Both ERK and Wnt/β-catenin pathways are involved in Wnt3a-induced proliferation

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Summary

The Wnt family of proteins regulates development and cell growth. We identified Wnt3a-based regulatory mechanisms for cell proliferation in NIH3T3 fibroblast cells. The degree of Wnt3a-induced proliferation was reduced by β-catenin small interfering RNA (siRNA) and extracellular signal-regulated kinase (ERK) siRNA, indicating that both the ERK and Wnt/β-catenin pathways are involved in Wnt3a-induced proliferation. Wnt3a immediately and transiently activated the Raf-1-MEK-ERK cascade in a manner distinct from that of the β-catenin increase seen in cells treated with Wnt3a. Wnt3a-induced ERK activation was maintained even though basal ERK activities were reduced by β-catenin siRNA, indicating that Wnt3a may activate the ERK pathway independently of β-catenin. The ERK pathway was however, activated by β-catenin transfection, which was abolished by co-transfection with dominant-negative Tcf-4. Therefore, ERK pathway activation by Wnt signaling could occur at multiple levels, including β-catenin-independent direct signaling resulting from a Wnt3a and β-catenin/Tcf-4-dependent post gene transcriptional event. Wnt3a stimulated the G1 to S phase cell cycle progression. This stimulation was reduced by the ERK pathway inhibitor, indicating that Wnt3a promotes proliferation by stimulating the ERK pathway. Wnt3a therefore stimulates the proliferation of fibroblast cells, at least in part, via activation of the ERK and Wnt/β-catenin pathways.

Key words: Wnt3a, Wnt signaling, β-catenin, Proliferation, ERK pathway

Introduction

Wnt is part of a large family of secreted glycoproteins, the members of which are involved in differentiation, organogenesis, cell migration and growth (Wodarz and Nusse, 1998; Moon et al., 1997; Miller, 2002). Upon binding to a receptor encoded by *Frizzled* genes (Sagara et al., 1998; Wang et al., 1996), Wnt activates the cytoplasmic Disheveled (Dsh) protein leading to inhibition of glycogen synthase kinase 3β (GSK3β) (Lee et al., 1999). Inhibition of GSK3β protects β-catenin against degradation by protein complexes, consisting of GSK3β, axin and adenomatous polyposis coli (APC) (Brown and Moon, 1998; Akiyama, 2000; Ikeda et al., 1998). In the resting state, very little β-catenin is present in either the cytoplasm or the nucleus because of rapid degradation by the proteasome promoted by the protein complex (Ikeda et al., 1998; Sakanaka et al., 1998). After cytoplasmic accumulation as a result of Wnt signaling, β-catenin is translocated into the nucleus where it cooperates with Tcf/Lef transcription factors for activation of target genes, such as *c-Myc* and *cyclin D1* (He et al., 1998; Tetsu and McCormick, 1999). Regulation of cell proliferation by Wnt signaling relies on activation of *c-Myc* and *cyclin D1*, followed by stimulated progression of the cell cycle (Lustig and Behrens, 2003). Aberrant regulation of the Wnt/β-catenin signaling pathway, including upregulation of β-catenin, is one of the major causes of colorectal and other cancers (Giles et al., 2003; Dimitriadis et al., 2001; Polakis, 2000; Miller, 1999; Yamada et al., 2003).

The extracellular signal-regulated kinase (ERK) pathway is a major transforming growth regulatory signaling pathway (Blenis, 1993; Sebolt-Leopold, 2000; Shapiro, 2002). The MAP kinase module kinases [Raf-1, mitogen activated protein kinase kinase (MEK) and ERK] of the ERK pathway (Cobb and Goldsmith, 1995) are activated by both Ras-dependent and independent mechanisms, and the Ras→Raf-1→MEK→ERK cascade is a common ERK activation route (Hilger et al., 2002; Marais et al., 1998). Although both the Wnt/β-catenin and the ERK pathways are major signaling pathways for cellular transformation, no significant interactions between these pathways have been identified.

We analyzed crosstalk between the Wnt/β-catenin and the ERK pathways in the regulation of cell proliferation. Evaluation of cross-regulation in the ERK pathway owing to Wnt/β-catenin signaling led to analysis of the ERK regulatory function of Wnt3a (Roelink and Nusse, 1991; Roelink et al., 1990) in fibroblast cells. Wnt3a is involved in development (Barrow et al., 2003; Galceran et al., 1999) and cytoskeletal reorganization (Shibamoto et al., 1998) and is expressed in the early mouse embryo in regions that also express Lef1 (Takada et al., 1994). Wnt3a induces transcription of the *LEF-1* promoter through both β-catenin-dependent and LEF-1-independent mechanisms (Filali et al., 2002). Recent studies identified the involvement of Wnt3a in the proliferation of several cell types without significant characterization of the mechanism itself (Boer et al., 2004; Derksen et al., 2004; Galli

et al., 2004). Wnt3a activates the Wnt/β-catenin signaling pathway in other cell lines, including mouse mammary epithelial cell line C57MG (Shibamoto et al., 1998; Haertel-Wiesmann et al., 2000) and mouse fibroblast NIH3T3 and L cells (Lee et al., 1999).

We investigated the mechanism of Wnt3a-induced cellular proliferation in mouse fibroblast cells and identified roles of both Wnt/β-catenin and ERK pathways. Wnt3a conditioned medium (Wnt3a-CM) and newly available recombinant Wnt3a proteins were used. Involvement of the Wnt/β-catenin (Moon et al., 2002) and ERK pathways in Wnt3a-induced proliferation was investigated by measuring the effect of cells transfected with β-catenin small interference RNA (siRNA) (Elbashir et al., 2001) on BrdU incorporation. We used MEK inhibitors and dominant-negative forms of the ERK pathway components to investigate ERK activation by Wnt3a via the Ras-Raf-1-MEK-ERK cascade. Differential kinetics of ERK activation and changes in the level of β-catenin, as a result of a Wnt3a time course treatment, indicate that Wnt3a-induced direct ERK activation occurs independently of β-catenin. Independence of the Wnt/β-catenin pathway (Akiyama, 2000; Brown and Moon, 1998; Ikeda et al., 1998) in Wnt3a-induced ERK pathway activation was further identified by measurement of the effects of β-catenin siRNA on Wnt3a-induced ERK activation. We found that Wnt3a stimulates proliferation of fibroblast cells via activation of the ERK and Wnt/β-catenin pathways.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics and Lipofectamine Plus reagent were purchased from Life Technologies (Grand Island, NY). ERK, Phospho-ERK (p-ERK), Myc (9E10), Flag, β-catenin and cyclin D1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-MEK (p-MEK), MEK, and horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibodies were products from Cell Signaling Biotechnology (Beverly, MA). Raf-1 antibody was purchased from transduction laboratories (Lexington, KY) and HRP-conjugated goat anti-rabbit IgG antibody was obtained from Bio-Rad laboratories (Herculus, CA). Anti-phospho-Raf-1/Ser-338 (p-Raf-1) antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and both α-tubulin and pan-Ras antibodies were obtained from Oncogene Research Products (San Diego, CA). Recombinant mouse Wnt3a expressed in Chinese hamster ovary cells was from R&D Systems (Minneapolis, MN). An enhanced chemiluminescence (ECL) system was obtained from Amersham Pharmacia (Uppsala, Sweden). A Luciferase assay kit was a Promega product (Madison, WI), and a Silencer™ siRNA construction kit was obtained from Ambion (Austin, TX). U0126 was purchased from Calbiochem (La Jolla, CA) and a protein assay solution was obtained from Bio-Rad Laboratories (Hercules, CA). 4′,6′-diamidine-2′-phenylindole dihydrochloride (DAPI) was from Boehringer Mannheim (Mannheim, Germany), goat anti-mouse-Cy™2- and goat anti-rabbit Rhodamine Red™-X-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories (West Grove, PA) and anti-BrdU monoclonal antibody was purchased from DAKO (Carpinteria, CA). An Amicon Centriprep concentrator was supplied by Millipore Corporation (Bedford, MA) and all other chemicals were purchased from Sigma (St. Louis, MO). pTOPFLASH and pFOPFLASH (Korinek et al., 1997) were provided by B. Vogelstein and K. Kinzler of Johns Hopkins Oncology Center, Baltimore. Flag-β-catein-pcDNA3.0 (Kolligs et al., 1999) and dominant-negative ΔN-Tcf-4E (Tetsu and McCormick, 1999) vectors were obtained from Eric R. Fearon of the University of Michigan School of Medicine, Ann Arbor. pSVSport1-dn-Raf was obtained from J. H. Kim of Korea University (Seoul, Korea). pCMV-MEK2A was obtained from G. Johnson of the National Jewish Medical Research Center (Denver, CO). pMTSM-MycMKP3 (Camps et al., 1998) was provided by Montserrat Camps of Serono Pharmaceutical Research Institute (Geneva, Switzerland). pcDNA3.1-H-Ras N17 (Cai et al., 1990) was obtained from David Stokoe of ONYX Pharmaceuticals (Richmond, CA). Wnt3a-producing L cells and control L cells (Shibamoto et al., 1998) were obtained from E.-H. Jho, University of Seoul, Seoul, Korea.

