Nuclear F-actin enhances the transcriptional activity of β-catenin by increasing its nuclear localization and binding to chromatin

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ABSTRACT

Actin plays multiple roles both in the cytoplasm and in the nucleus. Cytoplasmic actin, in addition to its structural role in the cytoskeleton, also contributes to the subcellular localization of transcription factors by interacting with them or their partners. The transcriptional co-factor β-catenin, which acts as an intracellular transducer of canonical Wnt signaling, indirectly associates with the cytoplasmic filamentous actin (F-actin). Recently, it has been observed that F-actin is transiently formed within the nucleus in response to serum stimulation and integrin signaling, and also during gene reprogramming. Despite these earlier observations, information about the function of nuclear F-actin is poorly defined. Here, by facilitating accumulation of nuclear actin artificially, we demonstrate that polymerizing nuclear actin enhanced the nuclear accumulation and transcriptional function of β-catenin. Our results also show that the nuclear F-actin co-localizes with β-catenin, and enhances the binding of β-catenin to the downstream target genes of the Wnt/β-catenin signaling pathway, including the genes for the cell cycle regulators c-myc and cyclin D, and the OCT4 gene. Nuclear F-actin itself also associated with these genes. Since Wnt/β-catenin signaling has important roles in cell differentiation and pluripotency, our observations suggest that nuclear F-actin formed during these biological processes is involved in regulating Wnt/β-catenin signaling.

INTRODUCTION

Polymerized actin (F-actin), a major component of the cytoskeleton, plays central roles in cell shape, cell motility, and cell division. In addition, the cytoplasmic F-actin is involved in the subcellular localization of transcription factors. For example, it was shown that the cytoplasmic F-actin retained the transcription factor Nrf2 in the cytoplasm by associating with it, via the Nrf2-binding protein Keep1, and inhibited the transcriptional function of Nrf2 (Kang et al., 2004). This observation was consistent with the observation that rearrangement of actin cytoskeleton affected the transcriptional function of Nrf2 (Kang et al., 2002). The co-transcription factor β-catenin is also known to associate with the cytoplasmic F-actin, by forming a complex with the F-actin binding protein α-catenin and E-cadherin (Buckley et al., 2014). The unbound form of β-catenin shuttles between the nucleus and the cytoplasm (Krieghoff et al., 2006); however, in the absence of Wnt signal, the transcriptional function of β-catenin is suppressed, as it gets degraded in the cytoplasm (Wu et al., 2010). Wnt ligands inactivate the β-catenin destruction machinery, allowing β-catenin to translocate to the nucleus, form a complex with the TCF/LEF transcription factor and activate target genes (Park et al., 2005; Huang and He, 2008). Through this signaling system, β-catenin controls the canonical Wnt signaling pathway, which contributes to multiple biological phenomena including cell proliferation, differentiation, migration, and division (Klaus and Birchmeier, 2008; Kuhl and Kuhl, 2013).

Actin also plays various roles in transcription regulation in the nucleus. Although the amount of actin in the nucleus is low relative to cytoplasm, it is actively regulated by nucleo-cytoplasmic shuttling, which is mediated by importin 9 and exportin 6 (Stüven et al., 2003; Dopie et al., 2012). In the nucleus, the monomeric G-actin is known to be a component of the multiple chromatin remodeling and histone modification complexes (Oma and Harata, 2011). Consistent with this observation, monomeric actin was shown to be required for the function of INO80 chromatin remodeling complex (Kapoor et al., 2013). Another paradigmatic example of the function of nuclear

actin in transcriptional regulation is the binding of nuclear G-actin to the myocardin-related

transcription factor (MRTF), which is a co-activator of serum serum response factor (SRF) (Shaw et

al., 1989; Wang et al., 2002). Thus, the binding of nuclear G-actin to MRTF induced nuclear export

of MRTF and inhibited SRF dependent transcriptional activation (Vartiainen et al., 2007; Knöll,

2010; Baarlink et al., 2013). In addition, it was shown that polymerized actin cooperated with

nuclear myosin I in transcriptional activation (Ye et al., 2008).

