5. Immunocytochemistry

Immunocytochemistry was performed essentially as described previously [9]. Briefly, cell fixation was performed in 4% paraformaldehyde, followed by a PBS wash and treatment with 0.1% Triton X-100 in PBS for 5 min at RT. Cells were blocked by 1% BSA in PBS for 20 min at RT, and then incubated with Nestin antibodies (1:500; Sigma) or NeuN antibodies (1:100) for 2 h at RT, followed by Alexa Fluor 488-conjugated anti-rabbit IgG for 2 h at RT. Cell nuclei were stained with 4-6-diamidino-2-phenylindole (DAPI). Fluorescence images were observed using a BZ-X710 microscope (KEYENCE, Osaka, Japan). Quantification of fluorescence intensity was performed using Hybrid cell count BZ-H3C software (KEYENCE). Fluorescence intensity was measured per cell in DAPI-positive cells. Nestin-positive or NeuN-positive cells were divided by the number of DAPI-positive cells.

2.6. Antibody array analysis

Akt signaling was detected by the PathScan®Akt Signaling Antibody (CST). This array kit enables simultaneous detection of 16 phosphorylated proteins predominantly belonging to the Akt signaling network. The analysis was performed according to the manufacturer's protocol. In brief, P19 cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 1% Triton X-100, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) containing 1×protease inhibitor cocktail (Nacalai Tesque), 1×phosphatase inhibitor cocktail (Nacalai Tesque) and 1 mM phenylmethylsulfonyl fluoride. In total, 37.5 μg of lysate were added onto a slide coated with target-specific capture antibodies. HRP and LumiGLO reagent were used to visualize the bound antibodies by chemiluminescence. Chemiluminescence signals were detected by LAS-4000 mini and phosphorylation levels were quantified with Image J (National Institutes of Health, Bethesda, MD, USA).

2.7. Statistical analysis

Differences between two points were analyzed by paired student's *t* test. Multiple comparison analyses were conducted by one-way ANOVA (Tukey's test). Quantitative data were obtained from at least three independent experiments and expressed as mean ± SEM. $P < 0.05$ was considered statistically significant.

3. Results

3.1. YKS promotes the level of neuronal lineage markers in differentiated P19 cells

To investigate whether YKS affects neuronal differentiation, we used P19 cells that can differentiate into neuronal cells, which is induced by floating culture and RA stimulation. We used two conditions for YKS treatment. Fig. 1A shows a schematic illustration of the neuronal

differentiation protocol used. In condition 1, YKS was administered during the cell re-adhesion period (4–6 Div), which is the phase of neuronal differentiation. We assessed the effect of YKS on the expression level of NeuN, a mature neuron marker [10], and Nestin, a marker of neural stem cells [11]. The NeuN expression level significantly increased by approximately 1.4-fold after 10 μg/ml YKS treatment compared with the control cells (Fig. 1B and C). However, when we conducted NeuN immunostaining (data not shown) and counted the number of NeuN-positive cells, the number of positive cells was not altered by YKS (Fig. 1D). Moreover, the Nestin expression level significantly increased by approximately 1.7-fold upon YKS treatment as assessed by immunocytochemistry analysis (Fig. 1E and F), but YKS did not increase the number of Nestin-positive cells (Fig. 1G). These results indicated that YKS treatment increased the protein expression level of neural stem cell and neuron markers, but it did not enhance neuronal differentiation in P19 cells.

Next, we examined the effect of YKS treatment on cells in suspension culture (0–4 Div, condition 2). A flow chart of the experiment is presented in Fig. 2A. In this experiment, the NeuN expression was also upregulated by approximately 1.5-fold by 10 μg/ml YKS treatment (Fig. 2B and C). Moreover, immunostaining revealed that the expression of Nestin increased by approximately 1.9-fold by 10 μg/ml YKS treatment (Fig. 2D and E). These results indicated that YKS affected the NeuN and Nestin expression level. However, combining conditions 1 and 2 did not result in an additive or synergistic effect of YKS treatment (data not shown).

YKS stimulation increased the level of neuronal lineage markers in differentiated P19 cells in both condition 1 and 2; we used condition 1 hereafter.

