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ROCK2 regulates bFGF-induced proliferation of SH-SY5Y cells through GSK-3β and β-catenin pathway

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Abstract

Increased neurogenesis by promoting proliferation of neural precursor cells in the adult dentate gyrus might be beneficial for the treatment of psychiatric disorders. Results demonstrate that bFGF is necessary for the proliferation of neural precursor cells and that the glycogen synthase kinase-3β (GSK-3β) and β-catenin pathway plays a role in it. However, the detailed mechanism of proliferation of neural precursor cells remains unclear. To elucidate that mechanism, we investigated the role of Rho-associated coiled-coil kinase (ROCK) in bFGF-induced proliferation using SH-SY5Y cells as a model of neural precursor-like cells. Y27632, a specific inhibitor of ROCK, decreased bFGF-induced proliferation. Lithium (Li), an inhibitor of GSK-3β, recovered Y27632-decreased proliferation and quercetin (Que), an inhibitor of β-catenin pathway, reversed the recovery effect of Li. Both nuclear β-catenin and cyclin D1 expression were altered by bFGF, Y27632, Li, and Que in parallel with the case of proliferation. Furthermore, bFGF inactivated GSK-3β through increasing the phosphorylation of Ser^{9} on GSK-3β, which is reversed by Y27632 through increased phosphorylation of Tyr^{216} on GSK-3β. ROCK has two subtypes: ROCK1 and ROCK2. Investigation with siRNA for ROCKs showed that ROCK2 is involved in bFGF-induced proliferation, but not ROCK1. These results suggest that ROCK2 might mediate bFGF-induced proliferation of SH-SY5Y cells through GSK-3β and β-catenin pathway. Further investigation of detailed mechanisms regulating the ROCK2/GSK-3β/β-catenin pathway might engender the development of new therapeutic targets of psychiatric disorders.

Keywords: ROCK, bFGF, GSK-3β, β−catein, Y27632, SH-SY5Y cell

Abbreviations: DG, dentate gyrus; bFGF; basic fibroblast growth factor; GSK-3β, glycogen synthase kinase-3β; ROCK, Rho-associated coiled-coil kinase; Li,lithium; Que; quercetin; siRNA, short interfereing RNA

1. Introduction

Neurogenesis occurs in the dentate gyrus (DG) of the adult human brain (Eriksson et al, 1998). Neurogenesis in DG is decreased in adult rodent models for stress-related disorders (Gould et al, 1997; Malberg et al, 2003; Pham et al, 2003). The administration of drugs commonly used in treatments of stress-related disorders increase neurogenesis in DG of adult rodents (Chen et al, 2000; Malberg et al, 2000; Hao et al, 2004). These results of studies suggest that increasing neurogenesis might be beneficial for the treatment of stress-related disorders and that the development of ways to increase neurogenesis in adult DG might contribute to the identification of new therapeutic targets for stress-related disorders.

One means to increase neurogenesis is to promote the proliferation of neural precursor cells. Some growth factors are involved in regulating proliferation of neural precursor cells (Haag, 2007). Especially, basic fibroblast growth factor (bFGF) is regarded as playing a pivotal role in the proliferation of neural precursor cells (Vescovi et al, 1993; Tao et al, 1997). bFGF binding to FGF receptors activates multiple signal transduction pathways, including glycogen synthase kinase-3β

(GSK-3β) and β-catenin pathway (Dailey et al, 2005). β-catenin pathway, also known as canonical Wnt pathway or β-catenin/TCF pathway, mediates cell proliferation by regulating cyclin D1 expression (Shtutman et al, 1999; Tetsu et al, 1999). In addition, recent studies including ours have shown that GSK-3β and β-catenin pathway are involved in the proliferation of neural precursor cells in both embryo and adult brains (Lie et al, 2005; Adachi et al, 2007; Hirsch et al, 2007; Boku et al, 2009). These results of studies suggest that bFGF might regulate proliferation of neural precursor cells through GSK-3β and β-catenin pathway. GSK-3β and β-catenin pathway might be involved in bFGF-induced proliferation of tumor-derived cells (Holnthoner et al, 2002; Cohen et al, 2007; Pai et al, 2008). However, it remains unclear how bFGF regulates proliferation of neural precursor cells.

Rho-associated coiled-coil kinases 1 and 2 (ROCK1 and ROCK2) are serine/threonine kinases and work as an effector of RhoA, a small GTPase (Ishizaki et al, 1996; Matsui et al, 1996; Nakagawa et al, 1996). RhoA/ROCK pathway has been shown to work multifunctionally in various cell behaviors (Riento and Ridley, 2003). Especially, RhoA/ROCK pathway plays a pivotal role in the regulation of the actin cytoskeleton (Amano et al, 1996; Kimura et al, 1996; Maekawa et al, 1999). ROCK is also involved in cell proliferation by increasing the expression of cyclin D1, a common cell cycle regulator and a transcriptional target of β-catenin pathway (Iwamoto et al, 2000; Croft and Olson, 2006; Chen et al, 2008). However, it remains unclear whether ROCK is involved in proliferation of neural precursor cells and how ROCK activates β-catenin pathway. Moreover, little is known about the functional difference between ROCK1 and ROCK2.

Here we investigated the involvement of ROCK, GSK-3β, and β-catenin pathway in bFGF-induced cell proliferation, using human neuroblastoma-derived SH-SY5Y cells as a model of neural precursor-like cells.

2. Results

2.1 bFGF increases cell proliferation

bFGF has been shown to increase proliferation of neural precursor cells in various culture systems (Murphy et al, 1990; Ray et al, 2006; Babu et al, 2007; Boku et al, 2009). Therefore, we first examined, using Alamar Blue assay, whether bFGF increases proliferation of SH-SY5Y cells as in the case of *in vitro* culture systems of neural precursor cells previously reported. Alamar Blue assay is a rapid and simple non-radioactive assay to measure the number of cells. Alamar Blue dye is a fluorogenic redox indicator that is converted from the oxidized form to the reduced form in cells. The reduced form of Alamar Blue dye is highly fluorescent. Its fluorescence reflects the number of cells (Ahmed et al, 1994). Results show that bFGF increases the number of cells in a dose-dependent manner in proliferation medium (Fig. 1A). Therefore, bFGF increases cell proliferation, as in the cases of *in vitro* culture systems of neural precursor cells. Reportedly, SH-SY5Y cells have both proliferation and differentiation potency (Encinas et al, 2000). Therefore, for this study, we used SH-SY5Y cells as a model of neural precursor-like cells for investigating the detailed mechanisms of bFGF-induced proliferation of neural precursor cells.

2.2 Y27632 decreases bFGF-induced cell proliferation

Y27632 is a specific inhibitor of ROCK (Uehata et al, 1997). To investigate whether ROCK is involved in proliferation of SH-SY5Y cells, we examined the effect of Y27632 on cell proliferation with Alamar Blue assay. Results showed that Y27632 decreased the number of cells in a dose-dependent manner in the presence of 20 ng/ml bFGF (Fig. 1B). However, Y27632 had no effect on cell proliferation in the absence of 20 ng/ml bFGF (Fig. 1B). A decrease of the number of cells in Alamar Blue assay can mean not only decreased cell proliferation, but also accelerated apoptosis and induced neuronal differentiation. We performed dUTP-nick end labeling (TUNEL) assay to test the possibility of accelerated apoptosis and immunocytochemistry with anti-Tuj1 antibody to test the possibility of induced neuronal differentiation. No TUNEL positive cells and Tuj-1 positive cells were visible (data not shown). Taken together, these results show that Y27632 decreases bFGF-induced proliferation of SH-SY5Y cells.

2.3 Lithium recovers the inhibitory effects of Y27632 on bFGF-induced cell proliferation

Lithium (Li), commonly used as a mood stabilizer, is a specific inhibitor of GSK-3β (Klein and Melton, 1996; Stambolic et al, 1996). To investigate whether ROCK regulates bFGF-induced cell proliferation through GSK-3β, we examined the effects of Li on the inhibitory effects of Y27632 on proliferation of SH-SY5Y cells with Alamar Blue assay. Li recovered cell proliferation decreased by Y27632 in the presence of 20 ng/ml bFGF (Fig. 1C). However, Li had no effect on cell proliferation without Y27632 in the presence of 20 ng/ml bFGF (data not shown). Consequently, these results show that ROCK is involved in bFGF-induced proliferation of SH-SY5Y cells through GSK-3β.

2.4 Quercetin decreases bFGF-induced cell proliferation

GSK-3β works as a negative regulator of the β-catenin pathway (Aberle et al, 1997; Orford et al, 1997). Therefore, Li is a stimulator of the β-catenin pathway. We have shown that β-catenin pathway is involved in bFGF-dependent proliferation of adult DG-derived neural precursor cells (Boku et al, 2009). To investigate whether β-catenin pathway is also involved in bFGF-induced proliferation of SH-SY5Y cells, we examined the effects of quercetin (Que), an inhibitor of β-catenin pathway (Park et al, 2005), on cell proliferation with Alamar Blue assay. Results showed that Que decreased cell proliferation in a dose-dependent manner in the presence of 20 ng/ml bFGF (Fig. 2A). However, Que had no effect on cell proliferation in the absence of bFGF (Fig. 2A). Therefore, β-catenin pathway might be involved in bFGF-induced proliferation of SH-SY5Y cells. In contrast, Li had no effect on cell proliferation decreased by Que in the presence of 20 ng/ml bFGF (Fig. 2B). Furthermore, Li had no effect on cell proliferation without both Y27632 and Que in the presence of 20 ng/ml bFGF (data not shown). In addition, Que abolished the recovery effect of Li on cell proliferation decreased by Y27632 (Fig. 2C), but had no effect on it without Li (data not shown). Taken together, these results suggest that GSK-3β and β-catenin pathway might be involved in bFGF-induced proliferation of SH-SY5Y cells and that ROCK might regulate GSK-3β.