The formation of nuclear F-actin is induced under various conditions, which include serum

stimulation, integrin signaling, stresses, DNA damage, and gene reprogramming (Nishida et al.,

1987; Miyamoto et al., 2011; Baarlink et al., 2013; Plessner et al., 2015; Belin et al., 2015). When

nuclear F-actin is formed, the G-actin pool in the nucleus is reduced, as a result of which the

function of G-actin is affected (Baarlink et al., 2013). In addition, artificial formation of nuclear

F-actin leads to an increase in the overall transcription level (Kalendova et al., 2014; Yamazaki et al.,

2015a, b). Nuclear F-actin is also shown to be essential in gene reprogramming of mouse nuclei

transplanted into *Xenopus* oocyte (Miyamoto et al., 2011). Despite all these interesting observations,

the role of nuclear F-actin is poorly defined.

Recently, it was shown that the nuclear accumulation of α-catenin requires β-catenin

(Daugherty et al., 2014). Moreover, artificial targeting of α-catenin to the nucleus correlates with the

formation of nuclear F-actin, which correlates with decreased transcription and changes in chromatin

structure (Daugherty et al., 2014). In the present study, we investigated the role of F-actin in the

nucleus on β-catenin. Our data suggest that, in addition to previously known effects of nuclear actin

on the level of transcription in general (Kalendova et al., 2014; Yamazaki et al., 2015a, b), nuclear

F-actin not only affects the subcellular localization and transcriptional function of β-catenin but also

plays a role in the regulation of Wnt/β-catenin signaling.

To determine the role nuclear F-actin in β-catenin function, we employed a strategy in which

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expression of actin fused to a nuclear localization signal (NLS) was previously shown to induce nuclear F-actin filament formation (Kokai et al., 2014; Kalendova et al., 2014; Yamazaki et al., 2015a). In this study, we found that nuclear F-actin tethers β-catenin in the nucleus and thereby increases the nuclear concentration of β-catenin. In addition to altering the subcellular localization of β-catenin, we demonstrated that nuclear F-actin activates transcriptions of β-catenin-targeting genes by increasing the binding of β-catenin to its binding sites on these genes. These novel findings support the contention that the nuclear F-actin has a role in regulating Wnt/β-catenin signaling.

RESULTS

Co-localization of β-catenin with induced nuclear F-actin bundles

To determine whether association with nuclear actin would affect the subcellular localization of β-catenin, we expressed a nuclear localization signal (NLS)-tagged actin construct in HeLa cells to induce nuclear accumulation of actin, immunostained these cells with an anti-β-catenin antibody and observed the subcellular distribution of β-catenin in these cells using laser-scanning confocal microscopy. As shown in Fig. 1A, nuclear actin bundles, which are stained with phalloidin (Yamazaki et al., 2015b), were visible in 1-5% of cells expressing NLS-actin, as was reported previously (Kalendová et al. 2014). In the nucleus of these cells, β-catenin, which was visualized with an Alexa568-conjugated secondary antibody, was found to be co-localized with F-actin bundles in cells expressing EYFP-NLS-actin (Fig. 1A, upper panels). Similarly, co-localization of β-catenin (visualized with an Alexa488-conjugated secondary antibody) with F-actin bundles (formed in cells expressing mCherry-NLS-actin) was also observed. Under the same experimental condition, bleed-through of EYFP-NLS-actin and mCherry-NLS-actin signals into the channel that was used for the detection of β-catenin signal was not observed (Suppl. Fig. S1). Although not proven, results described above support the idea that β-catenin binds to actin filaments regardless of the subcellular localization of actin.