3.2. The effect of YKS on signaling pathways in suspension cell culture

Next, we investigated the mechanism by which YKS promoted the level of neuronal lineage markers in P19 cells. Therefore, we examined

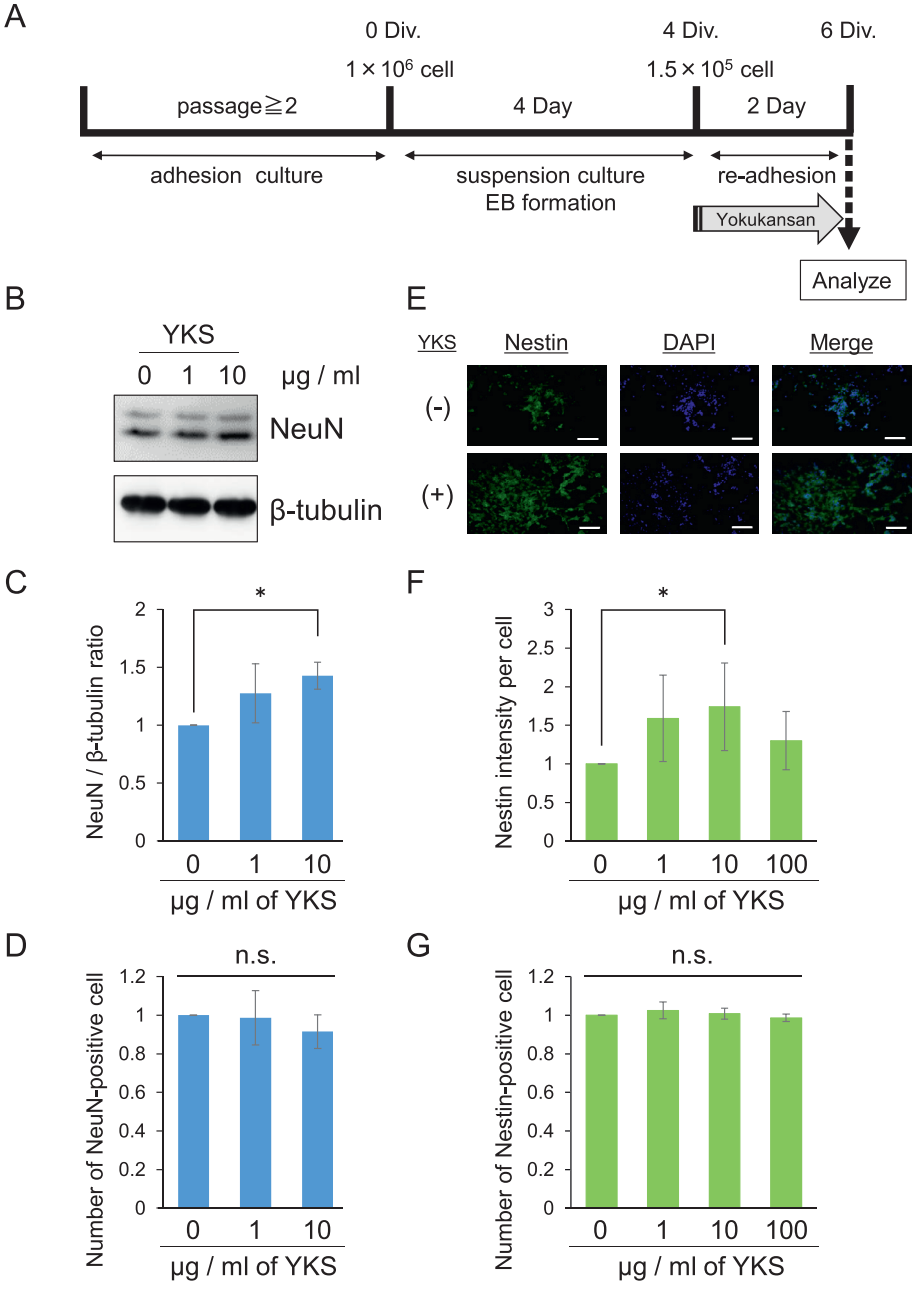

Fig. 1. The effect of YKS treatment during re-adhesion cell culture in P19 cells. (A) The experimental flowchart illustration showing YKS was administered during re-adhesion. (B) Western blot analysis of Neuronal nuclei (NeuN) and β-tubulin in P19 cells (Intact images are shown in Fig. S2A). (C) Western blot quantification of NeuN expression, normalized to β-tubulin in panel B. Data are expressed as mean ± SE (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$. (D) Quantification of NeuN-positive cells. Positive cells were divided by the number of DAPI-positive cells. Data are expressed as mean ± SE (n = 5). n.s.: not significant. (E) Representative immunofluorescence images of Nestin-positive cells (Green) and DAPI (Blue). Untreated cells (upper panel) and cells treated with 100 μg/ml YKS (lower panel) are shown. Scale bar: 100 μm. (F) Quantification of Nestin-positive cells intensity per DAPI-positive cells in (E). Data are expressed as mean ± SE (n = 5). $^{*}P < 0.05$. (G) Quantification of Nestin-positive cells. Positive cells were divided by the number of DAPI-positive cells. Data are expressed as mean ± SE (n = 5). n.s.: not significant.