2.5 Effects of bFGF, Y27632, Li and Que on nuclear β-catenin

β-catenin is translocated from cytosol to the nucleus, where it promotes the transcription of its target genes (Salic et al, 2000). We measured the effects of bFGF, Y27632, Li, and Que on nuclear β-catenin. Results show that bFGF increased nuclear β-catenin, Y27632 decreased nuclear

β-catenin increased by bFGF, Li recovered nuclear β-catenin decreased by Y27632, and Que abolished the recovery effect of Li (Fig. 3). Consequently, nuclear translocation of β-catenin is confirmed as altered in parallel with cell proliferation

2.6 Effects of bFGF, Y27632, Li and Que on cyclin D1 expression

To confirm the involvement of ROCK/GSK-3β/β-catenin pathway in bFGF-induced proliferation of SH-SY5Y cells, we investigated the effects of bFGF, Y27632, Li, and Que on the expression of cyclin D1, a transcriptional target of β-catenin pathway and a common cell cycle regulator, with quantitative RT-PCR and Western blotting. Results show that bFGF increased cyclin D1 expression, Y27632 decreased cyclin D1 expression increased by bFGF, Li recovered cyclin D1 expression decreased by Y27632, and Que abolished the recovery effect of Li in both mRNA (Fig. 4A) and protein (Fig. 4B) levels. These results indicate that cyclin D1 expression is altered in parallel with proliferation of SH-SY5Y cells. Therefore, ROCK, GSK-3β and β-catenin pathway might be involved in bFGF-induced proliferation of SH-SY5Y cells.

2.7 Both bFGF and Y27632 alter the phosphorylation states of GSK-3β

The results described above suggest that bFGF and Y27632 affects the activity of GSK-3β. The activity of GSK-3β is regulated by two phosphorylated residues: Ser^{9} to render it inactive (Cross et al, 1995) and Tyr^{216} to render it active (Hughes et al, 1993). Therefore, we investigated the effects of bFGF and Y27632 on the phosphorylation of these residues on GSK-3β with Western Blotting. bFGF increased the phosphorylation of Ser^{9}, but had no effect on that of Tyr^{216} (Fig. 5). However, Y27632 increased the phosphorylation of Tyr^{216}, but had no effect on that of Ser^{9} either in the

presence or absence of bFGF (Fig. 5). Li had no effect on either the phosphorylation of Ser^{9} or that of Tyr^{216} (data not shown). These results demonstrate that bFGF might inhibit GSK-3β through increased phosphorylation of Ser^{9} and that Y27632 might activate GSK-3β through increasing phosphorylation of Tyr^{216}.

2.8 ROCK2, but not ROCK1, is involved in bFGF-induced cell proliferation

Y27632 can inhibit both ROCK1 and ROCK2. To determine which of ROCK is involved in bFGF-induced proliferation of SH-SY5Y cells, we examined mRNA expression of cyclin D1 with small interfering RNAs (siRNAs), which can selectively knock down ROCK1 or ROCK2 mRNA expression. To rule out the possibility of the off-target effect, two independent siRNA was used for each ROCK. Both of two siRNAs for ROCK1 equally decreased mRNA expression of ROCK1, but not that of ROCK2 (Fig. 6A). In contrast, both of two siRNAs for ROCK2 equally decreased mRNA expression of ROCK2, but not ROCK1 (Fig. 6B). These results suggest that the effects of siRNAs we used are considered to be the specific effects on ROCK1 and 2 expressions, not the off-target effects. Therefore, one of two independent siRNAs was used for the knockdown of ROCK1 and 2 for investigating the effects of these siRNAs on mRNA expression of cyclin D1. The results showed that siRNA for ROCK2, but not that for ROCK1, decreased mRNA expression of cyclin D1 (Fig. 6C). Therefore, ROCK2, but not ROCK1, might be involved in bFGF-induced proliferation of SH-SY5Y cells.

3. Discussion

For this study, we used SH-SY5Y cells derived from human neuroblastoma as a model of neural precursor-like cells. The definitions of neural precursor cells are self-renewal and differentiation potency. SH-SY5Y cells have self-renewal activity and can differentiate into neurons (Påhlman et al, 1990). Moreover, bFGF increases proliferation of SH-SY5Y cells in a serum-free condition, as in the case of *in vitro* culture of neural precursor cells (Murphy et al, 1990; Ray et al, 2006; Babu et al, 2007; Boku et al, 2009). We have already shown that dexamethasone, a specific agonist of glucocorticoid receptor decreases proliferation of adult DG-derive neural precurosor cells and that lithium recovers dexamethasone-induced decrease of it (Boku et al, 2009). We also have shown that both dexamethasone and lithium have the same effects on proliferation of SH-SY5Y cells in our culture condition as in the case of adult DG-derived neural precursor cells (our unpublished data). Consequently, SH-SY5Y cells may fulfill the definitions of neural precursor cells and their proliferation may be similarly affected by bFGF, lithium and dexamethasone as in the case of proliferation of adult DG-derived neural precursor cells. Therefore, it might be reasonable to use SH-SY5Y cells as a model for neural precursor-like cells at least for the investigation of the mechanism of their proliferation.

ROCK, which is involved in some functions of the central nervous system, regulates axon outgrowth and growth cone dynamics (Arimura et al, 2000; Bito et al, 2000). It is involved in ischemic brain damage (Toshima et al, 2000), and it plays a role in long-term potentiation in CA1 region of hippocampus (ÓKane et al, 2004), and long-term spatial memory (Dash et al, 2004).

However, no report in the relevant literature has indicated the possibility that ROCK might be involved in neurogenesis. Consequently, our study is the first study of the role of ROCK in proliferation of neural precursor-like cells.

The mRNA expression of cyclin D1 is regulated by β-catenin pathway in tumor cell lines (Shtutman et al, 1999; Tetsu et al, 1999). β-catenin pathway is involved in regulating the proliferation of neural precursor cells both in embryo and adult brain (Lie et al, 2005; Adachi et al, 2007; Hirsch et al, 2007; Boku et al, 2009). Therefore, we hypothesized that ROCK regulates bFGF-induced proliferation of SH-SY5Y cells through β-catenin pathway. GSK-3β is a negative regulator of β-catenin pathway (Aberle et al, 1997; Orford et al, 1997). Li is an inhibitor of GSK-3β (Klein and Melton, 1996; Stambolic et al, 1996). In addition, Que functions as an inhibitor of β-catenin pathway through inhibition of the translocation of β-catenin from the cytosol to nucleus (Park et al, 2005). Consequently, using Li and Que, we investigated whether ROCK regulates bFGF-induced proliferation of SH-SY5Y cells through GSK-3β and the β-catenin pathway with Alamar Blue assay, quantitative RT-PCR and Western blotting. Li recovered Y27632-induced decrease of bFGF-induced proliferation of SH-SY5Y cells. In parallel, Li also recovered Y27632-induced decrease of the expression of cyclin D1 in both mRNA and protein levels, and Que reversed these recovery effects of Li. Moreover, bFGF increased the phosphorylation of Ser^{9}, which inactivates GSK-3β and which had no effect on that of Tyr^{216}, which activates GSK-3β. However, Y27632 had no effect on the phosphorylation of Ser^{9} and increased that of Tyr^{216}. A recent report has described that GSK-3β is rather activated when both Ser^{9} and Tyr^{216} are phosphorylated (Noël

et al, 2011). Taken together, these results suggest that bFGF might increase proliferation of SH-SY5Y cells through the inactivation of GSK-3β and following upregulation of β-catenin pathway and that ROCK2 might mediate bFGF-induced proliferation of SH-SY5Y cells through the activation of GSK-3β and following downregulation of the β-catenin pathway (Fig. 7). However, our results leave an important question unanswered: how does ROCK affect the phosphorylation states of GSK-3β ? In the following studies, we will answer this question.

β-catenin pathway is also well known as the canonical Wnt pathway. In addition to the canonical Wnt pathway, two non-canonical Wnt pathways exist: the planar cell polarity (PCP) pathway (Fanto and McNeill, 2004) and the calcium pathway (Slusarski and Pelegri, 2007). Especially, ROCK has been shown to mediate the PCP pathway (Marlow et al, 2002). However, both pathways are independent of β-catenin. Therefore, these pathways might be independent of bFGF-induced proliferation of SH-SY5Y cells.

Because Y27632 can inhibit both ROCK1 and ROCK2 (Ishizaki et al, 2000), investigations with Y27632 can not elucidate which of ROCK is involved in bFGF-induced proliferation of SH-SY5Y cells. Indeed, no specific inhibitor of ROCK can distinguish a subtype of ROCK, so that the functional difference between ROCK1 and ROCK2 is poorly understood. Results show that ROCK2, but not ROCK1, is involved in bFGF-induced proliferation of SH-SY5Y cells with siRNA: ROCK1 is expressed ubiquitously, but ROCK2 is expressed mainly in the brain, muscle, heart, lung, and placenta (Nakagawa et al, 1996). Moreover, ROCK2 is expressed abundantly in the cerebral cortex and hippocampus (Hashimoto et al, 1999). It is particularly interesting that DG, in

the hippocampus, is one region in which adult neurogenesis occurs (Eriksson et al, 1998). Therefore, our results and these results of prior studies suggest that ROCK2 regulates neurogenesis in adult DG. To confirm that implication, additional investigation using adult rodents and neural precursor cells derived from adult DG is needed. We have already established the culture system of adult rat DG-derived neural precursor cells (Boku et al, 2009). We are only beginning to investigate the role of ROCK2 in proliferation of adult rat DG-derived neural precursor cells.