Nuclear F-actin correlates with the accumulation of β-catenin in the nucleus under serum-deprived condition

In a previous study, we used Lifeact, an F-actin binding probe (Riedl et al., 2008) and demonstrated that majority of cells expressing NLS-actin contained F-actin in the nucleus regardless of whether or not any F-actin bundle was visible (Yamazaki et al., 2015a). It is possible that β-catenin also binds to

the nuclear F-actin that remains unbundled. To test this possibility, HeLa cells expressing EYFP, EYFP-actin or EYFP-NLS-actin was examined under a microscope to determine the subcellular localization of β-catenin (Fig. 1B). Since the amount of nuclear β-catenin is relatively high in proliferating cells, as the Wnt/β-catenin signaling is necessary for the expression of cycle regulators (Davidson and Niehrs, 2010), we cultured these cells for 24 h in the serum-free medium to reduce the background level of nuclear β-catenin before examining them under the microscope. Under this experimental condition, we found that significant amount of β-catenin translocated to the nucleus of cells expressing NLS-actin, but not to the nucleus of cells expressing actin (Fig. 1C).

Taken together with our observation that β-catenin co-localized with nuclear F-actin bundles (Fig. 1A), these results suggest that F-actin formed in the nucleus binds to β-catenin and increases the amount of β-catenin in the nucleus.

Nuclear accumulation of β-catenin by nuclear actin under normal culture condition

The NLS-actin-associated nuclear accumulation of β-catenin was less obvious in actively proliferating HeLa cells, which were grown in a serum containing medium, as compared to those grown under growth-suppressed condition (Fig. 2A, -XAV939/+serum). This is presumably because the relative abundance of β-catenin in the nucleus of actively proliferating cells concealed the actin-induced increase in nuclear β-catenin. To test whether nuclear actin induces nuclear translocation of β-catenin even under normal culture condition, the amount of endogenous β-catenin was reduced by treating cells with XAV939, which stabilizes axin and enhances degradation of β-catenin. As shown, after treatment with XAV939, significant increase in the nuclear β-catenin level was observed in HeLa cells expressing NLS-actin that were grown in serum containing medium (Fig. 2A, +XAV939/+serum).

We also used the human keratinocyte cell line HaCaT (Boukamp et al. 1988) to test whether

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actin would induce nuclear translocation of β-catenin when these cells are grown in serum containing medium. The growth of HaCaT cells was much slower than that of the HeLa cells (Suppl. Fig. S2). As expected, β-catenin was found predominantly in the periphery of cells expressing EYFP and was excluded from the nucleus even in cells grown in serum containing medium (Fig. 2B, EYFP). Furthermore, NLS-actin was found in the nucleus of HaCaT cells expressing EYFP-NLS-actin, and there was translocation of β-catenin to the nucleus of these cells (Fig. 2B, EYFP-NLS-actin). Statistical analysis revealed that the nuclear β-catenin level significantly increased by expressing NLS-actin in HaCaT cells (Fig. 2C). In 1% cells, NLS-actin formed short but visible F-actin bundles in the nucleus, and β-catenin co-localized with the nuclear F-actin bundles (Fig. 2B, lower panels of EYFP-NLS-actin). These results suggest that the translocation of β-catenin the nucleus that is promoted by nuclear actin polymerization is not restricted only to HeLa cells, but could also be observed in other cells that were grown under normal culture condition.

Nuclear actin activates Wnt/β-catenin targeting genes

Since the β-catenin destruction complex, which includes APC and axin, predominantly functions in the cytoplasm (Stamos and Weis, 2013), the β-catenin that translocates to the nucleus is not only expected to escape from being degraded, but is also expected to activate the transcription of its target genes. To test this possibility, we performed luciferase assay using a reporter plasmid that contained the TCF/LEF transcriptional response element (TRE). Consistent with our observation that β-catenin is accumulated in the nucleus by nuclear F-actin under serum-deprived condition, significantly increased promoter activity was observed in cells co-expressing the reporter and EYFP-NLS-actin constructsthan in cells co-expressing the reporter and either the EYFP or the EYFP-actin constructs (Fig. 3A). In contrast, the luciferase activity of the reporter construct lacking the TCF/LEF binding site did not increase by the expression of EYFP-NLS-actin (Suppl. Fig. S3). These results suggest

that nuclear F-actin enhances β-catenin-mediated activation of Wnt/β-catenin targeting genes.