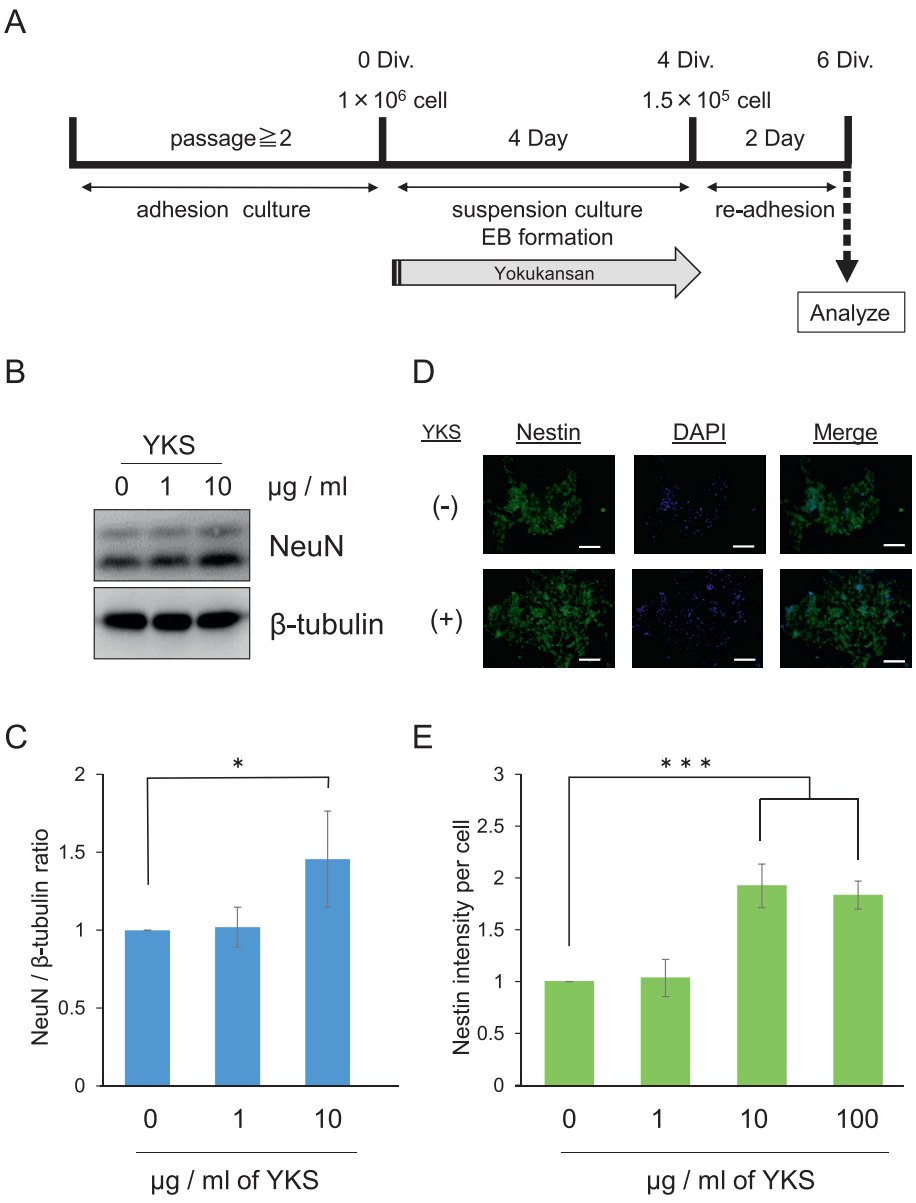

Fig. 2. The effect of YKS during suspension cell culture in P19 cells. (A) The experimental flowchart illustration showing YKS was administered during suspension. (B) Western blot analysis of NeuN and β-tubulin in P19 cells (Intact images are shown in Fig. S2B). (C) Western blot quantification of NeuN expression, normalized to β-tubulin in panel B. Data are expressed as mean ± SE (n = 4). $^{*}P < 0.05$, $^{**}P < 0.01$. (D) Representative immunofluorescence images of Nestin-positive cells (Green) and DAPI (Blue). Untreated cells (upper panel) and cells treated with 100 μg/ml YKS (lower panel) are shown. Scale bar: 100 μm. (E) Quantification of Nestin-positive cells intensity per DAPI-positive cells in (D). Data are expressed as mean ± SE (n = 5). $^{*}P < 0.05$, $^{***}P < 0.005$.

whether YKS affects intracellular signaling pathways. We investigated the phosphorylation level of PDK1, mTOR, GSK-3β and ERK1/2 in P19 cells under condition 1 by WB analysis (Fig. 3). There was a significant increase in phospho-PDK1 and phospho-mTOR(Ser-2448) expression (1.1-fold and 1.2-fold, respectively; Fig. 3A, C and E). However, there was no difference in phospho-GSK-3β (P-GSK-3β) in P19 cells (Fig. 3A and H). YKS also increased the level of phospho-ERK1/2, but this effect was not statistically significant (Fig. 3A and J). Therefore, we speculated that the mechanism by which YKS affects the level of neuronal lineage markers depends on the PDK1 and mTOR signaling pathways. Next, we examined whether inhibition of PDK1 and mTOR modulates the level of neuronal lineage markers in P19 cells. We used the selective inhibitors, OSU-03012 and Torin 1. P19 cells