Ectopic Expression of Axin Blocks Neuronal Differentiation of Embryonic Carcinoma P19 Cells*

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Axin regulates Wnt signaling through down-regulation of β-catenin. To test the role of Wnt signaling in neuronal differentiation, embryonal carcinoma P19 cells (P19 EC), which can be stimulated to differentiate into a neuron-like phenotype in response to retinoic acid (RA), were used. Reverse transcription-PCR and Western blot analysis showed that Axin is expressed in undifferentiated cells, whereas the level is clearly reduced during RA-induced neuronal differentiation. Interestingly, Axin levels were not reduced during endodermal differentiation of P19 EC cells and F9 EC cells by RA, suggesting that the reduction of the Axin level is a specific property of neuronal differentiation. Western analysis showed that the cytoplasmic level of β-catenin increased during neuronal differentiation of P19 EC cells. Indirect immunofluorescence with β-catenin antibody showed that the localization of β-catenin was changed from membrane in undifferentiated cells to nuclei in neuronal P19 EC cells. Induced expression of Axin during endodermal and early neuronal differentiation, using the Tet-On system, did not block normal differentiation. However, maintenance of the Axin level blocked neuronal differentiation and inhibited expression of a neuron-specific marker protein, βIII-tubulin. Also, ectopic induction of a β-catenin signaling inhibitor, ICAT, inhibited expression of βIII-tubulin. In contrast, addition of Wnt-3A-conditioned medium during the neuronal differentiation period enhanced the expression of βIII-tubulin. Overall, our data show that Wnt-3a/canonical β-catenin signaling through the down-regulation of Axin may play an important role in neuronal differentiation.

The Wnt signaling pathway has critical roles in embryonic development, differentiation, and tumorigenesis (1–4). Currently, 19 Wnt genes have been identified in humans and most of them have homologs in other organisms (5). The tightly controlled temporal and spatial expression patterns of Wnt genes have implied that different Wnts have specific roles in embryonic development, and ablation of specific Wnt genes in mice have shown that this is true (6, 7). In particular, targeted inactivation of Wnt-1, Wnt-3a, or Wnt-7a in mice suggest that they have critical roles in neural development and neural cell fate determination (8–10).

Wnt genes encode secreted glycoproteins that signal through the cell surface receptor Frizzled, which has at least 10 orthologs in mammals, and other coreceptors (for a review, see Ref. 11). Recent data suggest that heterotrimeric G-proteins are involved in the signaling between receptors and downstream pathways (12). Upon binding of Wnts to Frizzled, Dvl's are activated and antagonize the β-catenin degradation complex, which contains adenomatous polyposis coli, GSK-3β, and Axin, as well as other proteins (13–25). β-Catenin that escapes from the degradation complex enters into nuclei and interacts with Tcf/LEF factors to regulate expression of downstream target genes (26–34).

Axin was originally identified by an insertional mutation in transgenic mice (AxinTg), which caused developmental defects similar to those in mice carrying spontaneous mutations at the genetic locus called Fused (35, 36). Ectopic expression of Axin in Xenopus embryos showed that Axin blocked embryonic axis formation by inhibition of the canonical Wnt/β-catenin pathway (37), a finding that has been confirmed by genetic analysis in Drosophila (38, 39). The accumulating data have shown that Axin acts as a scaffolding protein, containing several domains that interact with adenomatous polyposis coli, GSK-3β, CKI, β-catenin, and other proteins (13–25). In the absence of a Wnt signal, Axin itself is phosphorylated and enhances phosphorylation of β-catenin by GSK-3β by bringing those proteins together, and the ubiquitin-proteasome-mediated pathway leads phosphorylated β-catenin to degradation (26–30). However, antagonizing GSK-3β activity upon binding of Wnts to Frizzled leads to an increase in hypophosphorylated Axin, which has lower affinity to interact with β-catenin. This results in the release of hypophosphorylated β-catenin from the degradation complex (40, 41). The released hypophosphorylated β-catenin is accumulated in the cytoplasm, translocated into nuclei, and then interacts with Tcf/LEF factors to regulate downstream target gene expression. Currently, about 50 target genes have been identified. The known function of several identified target genes explains some of the phenotypes that are caused by abnormal Wnt signaling. For example, induction of c-myc or

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The abbreviations used are: GSK-3β, glycogen synthase kinase 3β; EC, embryonal carcinoma; RA, retinoic acid; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; RT, reverse transcriptase; PMSF, phenylmethylsulfonyl fluoride; MAP2, microtubule-associated protein 2; GFP, green fluorescence protein; EGFP, enhanced GFP; CMV, cytomegalovirus; Dox, doxycycline; TcflEF, T cell factor/lymphoid enhancer factor; CKI, casein kinase I; dpc, days post-coitum; rtTA, reverse tetracycline controlled transactivator; ICAT, inhibitor of β-catenin and Tcf-4.
cycin D1 expression by Wnt signaling causes abnormal cell proliferation and results in tumors, whereas ectopic Xenopus axis induction by the injection of Wnts is due to enhanced expression of the dorsalizing homeobox gene stiemois, brachyury gene, and others (42–45).