Our report has described that ROCK2 regulates bFGF-induced proliferation of SH-SY5Y cells through GSK-3β and b-catenin pathway. This study is the first reported of the involvement of ROCK2 in GSK-3β and β-catenin pathway. Nevertheless, it remains unclear how ROCK2 regulates GSK-3β and β-catenin pathway and whether ROCK2 is actually involved in adult hippocampal neurogenesis and behaviors. The answers to these questions might engender better understanding of the molecular mechanism of adult neurogenesis and the development of new therapeutic targets of psychiatric disorders.

4. Experimental procedures

4.1 ***Cell culture*** SH-SY5Y cells (ATCC) were maintained in DMEM/F-12 Ham (Sigma, St. Louis, MO) with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA) and 100 μg/ml penicillin–streptomycin (Invitrogen Corp.) (maintenance medium). Treatments were performed in Neurobasal (Invitrogen Corp.), B27 (Invitrogen Corp.), 1 mM L-glutamine (Invitrogen Corp.), and 100 μg/ml penicillin–streptomycin (proliferation medium) with or without bFGF (Invitrogen

Corp.).

4.2 ***Cell counting*** 1×10^4 cells/well were put on 96-well plates in maintenance medium. After overnight incubation, cells were treated with bFGF, Y27632 (Calbiochem, San Diego, CA), lithium chloride (Li) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and/or quercetin (Que) (Calbiochem) at indicated concentrations in proliferation medium for 2 days. Then, 10 μl/well of Alamar Blue solution (Invitrogen Corp.) was added to medium, and cells were incubated at 37°C for 3 hr. The fluorescence intensity was measured using Fluoroscan (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) and calculated as described in the manufacturer's manual. Statistical analysis was performed using one-way ANOVA and Bonferroni's post hoc test. Significance was defined as $p < 0.01$ or 0.05. Data are expressed as means ± SEM.

4.3 ***RNA Extraction and quantitative RT-PCR*** 2×10^5 cells/well were put on 6-well plates in maintenance medium. After overnight incubation, cells were treated with or without 20 ng/ml bFGF, 30 μM Y27632, 3 mM Li, and 30 μM Que in proliferation medium for 2 days. Total RNA was extracted from cells with RNeasy extraction kit (Qiagen Inc., Hilden, Germany) and converted to cDNA with Quantitect Reverse Transcription kit (Qiagen Inc.). PCR was performed with SYBR GreenER qPCR SuperMix (Invitrogen Corp.) in ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster, CA). The conditions of PCR were: 50°C for 2 min and 95°C for 10 min with subsequent 40 cycles of 95°C for 15 s, and 60°C for 1 min. The sequences of forward and reverse primers used were: GGT GCA TGG CCG TTC TTA and TCG TTC GTT ATC GGA ATT AAC C for 18s rRNA, AGA ACA CGG CTC ACG CTT AC and

CAG ACA AAG CGT CCC TCA AG for cyclin D1, GCT GAT GAA ACT GAT GGT AAC CT and TTG CTG CTT ACC ACA ACA TAC TG for ROCK1, ACA GCT TGC CCC AAA CAA and TGG AAG AAT ACG ATC ACC TTG A for ROCK2. Also, 18s rRNA was used as a control. The results were analyzed using software (SDS 2.0; Applied Biosystems). Statistical analysis was performed by one-way ANOVA and Bonferroni's post hoc test. Significance was defined as $p < 0.05$. Data are expressed as the means ± SEM.

4.4 ***Western Blotting*** 2×10^5 cells/well were put on 6-well plates in maintenance medium. After overnight incubation, cells were treated with or without 20 ng/ml bFGF, 30 μM Y27632, 3 mM Li, and 30 μM Que in proliferation medium for 2 days. Preparation of total proteins was performed with Mammalian Cell Lysis Kit (Sigma). Preparation of nuclear proteins was performed with Nuclear Extract Kit (Active Motif). Protein concentration was measured using Protein Assay Kit (Pierce Biotechnology Inc., Rockford, IL), and equal amount of proteins (20 μl/well) were loaded onto a 10% SDS gel. The gel was transferred onto a nitrocellulose membrane (GE Healthcare, Milwaukee, WI) and incubated with primary antibodies, which were used at the following concentrations: mouse anti-cyclin D1 antibody (1:200; Santa Cruz Biotechnology, San Diego, CA), mouse anti-β-catenin antibody (1:200; Santa Cruz Biotechnology), rabbit anti-GSK-3β antibody (1:1000; Cell Signaling, Danvers, MA), rabbit anti-pGSK-3β (Ser^9) antibody (1:1000, Cell Signaling), rabbit anti-pGSK-3β (Tyr^{216}) antibody (1:500; Biosource, Camarillo, CA), mouse anti-GAPDH antibody (1:200; Santa Cruz Biotechnology). After washing, the membrane was

incubated with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit IgG antibody (1:10000; GE Healthcare). Protein expression was detected using Amersham ECL Plus Western Blotting Detection System (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare). The images were converted to digital files. Then the intensity of a band was analyzed using Image J (National Institutes of Health). Statistical analysis was performed by one-way ANOVA and Bonferroni's post hoc test. Significance was defined as $p < 0.01$ or 0.05. Data are expressed as means ± SEM.

4.5 ***RNA interference*** Double-strand, short interfering RNA (siRNA) of ROCK1 and ROCK2 were purchased from Invitrogen. 2×10^5 cells/well were put on six-well plates in proliferation medium with 5 ng/ml bFGF. After overnight incubation, transfection of siRNA was performed using Lipofectamine RNAi MAX (Invitrogen Corp.). Stealth RNAi negative control (Invitrogen Corp.) was used as a control. Cells were lysed 2 days after transfection. Then quantitative RT-PCR was performed as described above.

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Figure legends

FIGURE 1. **Effects of bFGF, Y27632 and Li on cell proliferation.**

A. bFGF increases proliferation of SH-SY5Y cells in a dose-dependent manner. Alamar Blue assay was performed 2 days after 20 ng/ml bFGF-treatment. Data are shown as means ± SEM of four independent cultures. * $p < 0.01$.

B. Y27632 decreased 20 ng/ml bFGF-increased proliferation of SH-SY5Y cells in a dose-dependent manner. Alamar Blue assay was performed 2 days after Y27632-treatment. Data are shown as the means ± SEM of four independent cultures. * $p < 0.01$.

C. Li reversed the inhibitory effect of 30 μM Y27632 on 20 ng/ml bFGF-increased proliferation of SH-SY5Y cells in a dose-dependent manner. Alamar Blue assay was performed 2 days after treatment. Data are shown as means ± SEM of four independent cultures. * $p < 0.01$.

FIGURE 2. **Effects of Que on cell proliferation.**

A. Que decreased 20 ng/ml bFGF-increased proliferation of SH-SY5Y cells in a dose-dependent manner. Alamar Blue assay was performed 2 days after Que-treatment. Data are shown as the means ± SEM of four independent

cultures. * $p < 0.01$.

B. Li had no effect on the inhibitory effect of 30 μM Que on 20 ng/ml bFGF-increased proliferation of SH-SY5Y cells in a dose-dependent manner. Alamar Blue assay was performed 2 days after treatment. Data are shown as means ± SEM of four independent cultures.

C. Que abolished the recovery effect of 3 mM Li on 30 μM Y27632-decreased proliferation of SH-SY5Y cells. Alamar Blue assay was performed 2 days after Li-treatment. Data are shown as means ± SEM of four independent cultures. # $p < 0.05$.

FIGURE 3. **Effects of bFGF, Y27632, Li and Que on nuclear β-catenin.**

A. Nuclear β-catenin was altered in parallel with the proliferation of SH-SY5Y cells by bFGF, Y27632, Li, and Que. Preparation of nuclear lysates and Western blot were performed 2 days after treatment. Concentrations of treated drugs in each lane are described as the table under the picture. 20 μg of nuclear lysates was loaded into each lane. Values are shown as the ratio of β-catenin vs. GAPDH. Data are shown as means ± SEM of three independent samples. # $p < 0.05$.

FIGURE 4. **Effects of bFGF, Y27632, Li, and Que on cyclin D1 expression.**

A. mRNA expression of Cyclin D1 was altered in parallel with the proliferation of SH-SY5Y cells by bFGF, Y27632, Li, and Que. RNA isolation and quantitative

RT-PCR were performed 2 days after treatment. The concentrations of treated drugs in each lane are described as the table under the graph. Values are shown as the ratio of cyclin D1 mRNA versus 18s rRNA. Data are shown as means ± SEM of three independent samples. # $p<0.05$.

B. Protein expression of Cyclin D1 was altered in parallel with the proliferation of SH-SY5Y cells by bFGF, Y27632, Li, and Que. Preparation of cell lysates and Western blot were performed 2 days after treatment. The concentrations of treated drugs in each lane are described as the table under the picture. 20 μg of cell lysates were loaded into each lane. Values are shown as the ratio of cyclin D1 versus GAPDH. Data are shown as the means ± SEM of three independent samples. * $p<0.01$, # $p < 0.05$.