were treated with these inhibitors during the re-adhesion period in condition 1. The PDK1 inhibitor, OSU-03012, partially suppressed the YKS effect on the level of neuronal lineage markers by approximately 1.4-fold (Fig. 4A and B). Furthermore, the mTOR inhibitor, Torin 1, abolished the YKS effect as it significantly reduced the level of neuronal lineage markers by approximately 2-fold (Fig. 4A and B). Moreover, Torin 1 and OSU-03012 treatments without YKS significantly decreased the level of neuronal lineage markers, but only slightly (Fig. 4C and D). These results suggest that YKS enhances the level of neuronal lineage markers by activating the mTOR pathway.

3.3. *YKS activates multiple pathways during cell proliferation*

In the previous experiments, we found that YKS treatment induced the phosphorylation of several proteins in differentiated P19 cells. However, as the phosphorylation levels were somewhat weak, we performed the experiment under simple adhesion culture conditions with YKS stimulation without RA. This culture system is not suitable for neuronal differentiation, but we simply explored the effect of YKS on signaling pathways. Fig. 5A shows a scheme of the experimental protocol. We investigated the phosphorylation level of ERK1/2 and GSK-3β in P19 cells by WB analysis. No difference was found in total GSK-3β and phosphorylated GSK-3β (P-GSK-3β) levels in P19 cells under YKS treatment alone (Fig. 5B and C). However, the phosphorylated ERK1/2 level increased by approximately 1.7-fold, without change in the total ERK1/2 levels (Fig. 5B and D). The increased phosphorylation levels of ERK1/2 induced by YKS treatment lasted for 24 h in cell culture (data not shown).