Therefore, we next analyzed the expression of Wnt/β-catenin targeting genes in cells expressing actin with or without an NLS-tag. It was previously shown that nuclear accumulated β-catenin accelerates cell growth by inducing cell cycle promoting factor genes (Nhieu et al., 1999). Particularly in human cells, c-myc, cyclin D, Tcf-1 and c-Jun genes were directly regulated by Wnt/β-catenin signaling (He et al., 1998; Tetsu and McCormick, 1999; Herbst et al., 2014). In addition, β-catenin was shown to bind to the β-catenin/TCF binding sites on the promoters of OCT4 gene (Tetsu and McCormick, 1999; He et al., 1998; Li et al., 2012). Consistent with the above discussed results, quantitative RT-PCR analysis revealed that the genes for c-myc, cyclin D, OCT4, and Tcf-1 (*MYC, CCND1, OCT4*, and *TCF-1*, respectively) were significantly activated in the cells expressing NLS-actin (Fig. 3B). On the other hand, the gene for c-Jun (*JUN*) was not activated by expressing NLS-actin (Fig. 3B). These results suggest that the nuclear actin affects a major portion, but not all, of Wnt/β-catenin targeting genes.

To confirm that nuclear actin indeed contributes to the transcriptional function of β-catenin, proliferation of cells expressing NLS-actin was analyzed. The growth of cells expressing NLS-actin was found to be significantly enhanced than the growth of cells expressing either EYFP or EYFP-actin (Fig. 3C), an observation that is in line with the activation of c-myc and cyclin D genes by nuclear actin (Fig. 3B). These results further support that the nuclear actin is involved in the regulation of Wnt/β-catenin signaling.

Nuclear F-actin facilitates the binding of β-catenin to chromatin

To further analyze the involvement of nuclear actin in the transcriptional function of β-catenin, we next performed chromatin immunoprecipitation (ChIP) assay. Consistent with the observed transcriptional activation results shown above (Fig. 3B), we found that the binding of β-catenin to

the β-catenin/TCF site of *CCND1* and *OCT4* increased in cells expressing NLS-actin (Fig. 4A and

B). To test whether the nuclear F-actin was indeed required for the binding of β-catenin to chromatin

regions, we expressed the G13R-actin mutant, which stabilizes G-actin and only forms unstable

F-actin (Posern et al., 2002), as a fusion protein with EYFP and NLS (EYFP-NLS-G13R-actin) in

HeLa cells. The binding of β-catenin to *CCND1* (Fig. 4A) and *OCT4* (Fig. 4B) was significantly

reduced in cells expressing the non-polymerizable NLS-G13R-actin. These results suggest that

nuclear F-actin, but not nuclear G-actin, contributes to the binding of β-catenin to Wnt/β-catenin

targeting genes.

Nuclear F-actin associates with the β-catenin binding regions on chromatin

Several previous studies showed that the nuclear actin associates with the promoters of genes and

regulates their expression (Ferrai et al., 2009; Taylor et al., 2010; Xu et al., 2010). To explain the

underlying mechanism by which the nuclear actin is recruited to specific regions on chromatin,

Miyamoto et al (2011) proposed the involvement of WASP, a nucleator of actin polymerization.

WASP associates with the SP1 transcription factor, and this association results in recruiting F-actin

to the SP1 site of the *OCT4* promoter (Taylor et al., 2010; Miyamoto et al., 2011). Results obtained

from our ChIP analysis revealed significant binding of NLS-actin to the SP1 sites, but not to the

transcription start site (TSS) or to the exon 4 of the *OCT4* gene (Fig. 5A). Most importantly, the

non-polymerizable NLS-G13R-actin did not bind to the SP1 site, TSS, or the exon 4 of the OCT4

gene. Taken together, these results demonstrated that the F-actin binding sites could be detected by

ChIP analysis. When we analyzed the results of binding of NLS-actin to the β-catenin/TCF sites of

OCT4 (Fig. 5A) and *CCND1* (Fig. 5B), we observed 2- to 6-fold increase in binding of NLS-actin to

these sites, but no binding of NLS-actin to the TSS and an exon of *OCT4 (Fig. 5A)* as wells as to

those of *GAPDH* (Suppl. Fig. S4) was observed. In contrast, non-polymerizable NLS-G13R-actin

did not bind to the β-catenin/TCF sites of *OCT4* and *CCND1*, suggesting that nuclear F-actin binds

to the β-catenin/TCF site. These results suggest a possibility that the nuclear F-actin plays a role in

transcription regulation not only by enhancing the binding of β-catenin to chromatin, but also by

directly associating itself with the chromatin.