Cell culture and production of a Wnt3a-conditioned medium

NIH3T3, Wnt3a-producing L cells and L cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C. Wnt3a-producing cells were maintained in the presence of G418 (Lee et al., 1999; Korinek et al., 1997). L and Wnt3a-producing L cells were grown to 70% confluence in DMEM, then serum starved for 12 hours in preparation of conditioned media (Con-CM) and Wnt3a-conditioned media (Wnt3a-CM). Recovered cell culture supernatants were concentrated 40-fold on a 30 kDa cut off Amicon Centriprep.

Transfections and transient reporter assay

NIH3T3 cells were plated onto six-well plates at 50% confluence. Cell transfections were performed using Lipofectamine Plus transfection reagent (Life Technologies) according to the manufacturer's instructions. Transfection efficiencies were normalized by transfection of 50 ng of pCMV β-gal reporter (Clontech). 48 hours after transfection, cells were treated with 100 μl concentrated Con-CM, Wnt3a-CM, or 150 ng/ml of recombinant Wnt3a, then were incubated for an additional 8 hours. The cells were then rinsed twice in ice-cold phosphate-buffered saline (PBS) and resuspended in reporter lysis buffer for a Luciferase assay. Luciferase activities were normalized using β-galactosidase levels as an internal control.

Western blot analysis

NIH3T3 cells grown in DMEM with 10% FBS were treated with 0-200 μl of either Wnt3a-CM or Con-CM. For analyses using recombinant proteins, cells were treated with 150 ng/ml Wnt3a, then harvested at different times for western blot analysis (Oh et al., 2002). Where required, 20 μM U0126 was applied 1 hour before treatment with 100 μl Con-CM, Wnt3a-CM, or 150 ng/ml recombinant Wnt3a. For transient transfection analysis, NIH3T3 cells were transfected with pCMV, pcDNA3.0, pSVSport1-dn-Raf, pCMV-MEK2A, pcDNA3.1-H-rasN17, pMTSM-MycMKP3 or pcDNA3.0-Flag-β-catenin. Cells were harvested for western blot analysis 48 hours after transfection. Where required, 100 μl of either Con-CM or Wnt3a-CM was applied 30 minutes before harvesting. For preparation of proteins, attached cells were rinsed twice in ice-cold PBS, harvested, then lysed directly in Laemmli sodium dodecyl sulfate (SDS) sample buffer. Samples were then boiled and subjected to 8-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis using anti p-ERK, ERK, p-MEK, MEK, p-Raf-1 (Ser338), Myc (9E10), pan-Ras-, cyclin D1, β-catenin, α-tubulin or anti-Flag primary antibodies, followed by incubation with an appropriate horse radish peroxidase-conjugated secondary antibody. Protein bands were visualized using ECL detection.

siRNA preparation and treatment

Mouse β-catenin (GenBank accession number NM_007614), ERK1 (accession number NM_011952), and ERK2 (accession number NM_011949) mRNA target sequences were designed using a small

interfering RNA fragment (siRNA) template design tool (Ambion). The β-catenin mRNA target sequences were 5′-AAGGCTTTTC-CCAGTCCTTCA-3′ (300-320) and 5′-AAGATGATGGTGTGCC-AAGTG-3′ (1400-1420). The mouse ERK1 mRNA target sequences were 5′-AATGTTATAGGCATCCGAGAC-3′ (311-332) and 5′-AACCCAAACAAGCGCATCACA-3′ (957-978). The ERK2 mRNA target sequence was 5′-AAAGTTCGAGTTGCTATCAAG-3′ (354-375). Each siRNA was synthesized using a Silencer™ siRNA construction kit (Ambion). The resulting siRNAs were transfected into NIH3T3 cells using Lipofectamine Plus reagent at a concentration of 1.68 μg per 3.5 cm dish. Transfected cells were grown for 48 hours at 37°C in 5% CO_2, then harvested for western blot analysis.

Immunocytochemistry and BrdU incorporation

For immunocytochemical analysis, NIH3T3 cells were plated onto coverslips at 30% confluence, then placed in six-well plates and grown in DMEM supplemented with 10% FBS. The cells were treated with 150 ng/ml recombinant Wnt3a for 30 minutes and transferred to DMEM containing 10% FBS for 8 hours before immunocytochemical analysis (Oh et al., 2002; Park et al., 2002). The cells were washed twice with PBS, then fixed in a methanol/formaldehyde (99:1) mixture at –20°C for 20 minutes. Cells were permeabilized with 0.2% Triton X-100 at 4°C for 30 minutes, then gently washed three times with PBS. Goat anti-mouse Cy™2-conjugated or goat anti-rabbit Rhodamine-conjugated secondary antibody at a dilution of 1:200 was added and cells were incubated for 1 hour. After washing with PBS three times, cells were treated with 1 μM DAPI in PBS for 10 minutes and were then washed again three times with PBS. For a BrdU incorporation study, cells were plated onto coverslips at 40% confluence. Cells were then treated with 150 ng/ml recombinant Wnt3a in DMEM supplemented with 1% FBS. Where required, 20 μM U0126 was added for 1 hour before recombinant Wnt3a treatment. For siRNA treatment studies, cells were transfected both with and without 1.68 μg siRNA in a 3.5 cm dish. 24 hours after transfection, cells were treated with 150 ng/ml recombinant Wnt3a in DMEM supplemented with 1% FBS for 24 hours before immunocytochemical analysis. Cells were grown for 5 hours in DMEM containing 20 μM BrdU before immunocytochemical analysis, then washed twice in PBS, fixed in a methanol/formaldehyde (99:1) mixture, and permeabilized with PBS containing 0.2% Triton X-100. The cells were then fixed in 3.7% formaldehyde for 10 minutes before incubmation for 30 minutes in 2 N HCl. After blocking with PBS containing 1% bovine serum albumin (BSA), the cells were incubated with anti-BrdU monoclonal antibody at a dilution of 1:20 for 2 hours, then washed with PBS. Goat anti-mouse Cy™2-conjugated secondary antibody at a dilution of 1:200 was added and the cells were incubated for 1 hour. DAPI was then applied at a final concentration of 1 μM in PBS for 10 minutes and the cells were washed in distilled water. Samples were mounted for photography on a Radiance 2100 laser-scanning system (Bio-Rad, UK). Each analysis was performed at least three times.