To further identify the action point(s) of YKS, antibody array analysis

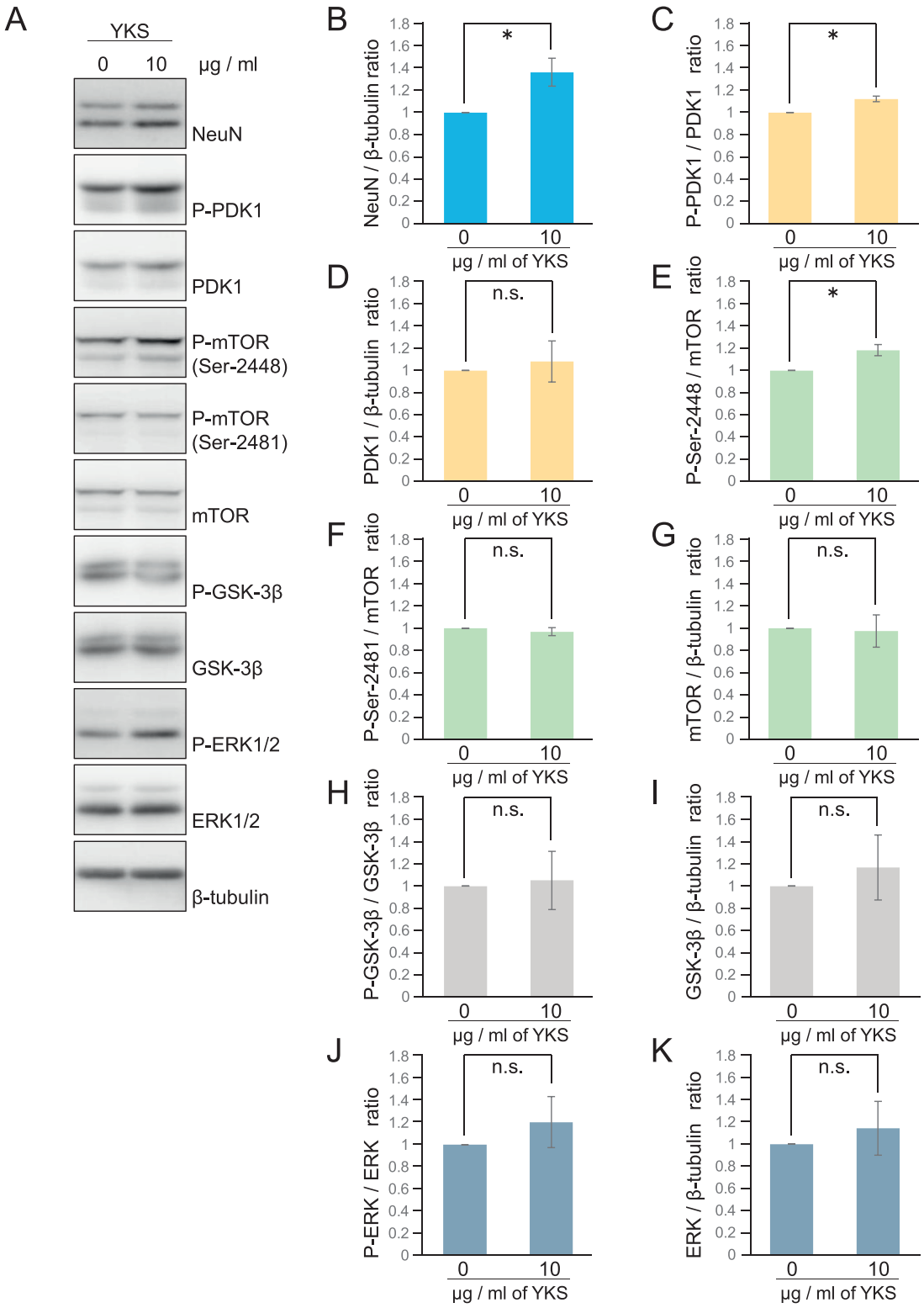

Fig. 3. The effect of YKS on signaling pathways in suspension cell culture. These experiments were conducted as in Fig. 1A. (A) Western blot analysis of NeuN and several signaling molecules in P19 cells (Intact images are shown in Fig. S2C). (B–K) Quantification of each WB signal intensity by Image J software. Data are expressed as mean ± SE (n = 3), $^*P < 0.05$.

simultaneously detecting 16 phosphorylated proteins was performed (Fig. S1A and B). As shown in Fig. 4E, YKS augmented 10 phosphorylated proteins among those tested with a 10%–40% increase, which included the signaling molecules, PDK1, Akt, AMPK, PRAS40, mTOR, p70 S6 kinase, GSK-3α, Bad and ERK1/2. These results suggest that YKS simultaneously activates multiple signaling pathways.