DISCUSSION

The results summarized above suggest that the formation of nuclear F-actin correlates with the enhancement of the nuclear translocation and transcriptional activation of β-catenin. In this study, cells overexpressing actin was used for the analysis. However, we observed that treatment of cells with 100 nM mycalolide B, an actin depolymerizing agent (Saito et al., 1994), decreased the expression of the Wnt/β-catenin target gene *TCF-1* (Suppl. Fig. S4). Thus, this result, together with the observed dominant effect of NLS-actin on Wnt/β-catenin signaling, supports the idea that nuclear actin is involved in Wnt/β-catenin signaling at physiological levels. It is noteworthy that F-actin is transiently formed in the nucleus in response to extracellular signals including serum stimulation and integrin-mediated mechanotransduction (Baarlink et al., 2013; Plessner et al., 2015). Thus, the induced formation of nuclear F-actin, in response to signaling, might have a role in cell cycle progression via transcriptional activation of cell cycle regulators (such as c-myc and cyclin D), which are targets of Wnt/β-catenin signaling.

Wnt/β-catenin signaling also regulates cell differentiation and development. Wnt/β-catenin signaling has been shown to contribute to self-renewal and pluripotency of stem cells and improves reprogramming of somatic cells to iPS cells (Marson et al., 2008; Kuhl and Kuhl, 2013). Therefore, modulation of Wnt/β-catenin signaling by transiently formed nuclear F-actin may contribute to determination of cell fates. Interestingly, it was previously shown that the amount of nuclear actin changes during cell differentiation (Bettinger et al., 2004), and actin is accumulated during differentiation of HL60 cells (Xu et al., 2010). Additionally, it was demonstrated that nuclear F-actin is formed and activates OCT4 expression during gene reprogramming process of mouse nuclei transplanted into *Xenopus* oocytes (Miyamoto et al., 2011). *OCT4* is a Wnt/β-catenin-targeting gene also in mammals, and we showed that nuclear F-actin enhances recruitment of β-catenin to the promoter site of *OCT4* (Fig. 4). Therefore, it is likely that nuclear F-actin has a role in gene

reprograming, at least partly, through the activation of Wnt/β-catenin signaling.

In addition to contributing to the binding of β-catenin to chromatin, we also showed that nuclear F-actin itself associates with the β-catenin/TCF binding sites. Since β-catenin/TCF complex was reported to bind to α-catenin that possesses F-actin-binding ability (Daugherty et al., 2014), it seems probable that the β-catenin/TCF/α-catenin complex mediates association of nuclear F-actin to the β-catenin/TCF binding sites. α-catenin has the ability to form actin bundles in vitro, and the expression of NLS-tagged α-catenin leads to the formation of nuclear actin bundles in ~25% of the expressing cells (Daugherty et al., 2014). Interestingly, the formation of nuclear actin bundles by NLS-α-catenin affects chromatin organization and correlates with reduced RNA synthesis (Daugherty et al., 2014). On the other hand, the expression of NLS-actin forms nuclear actin bundles only in a limited portion (1-5%) of expressing cells (Kalendová et al. 2014), but unbundled nuclear F-actin was detected with Lifeact in most of the expressing cells (Yamazaki et al., 2015a). Together with the observations that the expression of NLS-actin leads to an increase in the overall transcription level (Kalendová et al., 2014; Yamazaki et al., 2015a, b) and in the expression of Wnt/β-catenin targeting genes (Fig. 3), it is implied that nuclear actin affects gene expression differently depending on the modes of its organization.