Flow cytometry

NIH3T3 cells were grown to 50% confluence in DMEM containing 10% FBS and the cells were synchronized by double thymidine blocking (Park et al., 2002). The cells were treated with 150 ng/ml Wnt3a in DMEM containing 1% FBS for 24 hours before being subjected to fluorescence-activated cell sorting (FACS) analysis. In some cases, the cells were treated with 20 μM U0126 for 1 hour before Wnt3a treatment. Cells collected from six-well plates were rinsed twice in PBS and fixed by adding 70% ice-cold ethanol. The cells were then washed in PBS containing 1% FBS. Subsequently, DNA was stained with 100 μg/ml propidium iodide for 30 minutes at 37°C. The cell cycle profile was determined using a Becton Dickinson FACS Caliber. Data were analyzed using the ModFit LT 2.0 program (Verity Software House, Inc., ME) and the WinMDI 2.8 program created by Joseph Trotter of Scripps Research Institute, CA.

Results

Wnt3a activates the Wnt/β-catenin pathway

The β-catenin and cyclin D1 protein levels in NIH3T3 cells were significantly increased by Wnt3a treatment for 8 hours (Fig. 1A), indicating normal functionality of Wnt3a in the activation of the Wnt-β-catenin pathway (Akiyama, 2000; Brown and Moon, 1998). The ratio of the Luciferase activity from a Tcf-responsive reporter construct (pTOPFLASH) and a control Luciferase reporter gene construct (pFOPFLASH) representing Tcf/β-catenin mediated gene transcription as a result of Wnt/β-catenin pathway activation (Korinek et al., 1997), was also increased approximately twofold by Wnt3a treatment, compared to untreated control cells (Fig. 1B). Similar increases in β-catenin and cyclinD1 levels and activation of the Wnt3/β-catenin pathway reporter were also observed in cells treated with a Wnt3a conditioned medium (Wnt3a-CM). Cells treated with a control conditioned (Con-CM) medium were unaffected (data not shown).

Both the Wnt/β-catenin and ERK pathways are involved in Wnt3a-induced proliferation of NIH3T3

We measured BrdU incorporation resulting from Wnt3a treatment to investigate the role of Wnt3a in cell proliferation and found that the percentage of BrdU-incorporating cells was increased approximately twofold by Wnt3a treatment (Fig. 2A, left panel; representative data shown in Fig. 2B). NIH3T3 cells were transfected with β-catenin siRNA (Elbashir et al., 2001) and the change in cell proliferation was measured to characterize involvement of β-catenin in Wnt3a-induced proliferation. The percentage of BrdU-positive cells was reduced from 18% to 12% after transfection with β-catenin

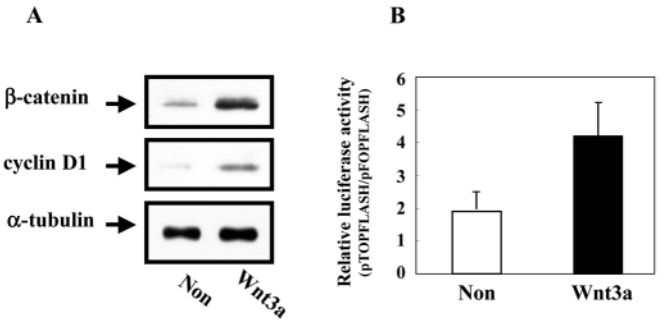

Fig. 1. The effects of Wnt3a on activation of the Wnt/β-catenin pathway. (A) NIH3T3 cells were grown to 70% confluence and either not treated or treated with 150 ng/ml of recombinant Wnt3a for 8 hours. Whole cell lysates were subjected to western blotting with β-catenin, cyclin D1 or α-tubulin primary antibodies. (B) NIH3T3 cells were grown to 50% confluence, then transfected with either 0.5 μg of pTOPFLASH or pFOPFLASH (Korinek et al., 1997). 48 hours after transfection, cells were harvested for a Luciferase assay. Where required, 150 ng/ml of recombinant Wnt3a was applied 8 hours before harvesting. The mean values and standard deviations of three independent experiments are shown.

siRNA, which caused a reduction in the β-catenin level (Fig. 2A, right panel). The percentage of BrdU positive cells was decreased more (from 43% to 30%) by β-catenin siRNA transfection in Wnt3a-treated cells compared to non-treated cells (Fig. 2A, left panel; representative data shown in Fig. 2B). Although Wnt3a-activated BrdU incorporation was reduced by β-catenin siRNA treatment, the level of inhibition was less than the reduction in the β-catenin protein level (Fig. 2A, right panel), indicating involvement of a pathway other than the Wnt/β-catenin pathway.

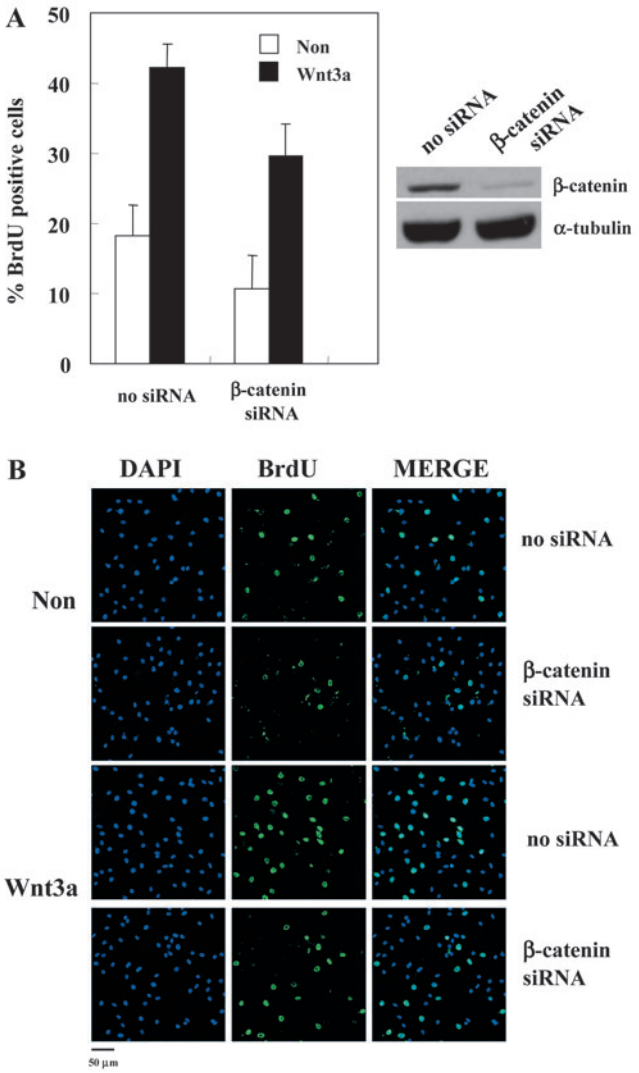

Fig. 2. Effects of β-catenin siRNAs on Wnt3a-induced BrdU incorporation. NIH3T3 cells were grown on coverslips in DMEM and either not transfected or transfected with 1.68 μg of β-catenin siRNA per 3.5 cm dish for 48 hours. Cells were labeled with 20 μM BrdU for 5 hours prior to cytochemical analysis. Where required, 150 ng/ml of purified Wnt3a was applied 24 hours before cytochemical analysis. Cell nuclei were stained with DAPI. Cells containing BrdU incorporated into the nucleus were scored as BrdU-positive. (A) Quantitative measurement of the percentage of BrdU-positive cells. Analyses were performed at least three times and 100 cells were counted in each case. Error bars indicate the standard deviations of three independent analyses. The right panel shows western blot analysis of β-catenin after transfection of NIH3T3 cells with β-catenin siRNA. (B) DAPI, BrdU and merged images of representative non-transfected and β-catenin transfected cells after Wnt3a treatment as summarized in A. Bar, 50 μm.