4. Discussion

To investigate the effect of YKS on neuronal differentiation, we evaluated the expression of proteins involved in neural stem cells and neurons. We found that YKS treatment in suspension culture for EB formation resulted in significantly increased levels of Nestin and NeuN

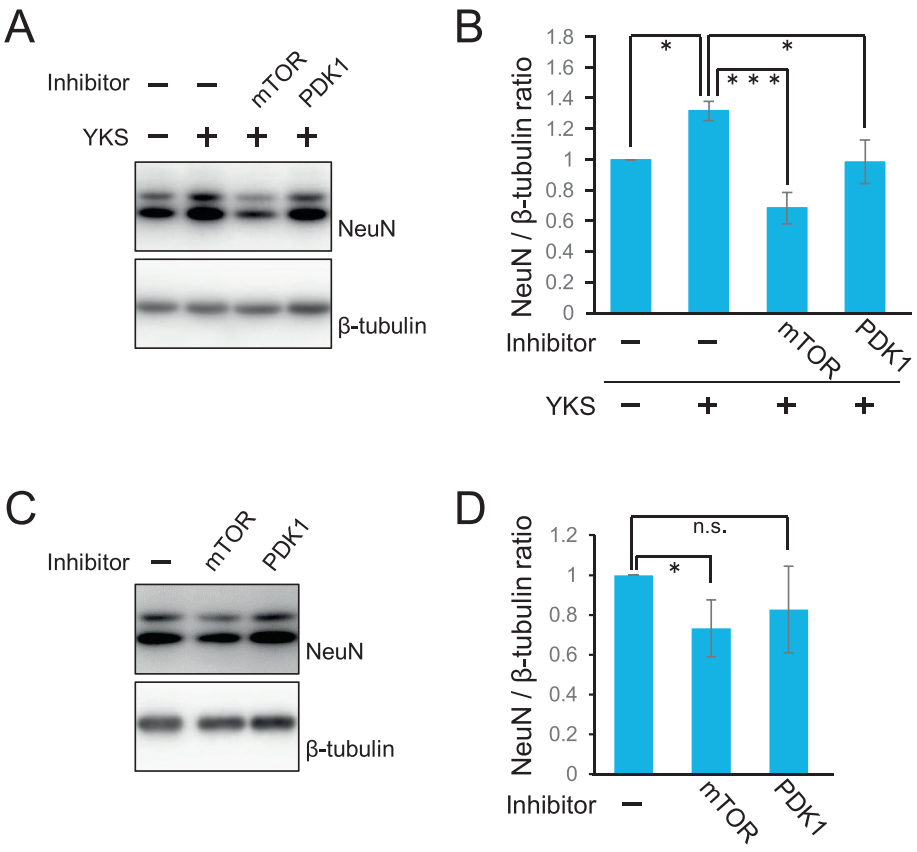

Fig. 4. The effect of Torin 1 and OSU-03012 during the differentiation period. (A–D) Effect of mTOR and PDK1 inhibitors (10 nM Torin 1 or 1 μM OSU-03012) during the re-adhesion period on cells treated with or without YKS [(4–6 days *in vitro* (Div)]. The NeuN and β-tubulin expression patterns are shown in panel A and C (Intact images are shown in Fig. S3A), and quantification of the NeuN/β-tubulin ratio is shown in panel B and D. Data are expressed as mean ± SE (n = 3). $^{*}P < 0.05$, $^{***}P < 0.005$. n.s.: not significant.

proteins with no change in the number of Nestin and NeuN-positive cells (Figs. 1 and 2). These results suggest that YKS did not promote differentiation into neurons through neural stem cells, but increased the level of neuronal lineage markers.

Nestin is an excellent marker for neural stem cells [11], which has been reported to be phosphorylated in adult neural stem/progenitor cells [12]. It has also been shown that Nestin phosphorylation is an important regulator of Nestin organization and dynamics during mitosis [13, 14]. It has been reported that Nestin plays a crucial role in the phosphorylation-dependent disassembly of vimentin intermediate during mitosis and that it is the target of cdk5 and p35 kinase during development [14, 15, 16]. In this study, to our surprise, YKS significantly increased the level of at least 10 phosphorylated proteins in P19 cells under proliferation conditions. We demonstrated that PDK1 and mTOR protein kinases, which are involved in neuronal cell differentiation [17, 18, 19, 20, 21], were significantly activated by YKS, though weakly. Thus, it is possible that Nestin is phosphorylated by protein kinases, such as PDK1 and/or mTOR.