FIGURE 5. **Effects of bFGF and y27632 on the phosphorylation states of GSK-3β.**

Phosphorylation states of GSK-3β were altered by bFGF and Y27632. Preparation of cell lysates and Western blot were performed 2 days after bFGF or Y27632-treatment. The bFGF concentration was 20 ng/ml, and that of Y27632 was 30 μM. 20 μg of cell lysates were loaded into each lane. Values are shown as the ratio of Ser9 or Tyr216 versus total GSK-3β. Data are shown as the means ± SEM of three independent samples. In the graph of Ser9, * $p<0.01$,

# $p < 0.05$.

FIGURE 6. **Effects of siRNA for ROCK on mRNA expression of cyclin D1.**

Selective decrease of mRNA expression of ROCK2 using siRNA decreased mRNA expression of cyclin D1, but the selective decrease of mRNA expression of ROCK1 did not alter the mRNA expression of cyclin D1. To rule out the possibility of the off-target effect, two independent siRNA for each ROCK was used for the check of the effects of siRNAs of ROCK1 and 2 on mRNA expression of ROCK1 and 2. Stealth RNAi negative control kit (Invitrogen Corp.) was used as a control for transfection, and 18s RNA was used as a control for quantitative RT-PCR. RNA isolation and quantitative RT-PCR were performed 2 days after transfection of siRNA. Values are shown as the ratio of cyclin D1 mRNA versus 18s rRNA. Data are shown as means ± S.E.M of three independent samples. # $p<0.05$.

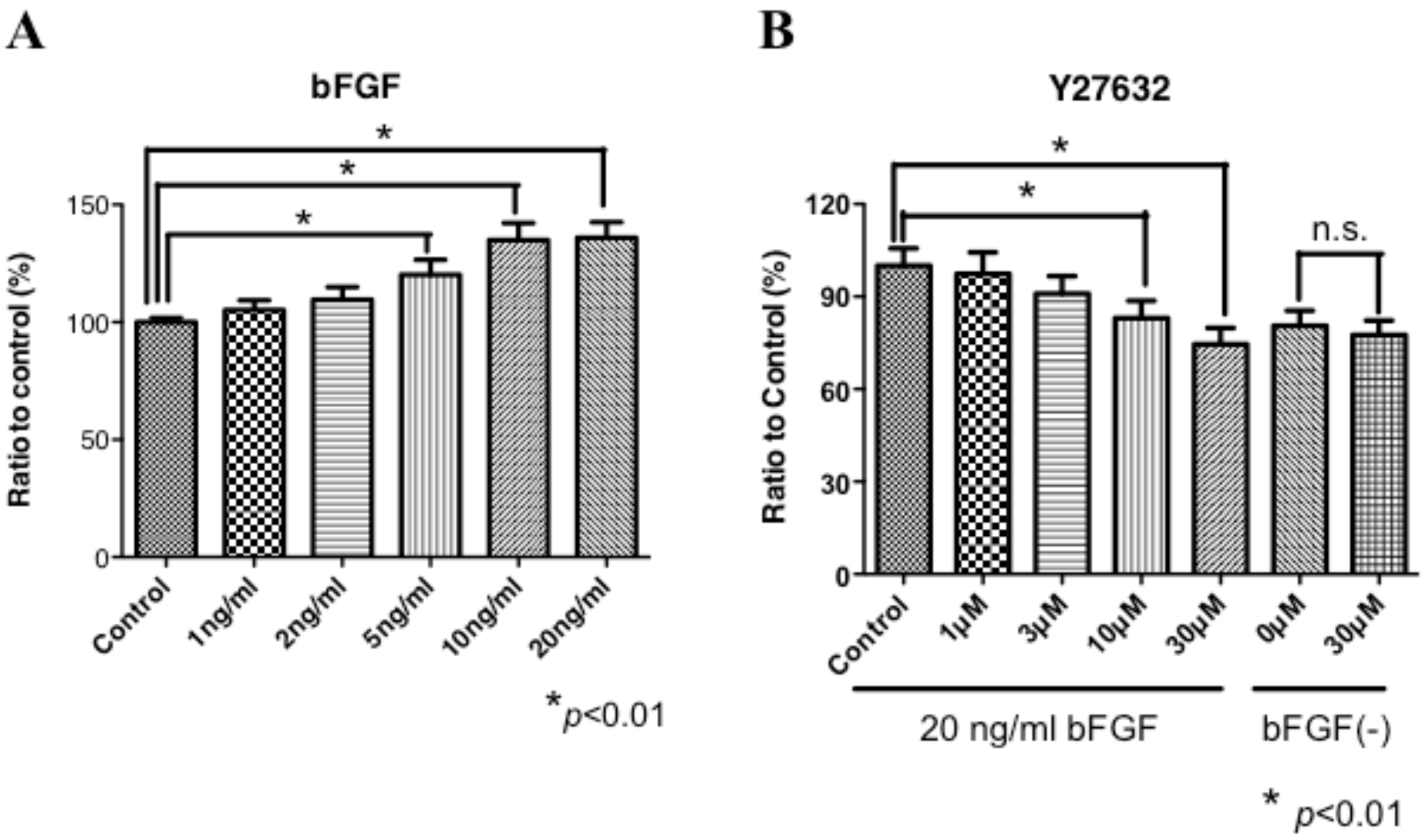
FIGURE 7. **Proposed schematic drawing for the action mechanism of bFGF and ROCK2 for the proliferation of SH-SY5Y cells through the GSK-3β and β-catenin pathway.**

A. bFGF inactivates GSK-3β through increased phosphorylation of Ser9 at GSK-3β. This inactivation of GSK-3β engenders promotion of the translocation of β-catenin from cytosol to the nucleus. In the nucleus, β-catenin promotes the

transcription of cyclin D1. ROCK2 might be involved in keeping the inactivation of GSK-3β through inhibition of the phosphorylation of Tyr216 at GSK-3β.

B. Y27632, a specific inhibitor of ROCK2, activates GSK-3β through the phosphorylation of Tyr216. This activated GSK-3β phosphorylates β-catenin, which engenders the degradation of β-catenin and the inhibition of its translocation from cytosol to the nucleus.

Figure 1


A
bFGF
Ratio to control (%)
150
100
50
0
Control
1ng/ml
2ng/ml
5ng/ml
10ng/ml
20ng/ml
*
*
*
*p<0.01
B
Y27632
Ratio to Control (%)
120
90
60
30
0
Control
1μM
3μM
10μM
30μM
0μM
30μM
n.s.
20 ng/ml bFGF
bFGF(-)
* p<0.01

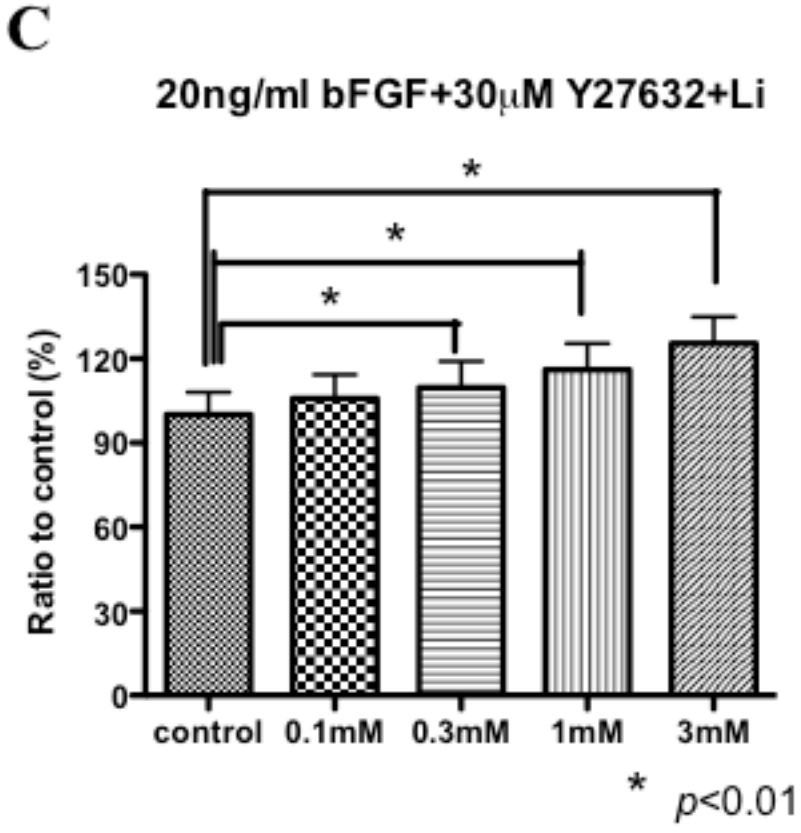
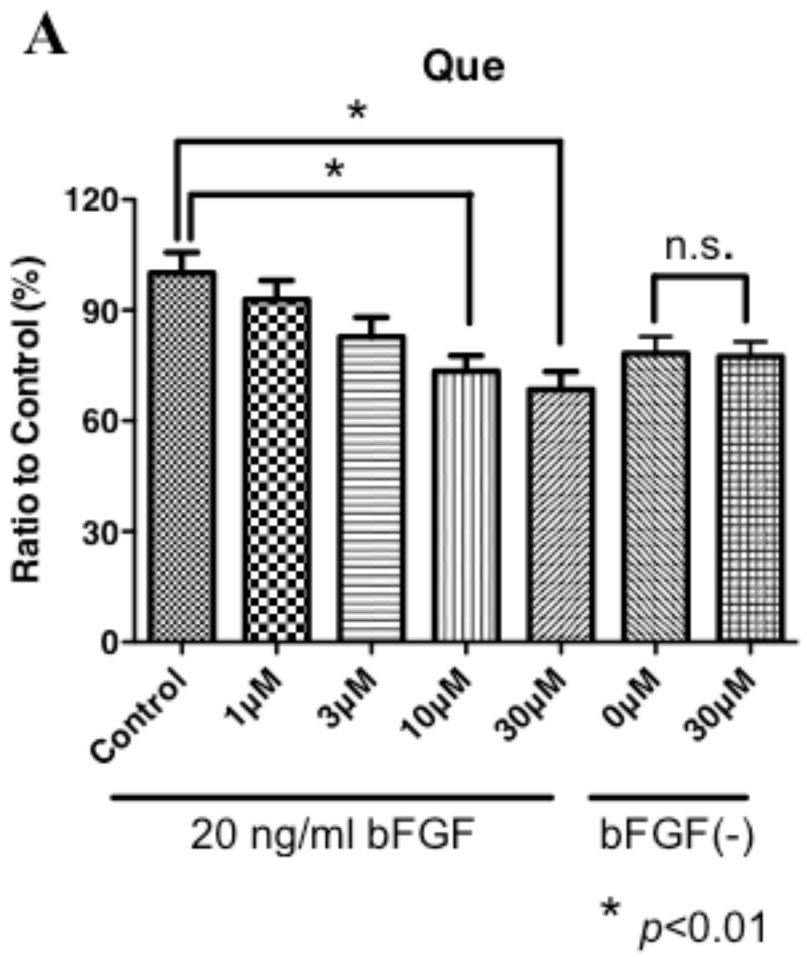
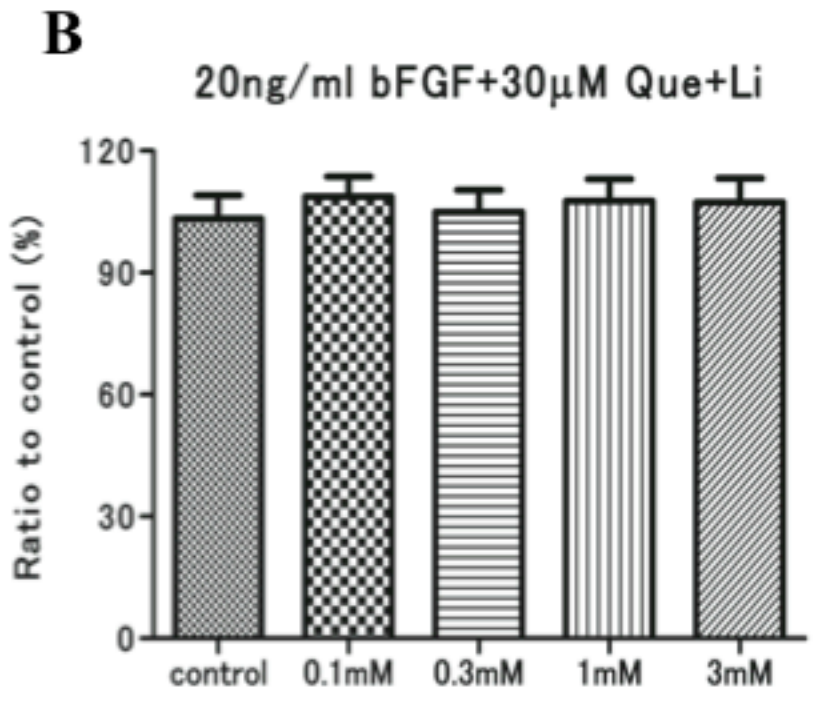