We also measured the effects of siRNA-induced reductions in ERK kinase levels in order to investigate involvement of the ERK pathway in Wnt3a-induced proliferation. The ERK1 and ERK2 proteins are 44 and 42 kDa proteins, respectively, and have overlapping functions in cell proliferation (Bokemeyer et al., 1997). siRNA transfection into NIH3T3 cells caused the percentage of BrdU-positive cells to drop from 18% to 12% for ERK1 and from 18% to 11% for ERK2 (Fig. 3A, left panel; representative data are shown in Fig. 3B). The percentage of BrdU-positive cells was similarly reduced by co-transfection with ERK1 and ERK2 siRNAs. The percentage of BrdU-positive cells was reduced from 43% to 38% for ERK1 siRNA and from 43% to 27% for ERK2 siRNA in cells stimulated with Wnt3a. The percentage of BrdU-positive cells was further reduced from 43% to 23% by ERK1 and ERK2 siRNA co-transfection in cells treated with Wnt3a (Fig. 3A). Therefore, the ERK1 and ERK2 proteins are involved in Wnt3a-induced proliferation of NIH3T3 cells. The reduction in ERK protein activity was visualized by western blot analysis, indicating that the ERK1 level is reduced less than the ERK2 level by siRNA treatment (Fig. 3A; right panel). ERK1 and ERK2 activities were reduced by ERK1 and ERK2 siRNAs, respectively. Similar patterns of BrdU incorporation levels were observed after Wnt3a-CM treatment in cells both treated and untreated with siRNAs for β-catenin and ERKs (data not shown), indicating that Wnt3a-CM functions in a similar manner to purified Wnt3a to induce the mechanism for proliferation of fibroblast cells.

Wnt3a induces transient activation of the Raf-1, MEK and ERK kinases in fibroblast cells

The activation status of the Raf-1, MEK and ERK kinases (Cobb and Goldsmith, 1995) was determined in NIH3T3 cells after Wnt3a treatment in order to investigate involvement of the ERK pathway in Wnt3a-induced growth stimulation. The p-ERK level, which represents the ERK activation status (Park et al., 2002), was increased within 30 minutes of treatment with recombinant Wnt3a. The level was then found to be decreased after 12 and 24 hours (Fig. 4A). The p-MEK level, which also represents the activation status of MEK (Park et al., 2002), was similarly increased 30 minutes after Wnt3a treatment, followed by a decrease. The p-Raf-1 (Ser338) level was also increased 30 minutes after Wnt3a treatment and remained increased after 12 and 24 hours (Fig. 4A). The position of the Raf-1 (Ser338) band, however, was somewhat higher after 30 minutes of Wnt3a treatment compared to that observed in untreated cells and cells treated with Wnt3a for 12 and 24 hours (Fig. 4A). Therefore, Raf-1 modification status is different in cells treated with Wnt3a for 30 minutes compared to cells treated with Wnt3a for 12 and 24 hours. The β-catenin protein levels were only slightly increased 30 minutes after treatment, but were significantly increased at both 12 and 24 hours after Wnt3a treatment (Fig. 4A).

To illustrate the kinetics of activation of the ERK pathway components after Wnt3a treatment, we treated cells with Wnt3a-CM and monitored the effects on activation of the ERK pathway components and β-catenin. The p-ERK level was increased within 5 minutes and then decreased thereafter (Fig.

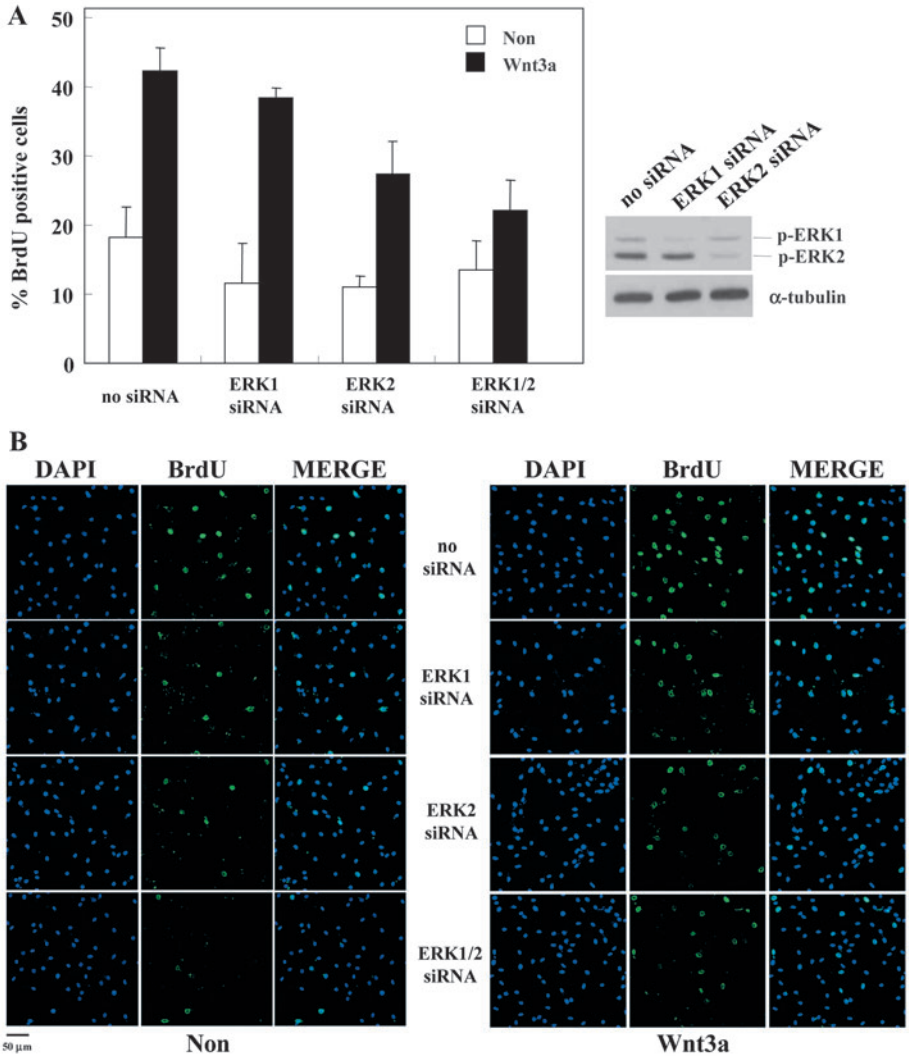

Fig. 3. Effects of ERK1 and ERK2 siRNAs on Wnt3a-induced BrdU incorporation. NIH3T3 cells were grown on coverslips in DMEM and either not transfected or transfected with ERK1 or ERK2 siRNAs, or ERK1/ERK2 siRNA for 48 hours. Cells were labeled with 20 μM BrdU 5 hours prior to cytochemical analysis. Where required, 150 ng/ml purified Wnt3a was applied 24 hours before cytochemical analysis. Cell nuclei were stained with DAPI. (A) Quantitative measurement of the percentage of BrdU-positive cells. Analyses were performed at least three times and 100 cells were counted in each case. Error bars indicate the standard deviations of three independent analyses. The right panel shows the western blot analysis of p-ERK1 and p-ERK2 after transfection of NIH3T3 cells with ERK1 and ERK2 siRNA, respectively (B) DAPI, BrdU and merged images of representative non-transfected and ERK1/2 transfected cells after Wnt3a treatment as summarized in A. Bar, 50 μm.