Moreover, the *Nestin* gene is controlled by epigenetic modifications such as DNA methylation/demethylation and histone acetylation [22]. In P19 cells, histone acetylation has been reported to be sufficient to mediate *Nestin* transcription, but DNA methylation was not [22]. Thus, YKS may interact with histone modification protein(s) such as histone deacetylase(s), and thereby control *Nestin* transcription.

The various effects of YKS on neurons has been reviewed recently [2]. We observed that YKS simultaneously augmented the level of 10 phosphorylated proteins including ERK1/2, AMPK, mTOR and PDK1 (Figs. 3 and 5E). In support of our observations, several studies have reported that YKS altered intracellular signaling. YKS activated ERK and phosphoinositide 3-kinase (PI3K)/Akt, and then promoted neurite outgrowth in PC12 cells [23, 24]. YKS-dependent activation of Akt signaling has also been reported in SH-SY5Y cells and in a mouse model of Alzheimer's disease [25, 26]. Thus, YKS activates several signaling molecules in various cell types. We also demonstrated that PDK1 and mTOR protein kinases were significantly activated by YKS. Consistently, we revealed that PDK1 and mTOR inhibitors diminished the YKS effect partially or completely, respectively (Fig. 4A and B). Therefore, our data suggest that YKS regulates the level of neuronal lineage markers via mTOR activation, however this is not enough to induce neuronal differentiation. Our observations are inconsistent with studies that showed that PDK1 and mTOR are involved in neuronal cell differentiation [17, 18, 19, 20, 21]. However, the upregulation mechanism of phosphorylated mTOR by YKS is a critical issue to be explored. mTOR kinase works in the complexes, mTORC1 and mTORC2 [27,28]. In our study, we did not show that YKS activated either or both of these complexes. Previous studies have shown that mTORC1 plays an important role in neuronal differentiation, which was associated with neuronal lineage markers expression [29, 30], and phosphorylation of its substrate Eukaryotic Translation Initiation Factor 4E Binding Protein 2 (4E-BP2) is the key event of the signaling [30]. However, because YKS stimulation did not increase 4E-BP1 phosphorylation (Fig. 5E), YKS may not activate both 4E-BP1 and 4E-BP2. Thus, insufficient activation of mTORC1 and 4E-BP1/2 by YKS may not enable full induction of neuronal differentiation. Nevertheless, YKS may activate the mTORC2 complex, because Ser-473 of Akt is an mTORC2 target and its phosphorylation level was slightly, though not significantly, elevated by YKS (Fig. 5E). Furthermore, the phosphorylation of the PDK1 target, Akt (Thr-308), was significantly increased by YKS (Fig. 5E). As these two phosphorylation sites are essential for complete activation of Akt [27], it appears that YKS affects Akt signaling.

YKS consists of seven kinds of crude drugs, of which β-Eudesmol derived from Atractylodes lancea rhizome has been suggested to be involved in neurite outgrowth in PC12 cells [31]. Saikosaponin D derived from Bupleurum root has been implicated in autophagy in neurons [32, 33]. Thus, YKS affects several neuronal functions, however, which of its components is responsible for upregulating the level of neuronal lineage markers is unknown [2, 4]. As YKS is a mixture of natural compounds at