C
20ng/ml bFGF+30μM Y27632+Li
Ratio to control (%)
150
120
90
60
30
0
control
0.1mM
0.3mM
1mM
3mM
*
*
*
* p<0.01

Figure 2


A
Que
*
*
n.s.
120
90
60
30
0
Ratio to Control (%)
Control
1μM
3μM
10μM
30μM
0μM
30μM
20 ng/ml bFGF
bFGF(-)
* p<0.01


B
20ng/ml bFGF+30μM Que+Li
120
90
60
30
0
Ratio to control (%)
control
0.1mM
0.3mM
1mM
3mM

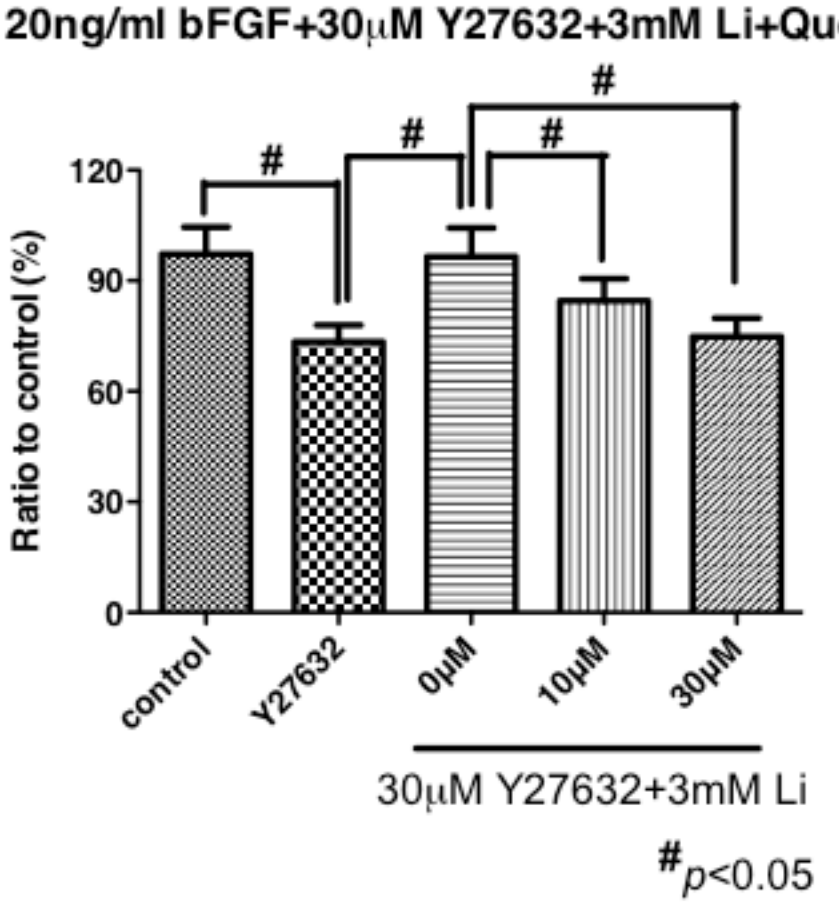
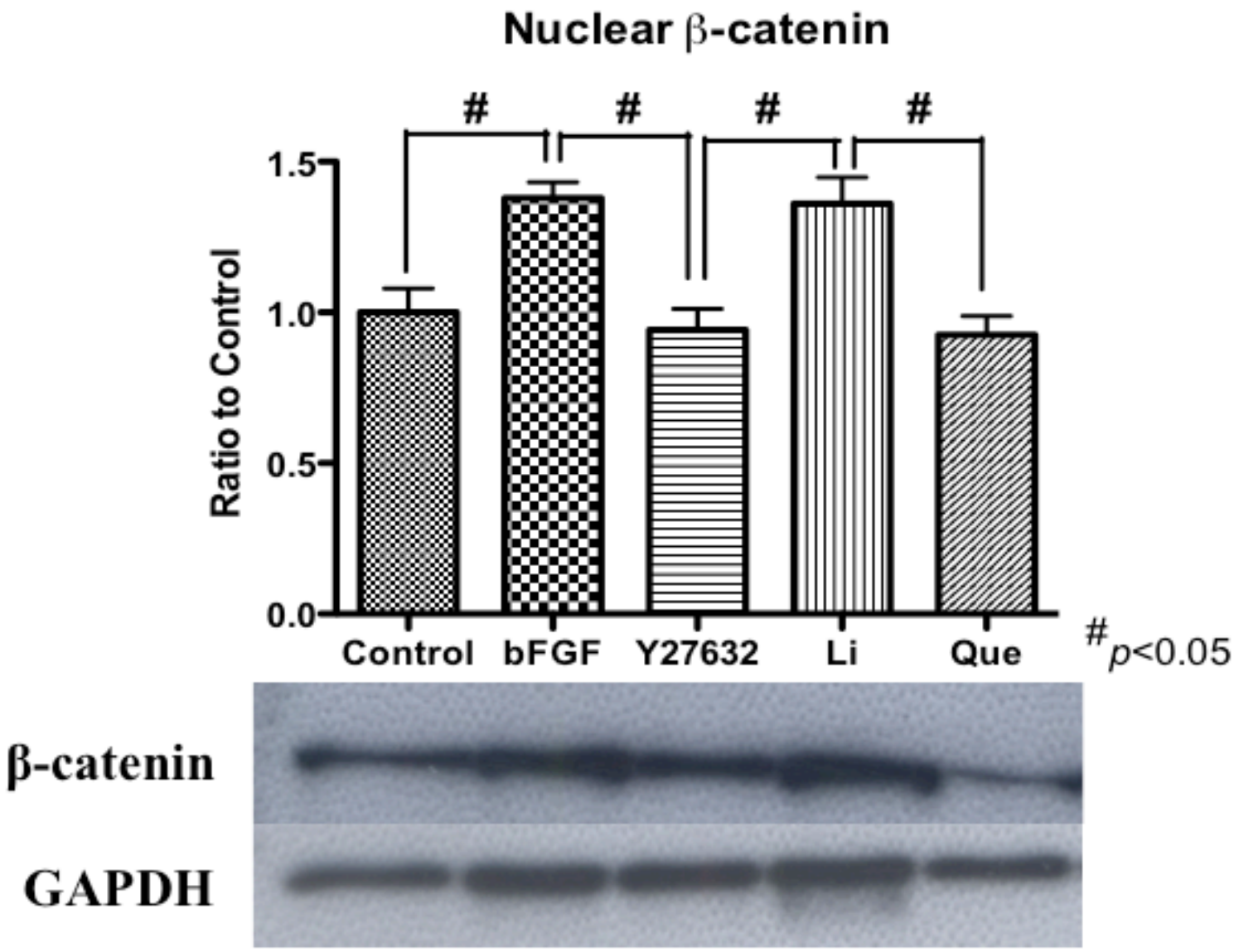