4B; left panel). The p-MEK and p-Raf-1 (Ser338) levels were similarly increased, followed by a decrease. β-catenin protein levels were increased in a step-wise manner up to 8 hours (Fig. 4B; left panel). Raf-1, MEK and ERK kinase activation and a β-catenin increase were not observed in cells treated with Con-CM (Fig. 4B; lower left panel), indicating specificity for Wnt3a-CM in activation of the MAP kinase module.

Wnt3a-CM-induced activation levels of Raf-1, MEK, and ERKs were dose-dependently upregulated by increasing the concentration of Wnt3a-CM (Fig. 4B; right panel). The β-catenin protein level was slightly increased 30 minutes after treatment with Wnt3a-CM at increased concentrations (Fig. 4B; right panel). However, Raf-1, MEK and ERK kinases were not activated by Con-CM treatment at any concentration (Fig. 4B; lower right panel), indicating specificity for Wnt3a-CM in activation of the MAP kinase module. We also observed transient activation of Raf-1, MEK and ERK kinases upon treatment of L cells with Wnt3a-CM (Fig. 4C; upper panel) whereas no activation was observed after treatment with Con-CM (Fig. 4C; lower panel).

Wnt3a induces transient nuclear accumulation of p-ERKs in NIH3T3 cells

The change in the sub-cellular localization of activated endogenous ERK protein was monitored by immunocytochemical analysis to investigate ERK pathway regulation by Wnt3a. Endogenous p-ERK levels were evenly distributed in both the cytoplasm and the nucleus in the resting status measured after Wnt3a treatment (Fig. 5). The p-ERK levels were highly enriched in the nucleus and the perinuclear area 30 minutes after treatment with purified Wnt3a. The p-ERK protein level was somewhat reduced within the nucleus and was redispersed to the cytoplasm at 8 hours after Wnt3a treatment (Fig. 5). The β-catenin protein level was also evenly distributed in both the cytoplasm and the nucleus in resting state cells. However, β-catenin was enriched in the nucleus 30 minutes after Wnt3a treatment, at levels similar to that of p-ERK (Fig. 5). However, unlike p-ERK, the β-catenin level was increased more in the nucleus 8 hours after Wnt3a treatment (Fig. 5).

Wnt3a activates the ERK pathway in NIH3T3 cells

NIH3T3 cells pre-treated with the MEK inhibitor U0126 were analyzed for the effect of Wnt3a on ERK activation in order to identify the mechanism of ERK activation by Wnt3a. Wnt3-induced (Fig. 6A; left panel) and Wnt3A-CM-induced (Fig. 6A; right panel) ERK activation was significantly reduced by pre-treatment with U0126. The effect of overexpression of dominant- negative (dn) forms of the Raf-1, MEK and ERK in Wnt3a-CM-induced ERK activation was also investigated to examine the mechanism of ERK activation by Wnt3a. Wnt3a-CM-induced ERK activation was significantly blocked by overexpression of dn-Raf-1, dn-MEK or dn-Ras (Fig. 6B; left and right panels). Levels of ERK activity inhibition were nearly equivalent to the level of inhibition resulting from