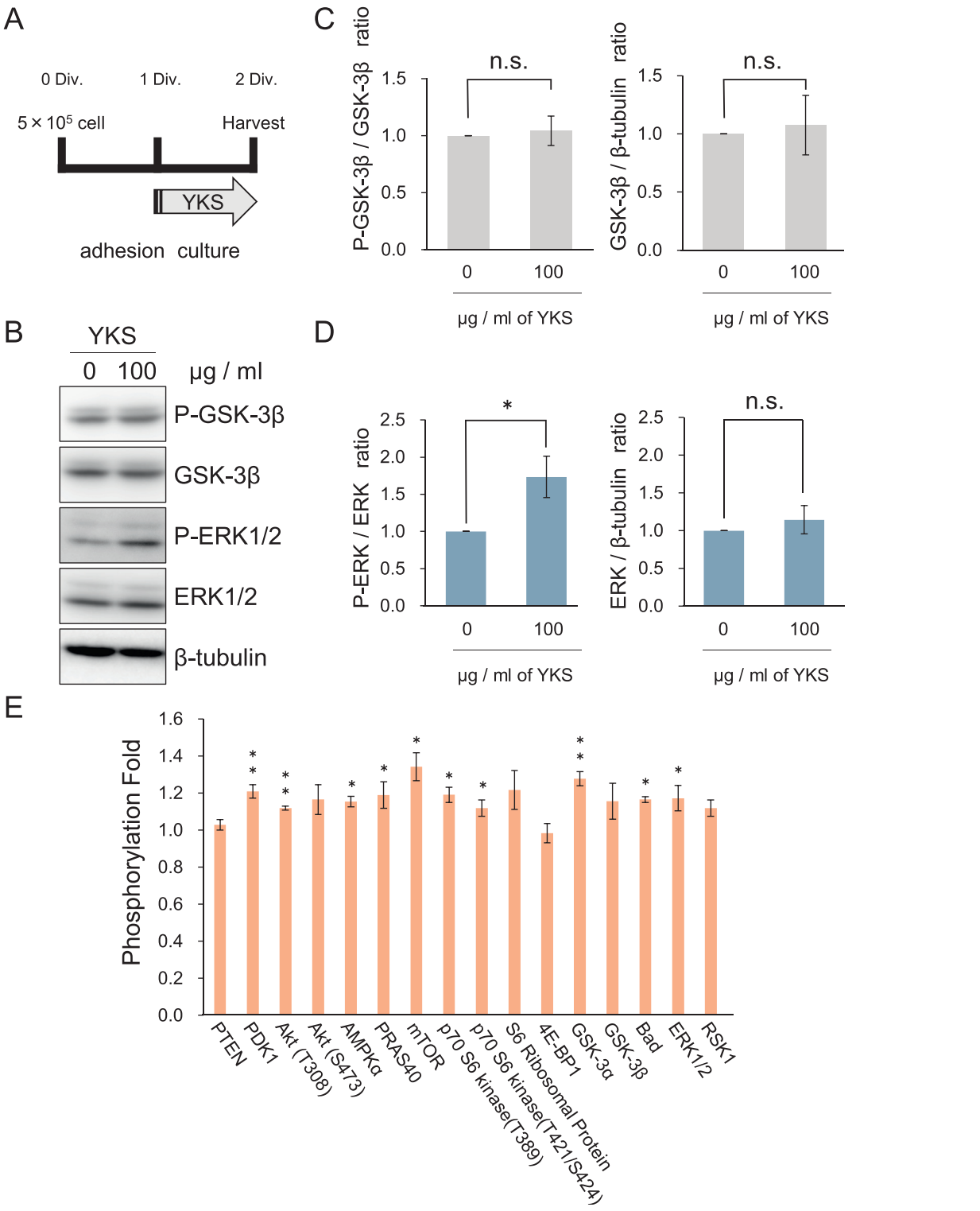

Fig. 5. The effect of YKS on multiple intracellular signaling pathways. (A) Illustration of the experimental protocol of adhesion cell culture. (B) Western blot analysis of phosphorylated GSK-3β (P-GSK-3β), GSK-3β, phosphorylated ERK1/2 (P-ERK1/2) and ERK1/2. β-Tubulin was used as a loading control (Intact images are shown in Fig. S3B). (C) Quantification of P-GSK-3β/GSK-3β and GSK-3β/β-tubulin ratios. Data are expressed as mean ± SE (n = 3). n.s.: not significant. (D) Quantification of P-

various concentrations, it may be hard to identify the exact point of action of YKS. The observed effects may be due to various points of action of YKS. Therefore, future studies are required to identify the active compound(s) in YKS that affect the level of neuronal lineage markers in differentiated P19 cells and neuronal differentiation and clarify the molecular mechanism, especially at the signal transduction level.

Declarations

Author contribution statement

Makoto Fukui, Syouichi Katayama: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the

data; Wrote the paper.

Yukinobu Ikeya: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Tetsuya Inazu: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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