C
20ng/ml bFGF+30μM Y27632+3mM Li+Que
#
#
#
#
120
90
60
30
0
Ratio to control (%)
control
Y27632
0μM
10μM
30μM
30μM Y27632+3mM Li
#p<0.05

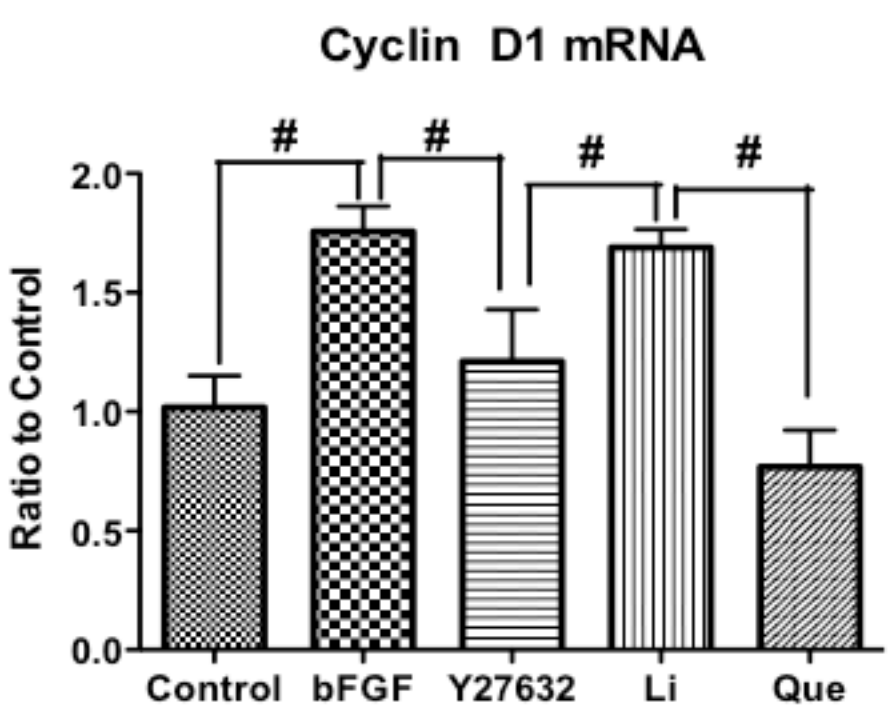
Figure 3



bFGF 20ng/ml	-	+	+	+	+
Y27632 30μM	-	-	+	+	+
Li 3mM	-	-	-	+	+
Que 30μM	-	-	-	-	+

Figure 4

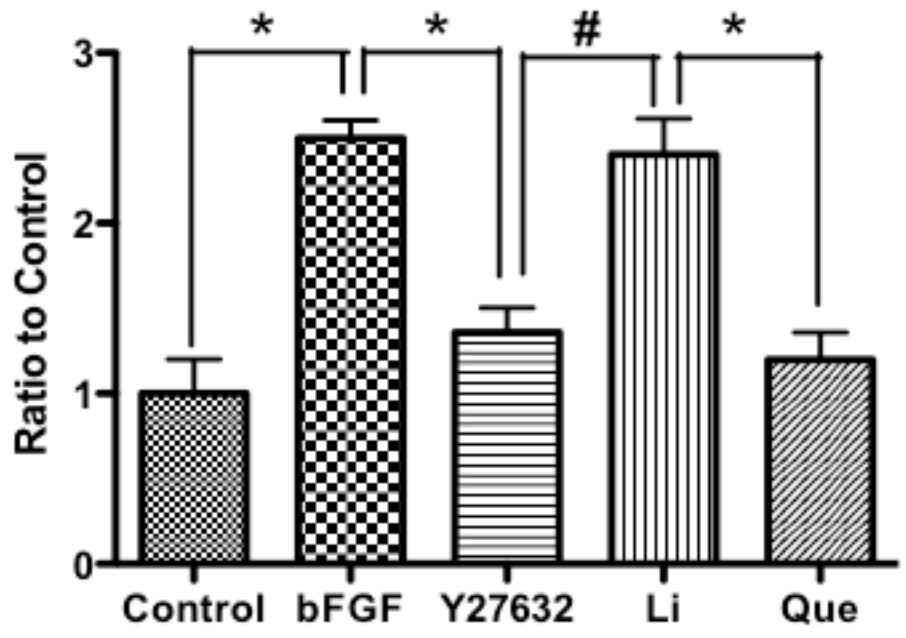
A



# $p<0.05$

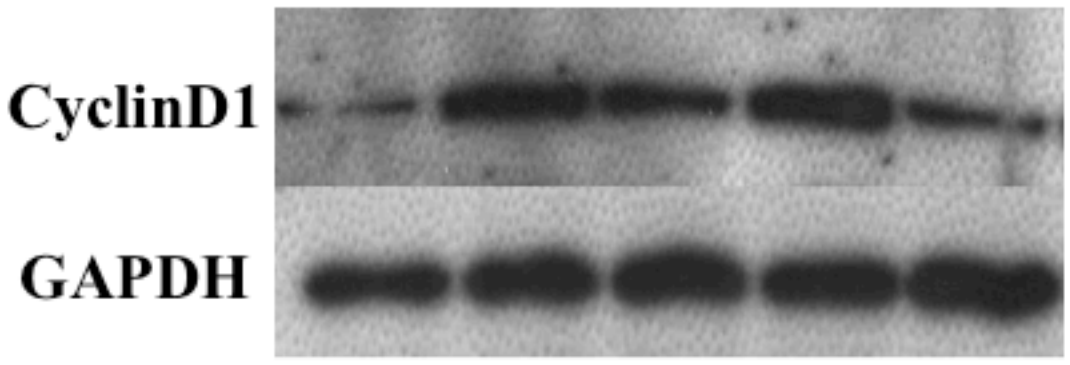
20ng/ml bFGF	-	+	+	+	+
Y27632 30μM	-	-	+	+	+
Li 3mM	-	-	-	+	+
Que 30μM	-	-	-	-	+

B



* $p<0.01$

# $p<0.05$



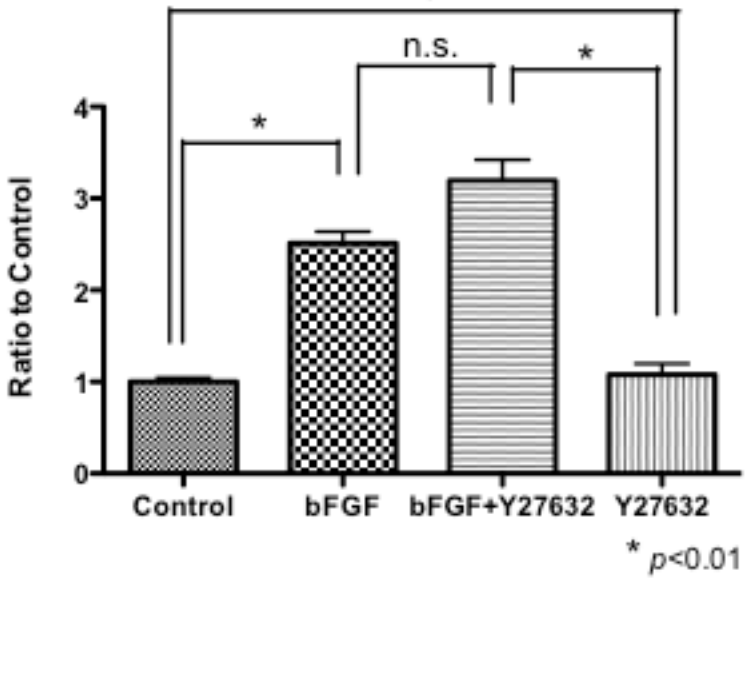
20ng/ml bFGF	-	+	+	+	+
Y27632 30μM	-	-	+	+	+
Li 3mM	-	-	-	+	+
Que 30μM	-	-	-	-	+

Figure 5

A


pGSK3β−Ser9
pGSK3β−Tyr216
Total GSK3β
Control
bFGF
bFGF + Y27632
Y27632

B


Ser9
n.s.
n.s.
*
*
Ratio to Control
0
1
2
3
4
Control
bFGF
bFGF+Y27632
Y27632
* p<0.01