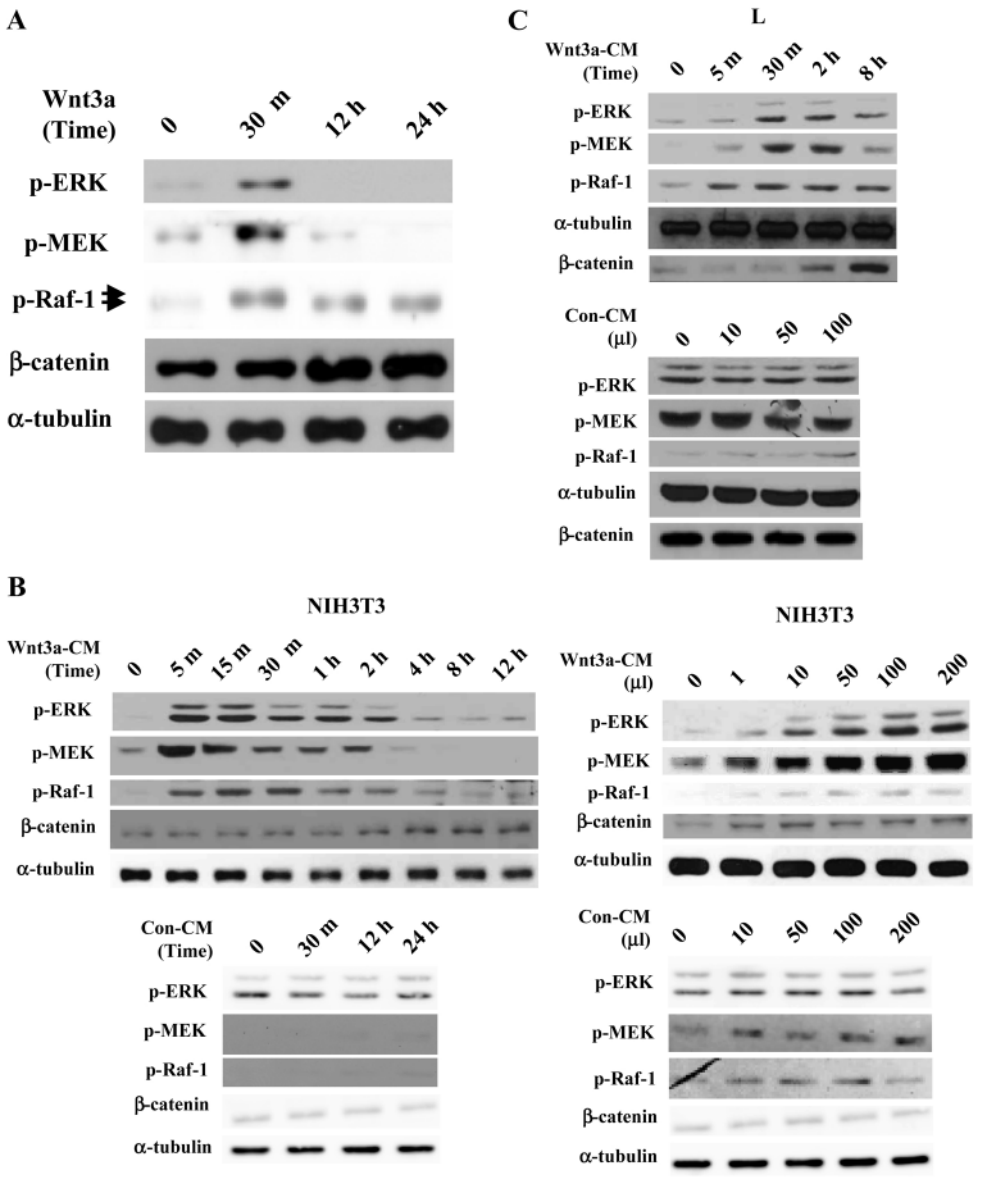

Fig. 4. Activation of ERK, MEK and Raf-1 kinases by Wnt3a in NIH3T3 and L cells. (A) NIH3T3 cells were grown in DMEM and treated with 150 ng/ml recombinant Wnt3a. Cells were harvested at 0, 30 minutes, 12 hours and 24 hours. p-ERK, p-MEK, p-Raf (Ser338), α-tubulin, β-catenin and cyclin D1 proteins were detected by western blot analysis. The p-Raf-1 (Ser338) bands differentially modified were marked with arrows. (B) Left panels, NIH3T3 cells were grown in DMEM and treated with 100 μl Wnt3a-CM (upper panel) or Con-CM (lower panel). Cells were harvested at 0, 5 minutes, 15 minutes, 30 minutes, 1 hour, 2, 4, 8, 12 and 24 hours. The p-ERK, p-MEK, p-Raf (Ser338), β-catenin and α-tubulin proteins were detected by western blot analysis (see A) Right panels, NIH3T3 cells were grown in DMEM and treated with different amounts of Wnt3a-CM (0, 1, 10, 50, 100 or 200 μl; upper panel) or Con-CM (0, 10, 50, 100 and 200 μl; lower panel). Cells were harvested 30 minutes after either Con-CM or Wnt3a-CM treatment for western blot analysis (see A). (C) Upper panel, L cells were grown in DMEM and treated with 100 μl Wnt3a-CM. Cells were harvested at 0, 5 minutes, 30 minutes, 2 and 8 hours after Wnt3a-CM treatment. Lower panel, L cells were grown in DMEM and treated with different amounts of Con-CM (0, 10, 50 or 100 μl). Cells were harvested 30 minutes after Con-CM treatment and western blot analyses were then performed.

overexpression of the potent in vivo ERK regulator MKP-3 (Camps et al., 1998; Fjeld et al., 2000) (Fig. 6B). MEK activities were reduced by overexpression of dn-MEK, dn-Raf-1, and dn-Ras but not by MKP-3 (Fig. 6B; left and right panels). The Raf-1 activity was slightly reduced by overexpression of dn-Raf-1 and dn-Ras, but was slightly increased by inhibition of the downstream component MEK by dn-MEK (Fig. 6B; left and right panels).

Wnt3a-induced ERK activation occurs independently of β-catenin

The effect of a reduction in the β-catenin level in Wnt3a-induced direct ERK activation was analyzed by using siRNA transfection to investigate involvement of the Wnt/β-catenin pathway in Wnt3a-induced ERK activation. Approximately 60% of the endogenous β-catenin protein level was lost from total cell extracts after β-catenin siRNA transfection (Fig. 7). Although ERK activities were reduced by β-catenin siRNA, the Wnt3a-induced ERK activation was maintained (Fig. 7A; left panel). The ERK activation effect of Wnt3a-CM was also maintained in cells treated with β-catenin siRNA (Fig. 7A; right panel). Therefore, Wnt3a-induced ERK activation may occur independently of the β-catenin level. The reduction of ERK activity by β-catenin siRNA indicates that endogenous β-catenin activates ERKs. Therefore, the effect of β-catenin overexpression on activation of ERK pathway components was analyzed. We found that Raf-1, MEK, and ERK activities were concomitantly increased by transient β-catenin overexpression (Fig. 8A). The β-catenin-induced ERK activation was mostly abolished by U0126 pre-treatment (Fig. 8B).

Activation of Wnt signaling leads to accumulation of nuclear β-catenin that is involved with a T-cell factor, such as Tcf-4, in the colorectal epithelium (Korinek et al., 1997). We co-transfected dominant-negative ΔN-Tcf-4E (Tetsu and McCormick, 1999) together with β-catenin and measured the effect of β-catenin on ERK activation to determine whether β-catenin activates ERK by β-catenin/Tcf-4-mediated gene

expression or by an alternative mechanism. ΔN-Tcf-4E significantly inhibited ERK activation by β-catenin overexpression (Fig. 8C). The β-catenin-mediated activations of Raf-1 and MEK kinases were similarly blocked by co-transfection with ΔN-Tcf-4E and β-catenin.

Wnt3a stimulates the G1 to S phase cell cycle progression involving ERK activation

The increase in BrdU incorporation upon addition of Wnt3a indicates that Wnt3a-induced cell proliferation probably occurs via stimulation of the cell cycle progression. We performed FACS analysis to investigate the role of Wnt3a in cell cycle stimulation. Wnt3a stimulated the G1 to S phase cell cycle progression of NIH3T3 cells with the relative proportion of cells in S phase increasing from 3.5% to 9.5% upon Wnt3a treatment (Fig. 9A). The induced G1 to S phase cell cycle progression after Wnt3a treatment was reduced from 9.5% to 7.5% by pre-treatment with the chemical MEK inhibitor U0126. The Wnt3a-induced increase in the number of BrdU-positive cells was also blocked by treatment with U0126 (Fig. 9B), as shown by cells treated with ERK siRNAs (Fig. 3).

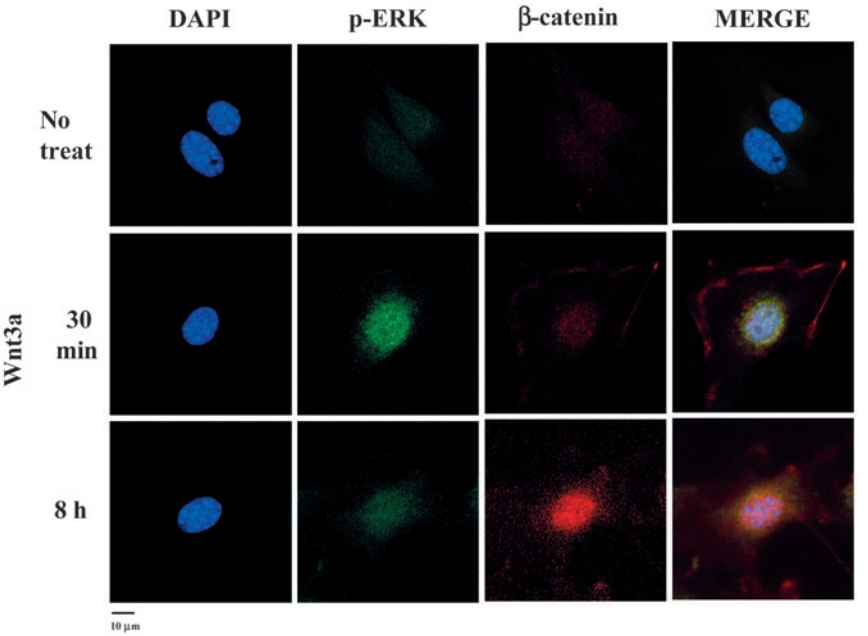

Fig. 5. Effects of Wnt3a on nuclear accumulation of the p-ERK and β-catenin proteins in NIH3T3 cells. NIH3T3 cells were grown in DMEM and treated with 150 ng/ml recombinant Wnt3a. Cells were subjected to immunocytochemical analysis at 0, 30 minutes and 8 hours after Wnt3a treatment. Cells were then treated with anti-p-ERK and anti-β-catenin antibodies. p-ERK proteins were detected as a green color using Cy^{TM}2-conjugated goat anti-mouse IgG, β-catenin proteins were detected as a red color using anti-rabbit-Rhodamine Red ^{TM}-X-conjugated secondary antibody. Cell nuclei were stained with DAPI. Bar, 10 μm.