Wnt/β-catenin signaling is known to be involved in a variety of diseases, including cancer, neurodegeneration, inflammatory diseases, and metabolic diseases (Kahn, 2014). As a result, various chemical compounds, targeted to factors involved in Wnt/β-catenin signaling, are being tested for therapeutic purposes. In fact, screening of chemical compounds targeted to actin has a long history, as a result of which various modulators (e.g. cytochalasin, latrunculin, and jasplakinolide), which can facilitate formation and stabilization of F-actin, have been isolated. Our findings open up the possibility of using these anti-actin compounds for modulating Wnt/β-catenin signaling. Elucidating the role of nuclear F-actin in Wnt/β-catenin signaling and screening of lead compounds affecting the

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signaling through the modulation of actin dynamics may contribute to the development of valuable

drugs, including regenerative medicine.

Acknowledgements

MATERIALS AND METHODS

Cell lines and plasmid constructs

HeLa and HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (penicillin and streptomycin) at 37°C in 5% CO2 humidified atmosphere. For observation under microscope, cells (1×10^5 cells) were grown on a glass cover-slip, which was placed inside the wells of a 6-well culture plate (BM Equipment). The growth of cells was determined by trypan blue exclusion method using the TC-20 automated cell counter (Bio-rad). Plasmid constructs EYFP (expressing enhanced yellow fluorescent protein EYFP), EYFP-actin (expressing EYFP fused to the N-terminal of β-actin), EYFP-NLS-actin (expressing EYFP and NLS-tagged β-actin, where an NLS is inserted between the EYFP and β-actin), and EYFP-NLS-G13R actin (expressing EYFP and NLS-tagged G13R-actin mutant) were described previously (Hofmann et al., 2009). Transfection of cells was carried out using 1-3 µg of a given plasmid and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) or Fugene HD (Promega).

Immunofluorescence staining and microscopy

Cells grown on glass slides to similar subconfluency were used for the immunofluerescence staining. Cells were fixed with 4% paraformaldehyde/phosphate-buffered saline (PBS) and were permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Permeabilized cells were treated with a rabbit β-catenin antibody (Abcam, ab16051) in 4% bovine serum albumin/PBS for 1 h at room temperature or overnight at 4°C. Cells were then treated with the anti-rabbit Alexa564 (Life Technologies) for 1 h at room temperature. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen). Slides were mounted in Vectasheld Mounting Medium (Vector Laboratories). Fluorescent images of cells were recorded and collected images were analyzed using a FV1000 confocal laser microscope (Olympus). For observation of cells under a confocal laser microscope, cells were excited with the argon laser (488 nm) for EYFP and Alexa488

or with the He-Ne laser (543 nm) for Alexa568 and mCherry. We used a dichroic mirror, DM488/543, a beam splitter, SDM560, and two emission filters, BA505-525 for EYFP and Alexa488 and BA560IF for Alexa568 and mCherry. Scanning was performed in sequential mode to minimize bleed-through signa. Data analysis was performed using the Olympus FluoView FV1000 software.

Real-time PCR analysis

Total RNA was extracted from cells using the Qiagen RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was reverse-transcribed to cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen). Real-time PCR was carried out using SYBR-Green PCR kit (Applied Biosystems) in a total volume of 10 μl. The amount of each target cDNA was normalized with respect to the endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the ΔΔCt method. At least three independent experiments were performed for each target cDNA. Sequences of the oligonucleotide primers used for this analysis are shown below:

GAPDH -F; 5-TGCACCACCAACTGCTTAGC-3

GAPDH -R; 5-GGCATGGACTGTGGTCATGAG-3

c-myc-F;5-CGCGCTGAGTATAAAAGCCG-3

c-myc-R; 5-CTATTCGCTCCGGATCTCCC-3

Cyclin D-F; 5-CTGGAGGTCTGCGAGGAACA-3

Cyclin D-R; 5-CCTTCATCTTAGAGGCCACGAA-3

OCT-4-F; 5-GACAACAATGAGAACCTTCAGGAGA-3

OCT-4-R; 5-CTGGCGCCGGTTACAGAACCA-3

TCF-1-F; 5-TGACCTCTCTGGCTTCTACT-3

TCF-1-R; 5-TTGATGGTTGGCTTCTTGGC-3

c-Jun-F; 5-ATGCCCTCAACGCCTCGTTCC-3

c-Jun-R; 5-CTGGGCAGCGTGTTCTGGCTGT-3

Dual-Luciferase reporter assay

The dual-luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, the TCF/LEF reporter plasmid construct (Promega) and one of the EYFP constructs (EYFP, EYFP-actin or EYFP-NLS-actin) were transfected into the cells using FuGENE HD (Boehringer). The pRL-TK renilla luciferase construct (Promega) was used as an internal control for the transfection efficiency. All experiments were performed at least three times using independently grown cell cultures.

Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using the ChIP Reagents kit (NipponeGene) according to the protocol provided by the manufacturer. Briefly, HeLa cells were transfected with EYFP-actin, EYFP-NLS-actin or EYFP-NLS-G13R actin, transfected cells were crosslinked by treatment with 1% formaldehyde and subsequently nuclear extracts were prepared from these cross-linked cells according to the supplied protocol. Actin- and β-catenin-bound chromatin fraction in the sonicated nuclear lysate, prepared using Bioruptor (BM Equipment), was immunoprecipitated with GFP-Trap beads (ChromoTek) and anti-β-catenin antibody (R&D Systems AF1329) bound to Dynabeadsprotein G (Invitrogen), respectively. Purified DNA from the immunoprecipitaed fraction was subjected to real-time PCR analysis using the primers shown below.

β-catenin binding site of CyclinD-F; 5'-AGGCGCGGCGGCTCAGGGATG-3'

β-catenin binding site of CyclinD-R; 5'-ACTCTGCTGCTCGCTGCTACT-3'

β-catenin binding site of c-myc-F; 5'-TTGCTGGGTTATTTTAATCAT-3'

β-catenin binding site of c-myc-R; 5'-ACTGTTTGACAAACCGCATCC-3'

β-catenin binding site of OCT4-F;5'-CATTGTCCTGCCCCCTTC-3'

β-catenin binding site of OCT4-R: 5'-CTGGGTGGGGAAAACCAG-3'

OCT4 transcription start site -F; 5'-TGAGTAGTCCCTTCGCAAGC-3'

OCT4 transcription start site -R; 5'-ATCACCTCCACCACCTGGA-3'

SP1 binding site of OCT4-F; 5'-CAAACATCCTTCGCCTCAGT-3'

SP1 binding site of OCT4-R; 5'-TGCGAAGGGACTACTCAACC-3'

OCT4 exon4-F; 5'-GGTCCGAGTGTGGTTCTGTAA-3'

OCT4 exon4-R; 5'-CTGAGAAAGGAGACCCAGCA-3'

GAPDH-TSS-F; 5'-AAGACCTTGGGCTGGGACT-3'

GAPDH-TSS-R; 5'-CAAAAGAAGATGCGGCTGAC-3'

GAPDH-exon-F; 5'-CGGCTACTAGCGGTTTTACG-3'

GAPDH-exon-R; 5'-AAGAAGATGCGGCTGACTGT-3'

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FIGURE LEGENDS

Figure 1. Effects of nuclear actin on the subcellular localization of β-catenin

(A) Actin, tagged with a nuclear localization signal and either with EYFP (EYFP-NLS-actin, upper panels) or with mCherry (mCherry-NLS-actin, lower panels), was expressed in HeLa cells, and cells were observed under a laser-scanning confocal microscope. The cell nucleus was stained with DAPI, and β-catenin was immunostained with a specific β-catenin antibody and a secondary antibody conjugated either with Alexa568 (upper panels) or with Alexa488 (lower panels). Images of cells after staining with DAPI and antibody were obtained as described in Materials and Methods. The arrowheads show the β-catenin that co-localized with the F-actin bundle in the nucleus. (B) Images of HeLa cells expressing EYFP, EYFP-actin, or EYFP-NLS-actin, which were cultured in the serum-free medium for 24 h and then observed under a wide field microscope. Bar=10 μm. (C) Signal intensities of β-catenin from the nucleus and cytoplasm of HeLa cells expressing EYFP, EYFP-actin, or EYFP-NLS-actin were measured after the cells were cultured in serum-free medium for 24 h. The relative amount of β-catenin in the nucleus was represented as the ratio of signal intensities of nuclear and cytoplasmic β-catenin, and is shown as box plot. The relative amount of actin in the nucleus of cells expressing EYFP was defined as 1.0. Data shown are mean ± s.e.m. of three independent experiments (n >30 cells/experiment). **, P<0.01 (in C).