C

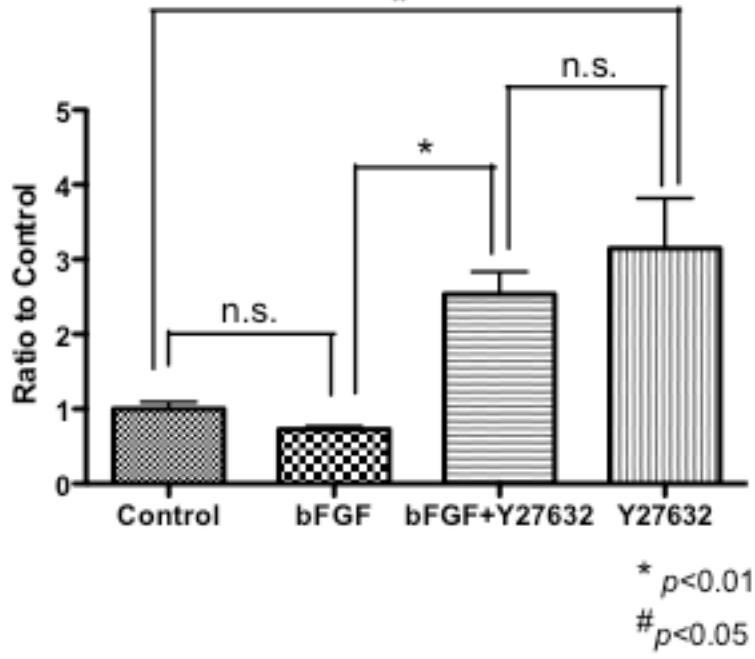
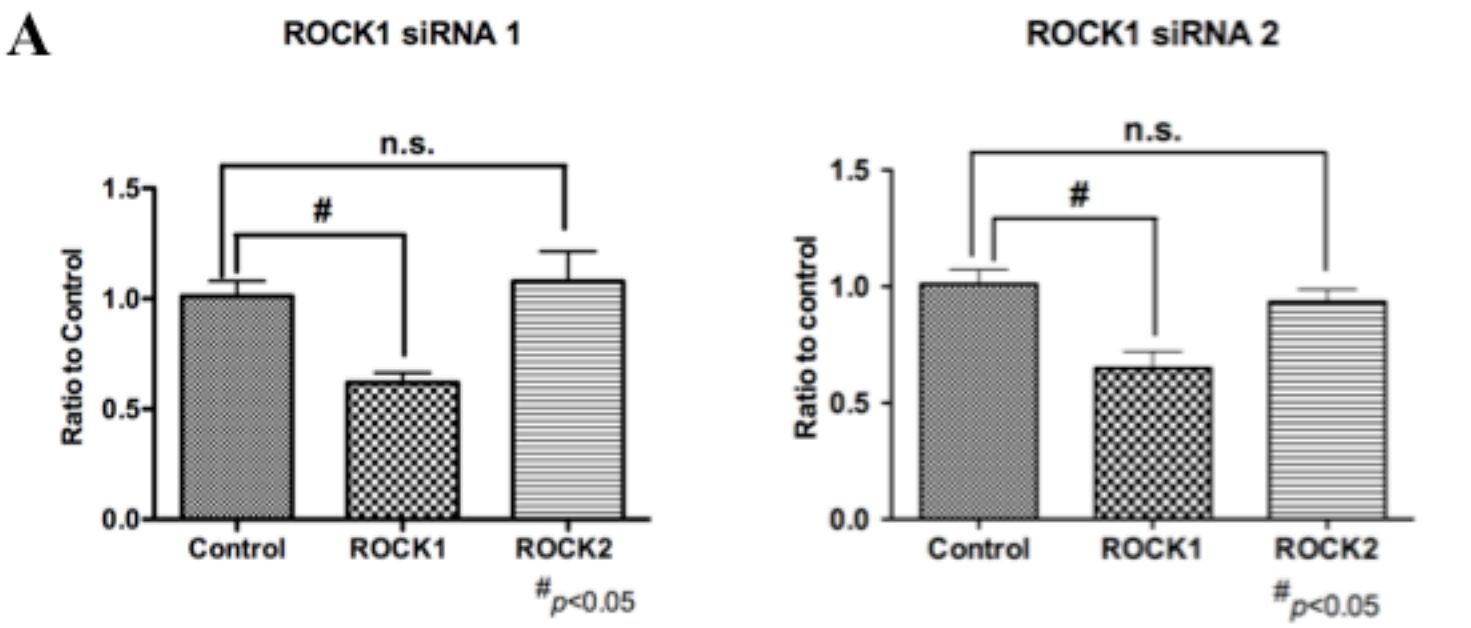
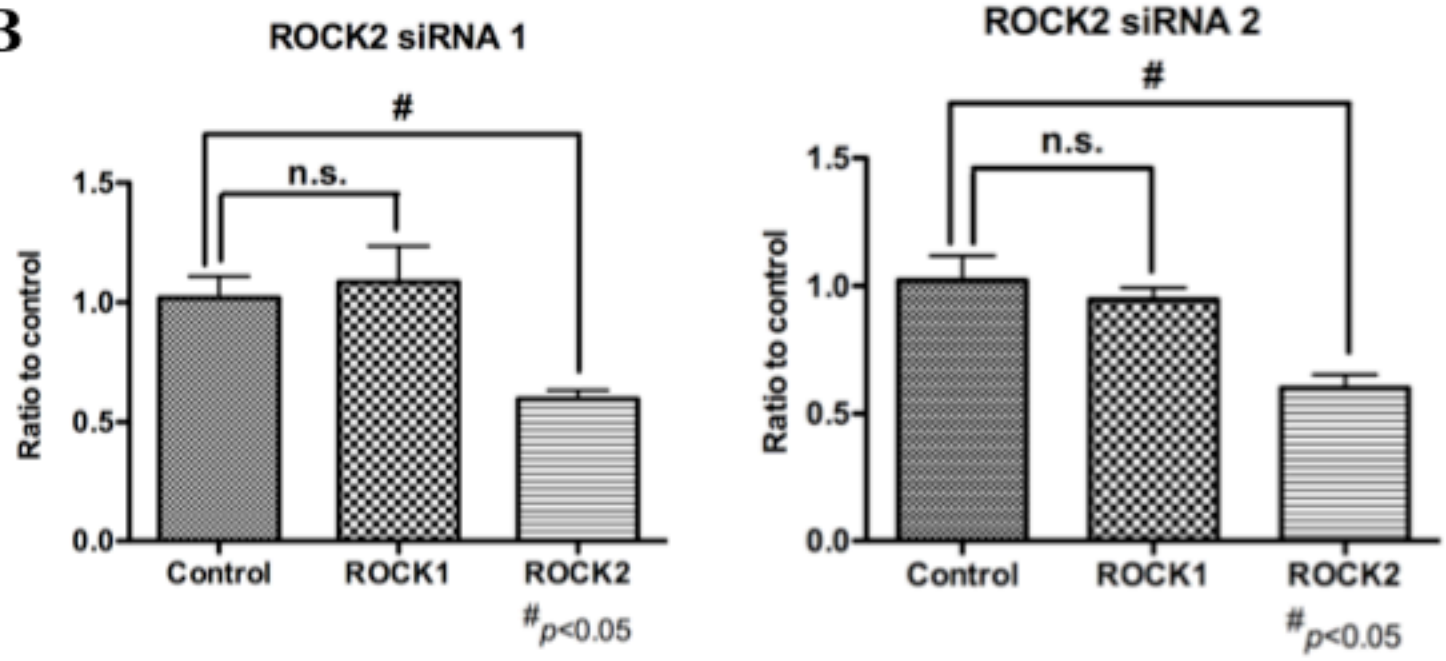

Tyr216
#
n.s.
*
n.s.
Ratio to Control
0
1
2
3
4
5
Control
bFGF
bFGF+Y27632
Y27632
* p<0.01
# p<0.05

Figure 6


A
ROCK1 siRNA 1
n.s.
#
Ratio to Control
1.5
1.0
0.5
0.0
Control
ROCK1
ROCK2
#p<0.05
ROCK1 siRNA 2
n.s.
#
Ratio to control
1.5
1.0
0.5
0.0
Control
ROCK1
ROCK2
#p<0.05


B
ROCK2 siRNA 1
#
n.s.
Ratio to control
1.5
1.0
0.5
0.0
Control
ROCK1
ROCK2
#p<0.05
ROCK2 siRNA 2
#
n.s.
Ratio to control
1.5
1.0
0.5
0.0
Control
ROCK1
ROCK2
#p<0.05