Discussion

The Wnt pathways are involved in regulation of differentiation, organogenesis, adipogenesis and proliferation (Akiyama, 2000; Polakis, 2000; Ross et al., 2000). The pathways are activated by various Wnt proteins, including Wnt1, Wnt2, Wnt2b, Wnt3a, Wnt5b, Wnt7b and Wnt8a (Dimitriadis et al., 2001; Adamson et al., 1994), several of which induce cell growth and transformation (Polakis, 2000; Wong et al., 1994; Shimizu et al., 1997). Wnt signals are transduced through at least three distinct intracellular pathways, including the canonical Wnt/β-catenin signaling pathway, the Wnt/Ca^{2+} pathway, and the Wnt/polarity pathway (reviewed by Miller,

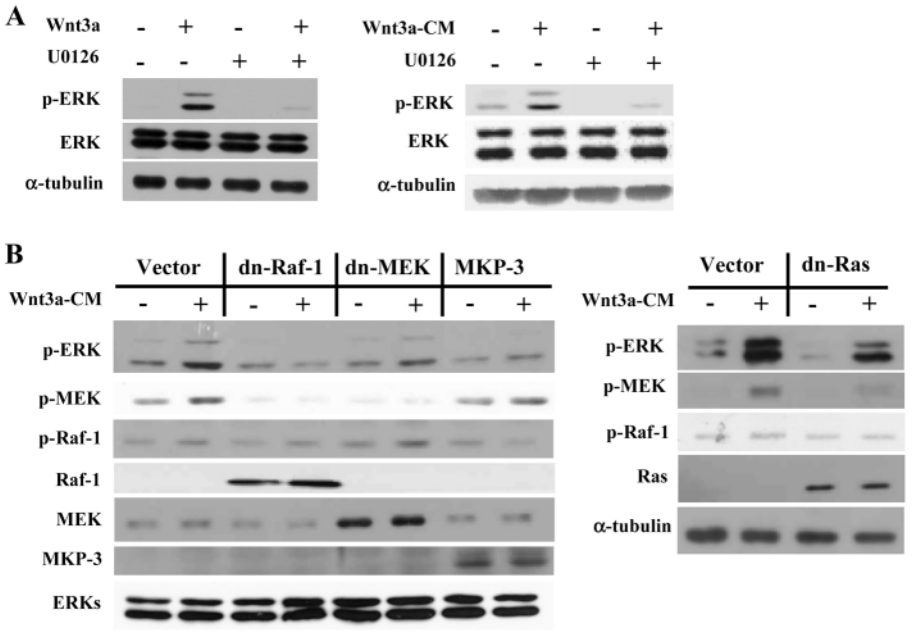

Fig. 6. The effects of ERK pathway inhibitors on Wnt3a-induced Raf-1, MEK, and ERK kinase activation. (A) NIH3T3 cells were grown in DMEM and either not treated or treated with 150 ng/ml recombinant Wnt3a (left panel) or with 100 μl Con-CM or Wnt3a-CM (right panel) for 30 minutes before harvesting. Where required, 20 μM U0126 was added 1 hour before the Con-CM or Wnt3a-CM treatment. (B) NIH3T3 cells were grown in DMEM and transfected with 0.5 μg pCMV vector, 0.5 μg pSVSport1-dn-Raf-1, 0.5 μg pCMV-MEK2A vector, 0.5 μg pMTSM-MycMKP-3 (left panel) and 0.5 μg pcDNA3.0 vector or pcDNA3.1 H-Ras N17 (right panel). 48 hours after transfection, the cells were treated for 30 minutes with 100 μl Con-CM or Wnt3a-CM. Cells were harvested and subjected to western blot analysis.

2002; Huelsken and Behrens, 2002). The Wnt/β-catenin pathway is activated by Wnt-1, Wnt-3, Wnt-3a, Wnt-7a, and Wnt-8, and is involved in transformation (Miller et al., 1999; Kolligs et al., 2002). Wnt3a involvement in the proliferation of chick somites (Galli et al., 2004), human mesenchymal stem cells (Boer et al., 2004) and multiple myeloma cells (Derksen et al., 2004) has been investigated. We investigated the mechanism of Wnt3a-mediated cell proliferation in mouse fibroblast cells associated with the Wnt/β-catenin (Akiyama, 2000; Brown and Moon, 1998; Ikeda et al., 1998; Moon et al., 2002) and the ERK transforming pathways (Blenis, 1993; Sebolt-Leopold, 2000; Shapiro, 2002).

Wnt3a-induced increases in the β-catenin level and Tcf-4/β-catenin responding reporter activity indicate that Wnt3a activates the Wnt/β-catenin pathway. The activities of Raf-1, MEK, and ERK kinases were concomitantly increased by

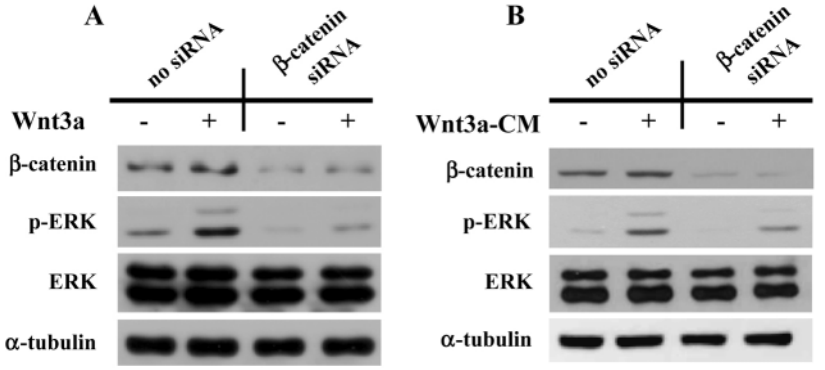

Fig. 7. The effects of β-catenin siRNA on Wnt3a-induced ERK activation. NIH3T3 cells were either not transfected or transfected with β-catenin siRNA for 48 hours. (A) Cells were either treated or not treated with 150 ng/ml recombinant Wnt3a for 30 minutes before harvesting. (B) Cells were treated with 100 μl Con-CM or Wnt3a-CM for 30 minutes before harvesting. Cell extracts were then prepared, and western blot analyses were performed.

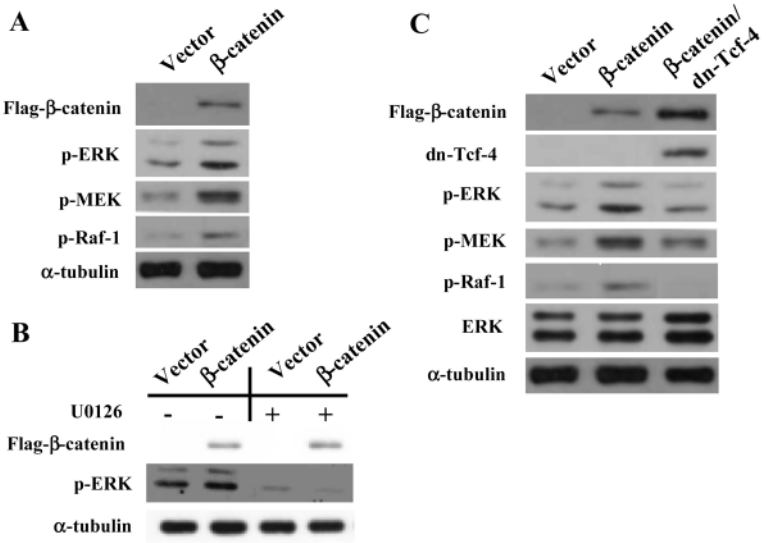

Fig. 8. Activation of the ERK pathway by β-catenin overexpression, and effects of dominant-negative Tcf-4 on β-catenin-induced ERK pathway activation. (A) NIH3T3 cells were grown in DMEM and transfected with either 0.5 μg pcDNA3.0 vector or 0.5 μg Flag-β-catenin-pcDNA3.0. Cells were harvested 48 hours after transfection. (B) NIH3T3 cells were grown in DMEM and transfected with either 0.5 μg pcDNA3.0 vector or 0.5 μg Flag-β-catenin-pcDNA3.0. Where required, cells were treated with 20 μM U0126 1 hour before harvesting. Anti-Flag antibody was used for detection of Flag-β-catenin. (C) NIH3T3 cells were grown in DMEM and transfected with either 0.5 μg pcDNA3.0 vector or 0.5 μg Flag-β-catenin-pcDNA3.0. Cells were co-transfected with 0.5 μg of vector or ΔN-Tcf-4E together with Flag-β-catenin-pcDNA3.0. The cells were harvested after 48 hours and western blot analyses were performed with β-catenin, Flag, ERK, p-ERK, p-MEK, Raf-1 or α-tubulin antibodies.