Figure 2. Nuclear actin-mediated translocation of β-catenin to the nucleus of cells grown in serum containing medium

(A) HeLa cells expressing EYFP, EYFP-actin or EYFP-NLS-actin were cultured in serum containing medium supplemented with (right) or without (left) 10μM XAV939 and amounts of nuclear β-catenin in these cells (box plot) were determined as described in the legend of Fig. 1C. (B)

HaCaT cells expressing EYFP, EYFP-actin or EYFP-NLS-actin were cultured in serum containing medium for 24 h and then these cells were observed under a wide field microscope. DNA and β-catenin were detected following staining with DAPI and immunostaining with an anti-β-catenin antibody, respectively. Bar=10 μm. (C) HaCaT cells expressing EYFP, EYFP-actin or EYFP-NLS-actin were cultured in serum containing medium for 48 h and amounts of β-catenin in these cells (box plot) were determined as described in the legend of Fig. 1C. Data shown are mean ± s.e.m. of three independent experiments (n>24 cells/experiment). **, P<0.01 (in A and C).

Figure 3. Nuclear actin enhanced transcriptional function of β-catenin

(A) The TCF/LEF reporter plasmid construct was introduced into HeLa cells expressing EYFP, EYFP-actin or EYFP-NLS-actin, and the cells were cultured in serum-free medium for 24 h. The TCF/LEF responsive luciferase activity was measured in each sample as described in Materials and Methods, and the observed activity was plotted relative to that in cells expressing EYFP, the value for which was assigned as 1.0. (B) Quantitative RT-PCR analysis of mRNAs of β-catenin-targeting genes. Expression levels of c-myc (*MYC*), cyclin D (*CCND1*), OCT4 (*OCT4*), TCF-1 (*TCF-1*) and c-Jun (*JUN*) in HeLa cells expressing EYFP-actin or EYFP-NLS-actin were quantified by a qRT-PCR assay. The expression level of each gene was normalized with respect to that of *GAPDH* gene and was expressed relative to that in HeLa cells expressing EYFP-actin.(C) Growth curves of HeLa cells expressing EYFP, EYFP-actin or EYFP-NLS-actin in the serum-free medium. Cell growth was measured using a TC20 automated cell counter (BioRad). *, P<0.05 (in A, B and C); **, P<0.01 (in A). Data shown are mean ± s.e.m. of at least three independent experiments.

Figure 4. Increased recruitment of β-catenin to its binding sites by expressing polymerizable

nuclear actin

The binding of endogenous β-catenin to its targeting sites in cyclin D (A, *CCND1*) and OCT4 (B, *OCT4*) genes was analyzed by ChIP assay using an antibody specific for β-catenin. The binding of β-catenin was quantified by real-time PCR analysis of total RNA prepared from HeLa cells expressing EYFP-actin, EYFP-NLS-actin, or EYFP-NLS-G13R-actin. The values shown are relative to the value in cells expressing EYFP-actin. Data shown are mean ± s.e.m. of at least three independent experiments. *, $P<0.05$ (in A and B); **, $P<0.01$ (in B).

Figure 5. Association of polymerizable actin to β-catenin binding sites.

The binding of exogenously expressed actin (EYFP-actin, EYFP-NLS-actin, and EYFP-NLS-G13R-actin) to OCT4 and GAPGH genes (A, *OCT4* and *GAPDH*) and cyclin D gene (B, *CCND1*) was analyzed by ChIP assay using anti-EYFP magnetic beads followed by quantitative PCR analysis. In A, the targeted sites of *OCT4* gene (promoter, SPI, TSS and exon 4) are indicated over the plot. The values shown are relative to that in cells expressing EYFP-actin. Data shown are mean ± s.e.m. of at least three independent experiments. *, $P<0.05$ (in A and B); **, $P<0.01$ (in A).