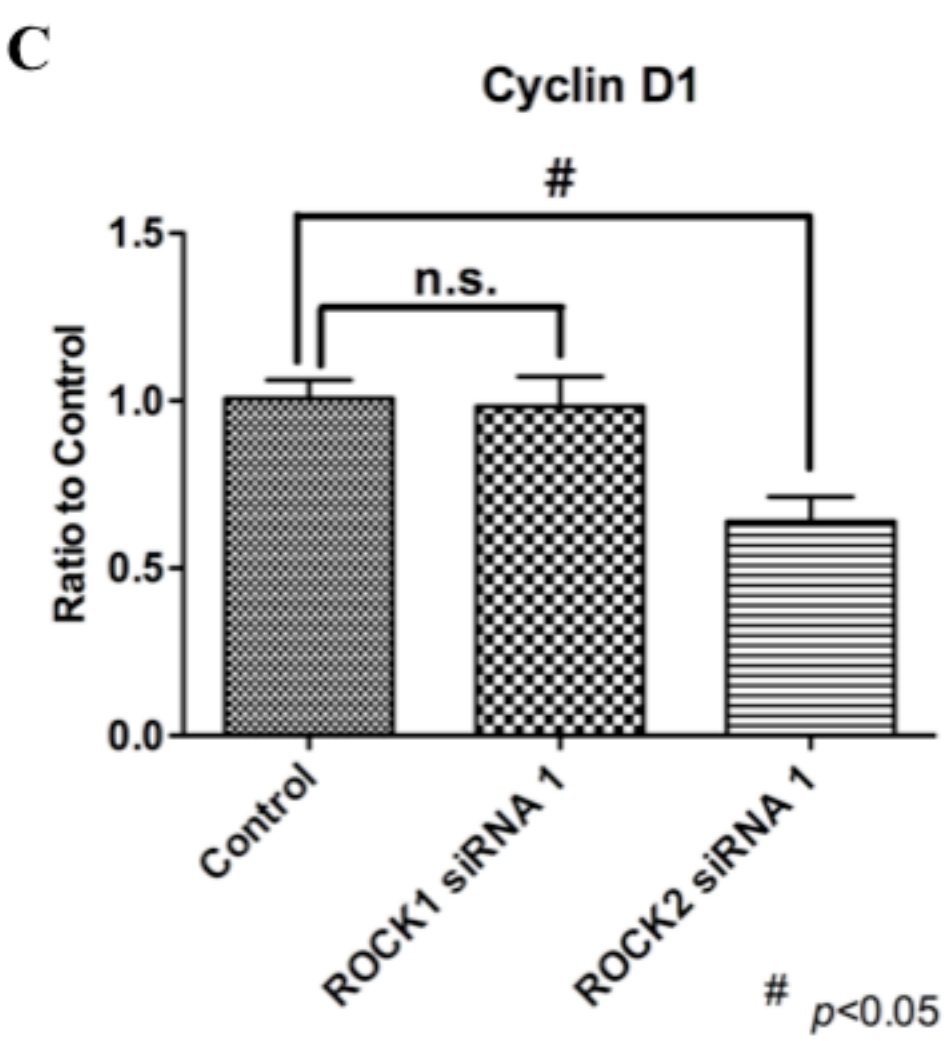
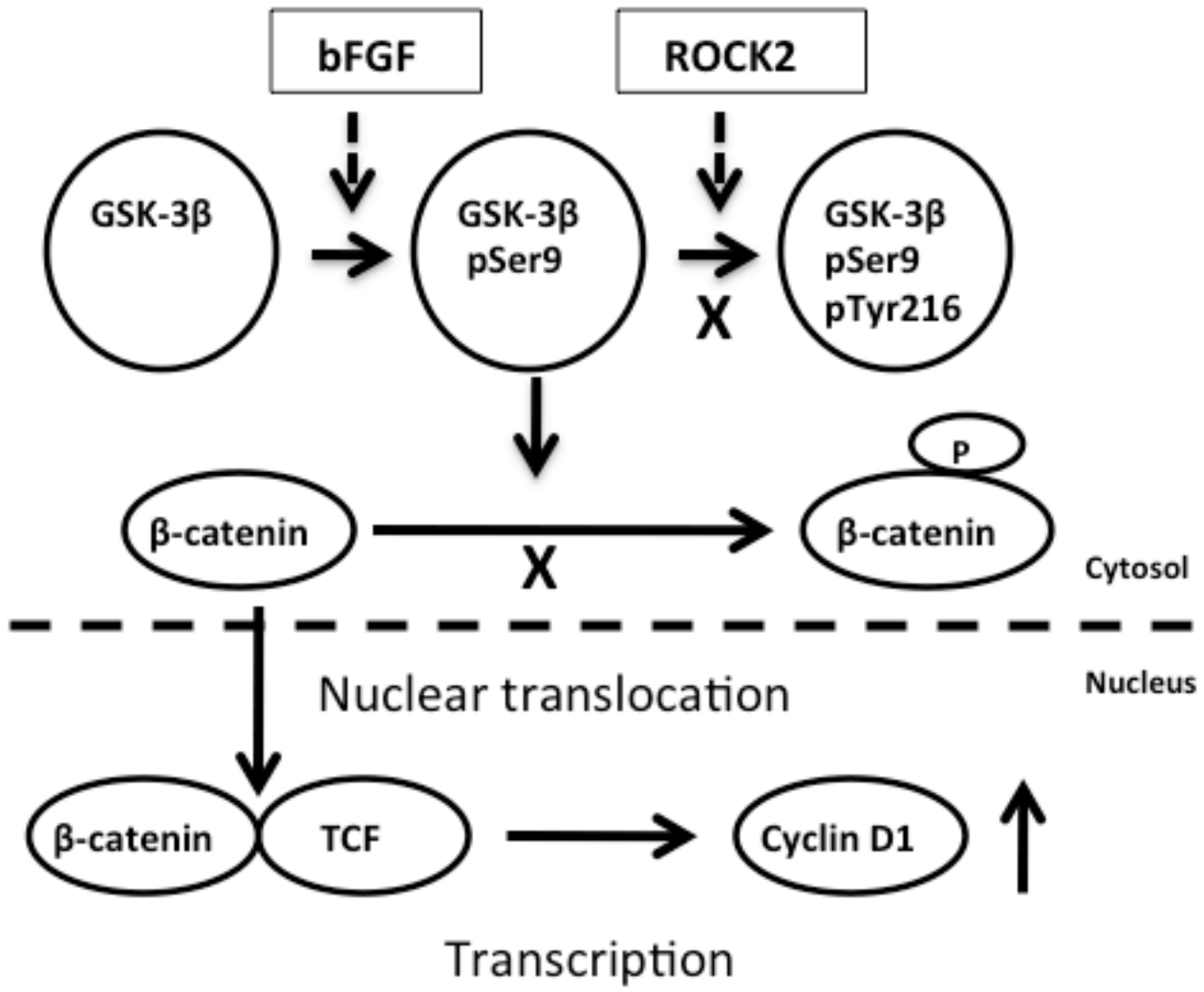
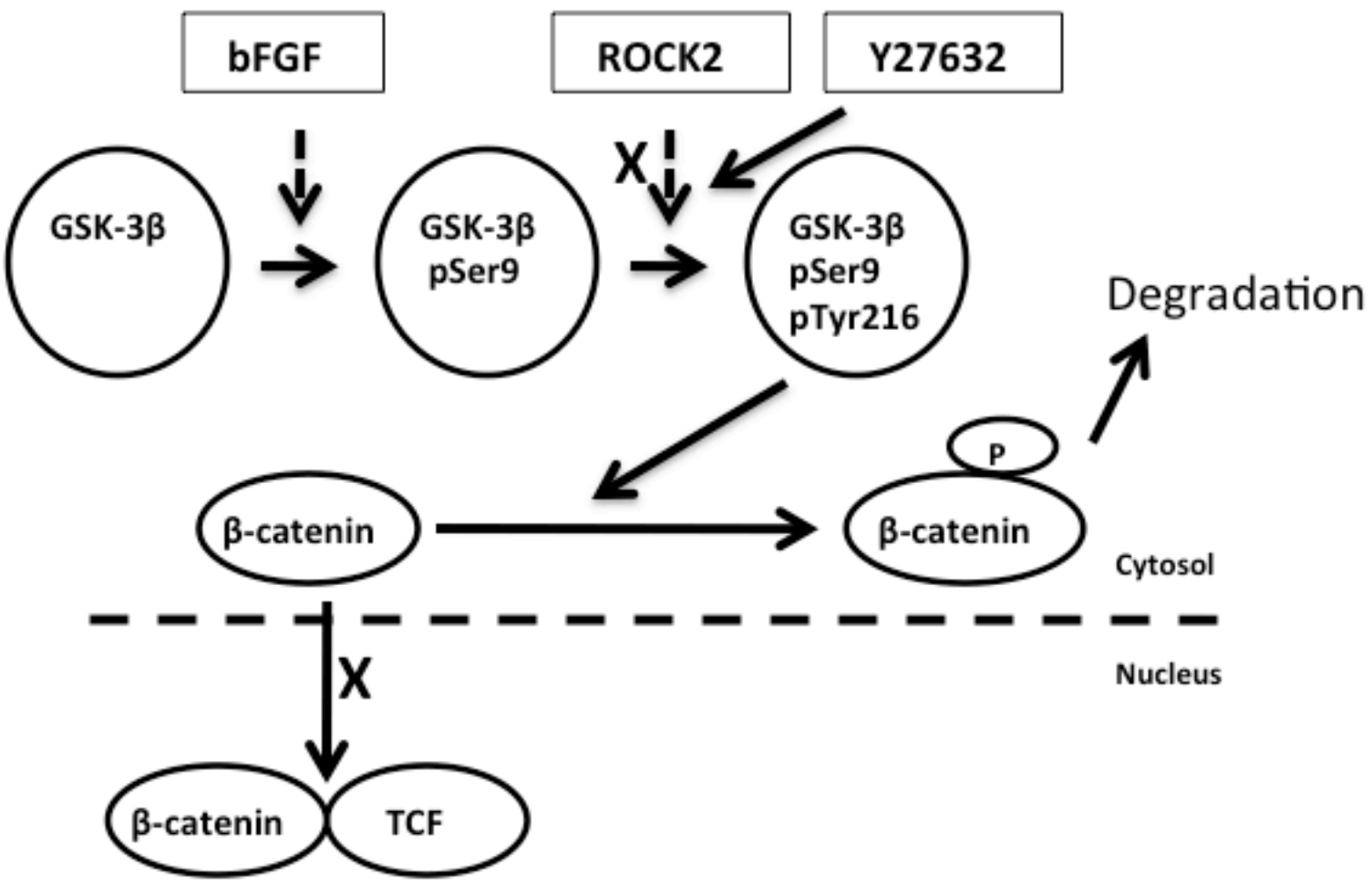

C
Cyclin D1
#
n.s.
Ratio to Control
1.5
1.0
0.5
0.0
Control
ROCK1 siRNA 1
ROCK2 siRNA 1
# p<0.05

Figure 7

A


bFGF
ROCK2
GSK-3β
GSK-3β pSer9
GSK-3β pSer9 pTyr216
X
P
β-catenin
β-catenin
X
Cytosol
Nucleus
Nuclear translocation
β-catenin
TCF
Cyclin D1
Transcription

B


bFGF
ROCK2
Y27632
X
GSK-3β
GSK-3β pSer9
GSK-3β pSer9 pTyr216
Degradation
P
β-catenin
β-catenin
Cytosol
Nucleus
X
β-catenin
TCF