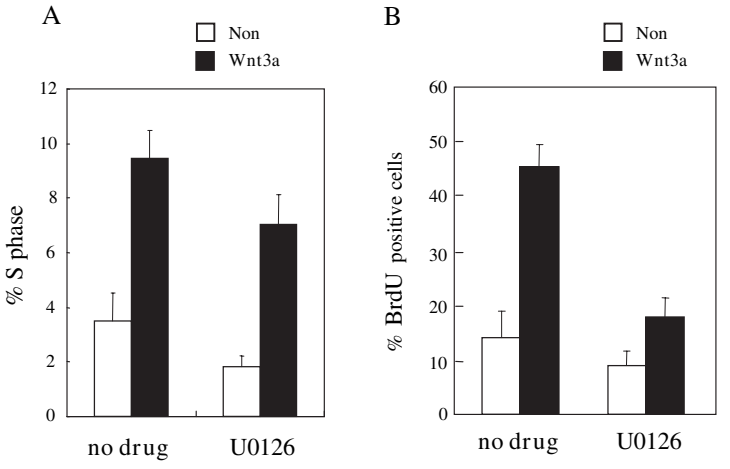

Fig. 9. The effect of Wnt3a on cell cycle progression and the effect of U0126 on NIH3T3 cells. (A) NIH3T3 cells were grown in DMEM and synchronized with double thymidine blocking (Park et al., 2002). Cells were either not treated or treated with 150 ng/ml recombinant Wnt3a for 24 hours before harvest for FACS analysis. Where required, 20 μM U0126 was added 1 hour before treatment with Wnt3a. Error bars indicate the standard deviations of three independent experiments. (B) The effect of U0126 on Wnt3a-induced BrdU incorporation in NIH3T3 cells. NIH3T3 cells were grown in DMEM and either not treated or treated for 24 hours with 150 ng/ml of recombinant Wnt3a in DMEM containing 1% FBS. Where required, 20 μM U0126 was applied 1 hour before treatment with Wnt3a. Cells were labeled with 20 μM BrdU for 5 hours prior to cytochemical analysis using anti-BrdU antibody. Cell nuclei were stained with DAPI. Cells containing BrdU incorporated into the nucleus were scored as BrdU positive and the relative percentage of BrdU-positive cells was determined. Analyses were performed at least three times and 100 cells were counted in each case. Error bars indicate the standard deviations of three independent analyses.

Wnt3a, indicating that Wnt3a regulates both the ERK and the Wnt/β-catenin pathways in fibroblast cells. The immediate activation of the Raf-1, MEK, and ERK kinases by Wnt3a indicates that ERK pathway activation is acquired by direct signaling rather than by post-gene transcription. The activation of Raf-1, MEK, and ERK kinases and the induction of β-catenin in L cells indicate that Wnt3a-induced ERK pathway activation is not uniquely limited to NIH3T3 cells. Wnt3a-induced ERK activation was blocked by pre-treatment of cells with the MEK inhibitor U0126. In addition, Wnt3a-induced ERK activation was also inhibited by overexpression of dn-Ras, dn-Raf-1 and dn-MEK. These results indicate that Wnt3a activates the ERK pathway via the Ras→Raf-1→MEK→ERK cascade.

To identify involvement of the Wnt/β-catenin pathway in ERK pathway activation, we reduced the β-catenin level using siRNA and found that ERK activities were inhibited. ERK activation, which occurs within 30 minutes of Wnt3a treatment, was retained even in cells with reduced β-catenin levels upon treatment with β-catenin siRNA (Fig. 7A,B). These results indicate that the immediate ERK activation by Wnt3a (Fig. 4) may occur independently of β-catenin. A comparison of the kinetics between ERK activation and β-catenin accumulation also indicates β-catenin independent ERK activation by Wnt3a (Fig. 3A; Fig. 4A). β-catenin was accumulated at a later stage of Wnt3a signaling, although ERK activities were increased in a transient pattern soon after treatment with Wnt3a. However, β-catenin may still be involved in ERK activation, although it may not be directly associated with immediate Wnt3a-induced ERK activation. This was indicated by reduction of ERK activation in cells with lowered β-catenin levels upon β-catenin siRNA treatment (Fig. 7A,B). Involvement of β-catenin in ERK activation was further indicated by activation of ERK activities by β-catenin overexpression (Fig. 8A). The Raf-1, MEK, and ERK kinases were concomitantly increased by β-catenin overexpression (Fig. 8A). The β-catenin-induced ERK activation level was also reduced by pre-treatment with U0126, indicating that β-catenin activates ERKs via activation of the MAP kinase cascade. Reduction of β-catenin-induced ERK activation by dn-*Tcf-4* transfection (Fig. 8C) indicates that ERK pathway activation by β-catenin transfection may occur by β-catenin/Tcf-4-mediated gene transcription (He et al., 1998; Testu and McCormick., 1999). However, immediate activation of the ERK pathway by Wnt3a can occur independently of β-catenin. Although activation of the ERK pathway by β-catenin was reduced by dn-Tcf-4, we could not exclude the presence of a direct signaling mechanism involving activation of the ERK pathway by β-catenin.

The Wnt/β-catenin pathway is involved in proliferation and is mediated by stabilization of β-catenin followed by activation of a heterodimeric Tcf-4/β-catenin transcription complex (Polakis, 2000; Kikuchi, 2000). We identified involvement of the ERK and Wnt/β-catenin pathways in the Wnt3a-induced proliferation of fibroblast cells. The reduction of Wnt3a-induced BrdU incorporation by β-catenin siRNA indicates involvement of the Wnt/β-catenin pathway in cell proliferation. Wnt3a-induced cell proliferation was reduced by both ERK1 and ERK2 siRNAs and by U0126, indicating that the ERK pathway is also involved in the proliferation of NIH3T3 fibroblast cells. The degree of cell proliferation was reduced more by co-treatment with ERK1 and ERK2 siRNAs, and the level of reduction was more significant than that level achieved with β-catenin siRNA treatment. These results indicate that both the ERK and the Wnt/β-catenin pathways are involved in Wnt3a-mediated proliferation. The ERK pathway is involved in cell proliferation by activation of the cell cycle progression and transient ERK activation is an important marker for cell proliferation (Park et al., 2002; Chang et al., 2003).

The amount of DNA at S phase was increased approximately threefold by Wnt3a treatment, indicating that Wnt3a is a strong promoter of G1 to S phase progression in NIH3T3 cells. This Wnt3a-induced G1 to S phase progression was reduced by pre-treatment with U0126, indicating involvement of the ERK pathway in Wnt3A-induced cell cycle progression. The ERK pathway is involved in cell proliferation by activation of the cell cycle progression, and transient ERK activation is an important marker for cell proliferation (Park et al., 2002; Chang et al., 2003). Both the ERK and the Wnt/β-catenin pathways are therefore involved in Wnt3a-induced proliferation of fibroblast cells.

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