Among the diverse biological roles of Wnts, their involvement in neural differentiation has been studied due to prominent expression of several Wnts in the developing central nervous system (6, 7). Indeed, absence of Wnt-1 and Wnt-3a leads to abnormally populated dorsal interneurons, implying that Wnt signaling has a role in the determination of neuronal cell fate (46). An elegant finding by Hall et al. (10), that secretion of Wnt-7a from postsyncytial granule cell neurons remodels the axons and growth cones of developing mossy fibers, is another good example for involvement of Wnt signaling in neuronal differentiation. However, it has not been shown that Wnt signaling plays any autocrine role in neurite extension.

The pluripotent P19 embryonal carcinoma (EC) cell line has been used as a model system to study neuronal differentiation, because these cells can be easily differentiated into neuronal cells that form neurite-like structures upon simple retinoic acid treatment (47, 48). It has been reported that the expression of diverse Wnts are regulated during neuronal differentiation, and it has therefore been suggested that different Wnts may have roles in this process (49). However, although many Wnts display a dynamic expression pattern during the neuronal differentiation of P19 cells, so far only Wnt1 has been tested for a potential role in this process (49). However, many Wnts have roles in this process (49). Indeed, absence of Wnt-1 and Wnt-3a expression of the dorsalizing homeobox gene axis induction by the injection of Wnts is due to enhanced proliferation and results in tumors, whereas ectopic cyclin D1 expression by Wnt signaling causes abnormal cell division in neuronal differentiation has been studied due to prominent expression of many Wnts during neuronal differentiation, it remains unknown whether canonical β-catenin signaling is involved in the neuronal differentiation of P19 cells (49, 50).

We show here that Axin is down-regulated and that β-catenin accumulates in the cytoplasm and the nucleus, during the neuronal differentiation of P19 cells. These data imply that canonical β-catenin signaling is involved in the neuronal differentiation of P19 cells. To test the significance of canonical β-catenin signaling for this process, the expression of Axin was induced during RA-induced neuronal differentiation, using the Tet-On-inducible system (51, 52). This treatment blocked the formation of neurite-like structures. In contrast, neuronal differentiation of P19 cell was enhanced in the presence of Wnt-3a-conditioned media (53). Our overall data lead us to conclude that Wnt-3a/canonical β-catenin signaling through the down-regulation of Axin may play an important role in neuronal differentiation.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—P19 embryonal carcinoma (EC) cells (obtained from ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, HyClone Laboratories Inc.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen), in humidified 5% CO2. To induce neuronal differentiation by P19 EC cells, they were aggregated in bacterial-grade Petri dishes with 1 ml G418 (Invitrogen). For preparation of Wnt-3a-conditioned media (CM), the Wnt-3a-expressing L cells were grown in serum-free medium (DMEM, Invitrogen) supplemented with 10% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 μg/ml leukemia inhibitory factor (Volunteer Services Co., Inc.), 1 μg/ml insulin, and 1 μg/ml heparin, in 1.2% agarose in PBS. The fixed cells were incubated with blocking solution (1% normal goat serum, 1% normal horse serum, 1% BSA, and 0.2% sodium azide, Amersham Bioscience) for 1 h, followed by a 1 h incubation with a mouse monoclonal antibody against SSEA-1 (Development Studies Hybridoma Bank), or anti-βIII-tubulin (BAbCO, anti-MAP2 (Sigma), anti-β-catenin, or anti-E-cadherin antibodies (Transduction Laboratories). After a rinse with PBS, the cells were incubated with rhodamine, or fluorescein isothiocyanate-conjugated (Jackson Immunoresearch Laboratories) secondary antibodies at room temperature for 1 h, mounted, and examined using a fluorescence microscope (Zeiss).

Northern Blot and RT-PCR—Total RNA was isolated using TRIzol (Invitrogen) from undifferentiated or differentiated P19 cells at different time periods. For Northern blot analysis, ~20 μg of total RNA was loaded on a 1.2% formaldehyde–agarose gel, blotted to nitrocellulose membranes (Amersham Biosciences) by using a TurboBlotter kit (Schleicher & Schuell). The inserts for the preparation of probes for mouse Wnt-1, Wnt-3a, and Wnt-5a were generated by PCR, using the pLNCX/Wnt-1, pLNCX/Wnt-3a, and pLNCX/Wnt-5a plasmid DNA (kind gift from Dr. Jan Kitajewski, Columbia University) as templates with primers derived from sequences in GenBank™. The inserts for the mouse β-actin was obtained by RT-PCR with total RNA of P19 EC cells. The sequences of all PCR products were confirmed by automated sequencing. All probes were labeled with [α-32P]dCTP using a Random Primed DNA Labeling kit (Roche Molecular Biochemicals) and hybridized to the membrane with ExpressHyb solution (Clontech) according to the manufacturer’s protocols. The Northern blot was hybridized and hybridized to β-actin CDNA probe for even RNA loading control.

The following primers were used for PCR: for Wnt-1, 5′-CAGTAGTGGCGCAGTGTG-3′ and 5′-ATGGTGTGGTTGTCACTG-3′; for Wnt-3a, 5′-TGGTAGCTGCCAGGCTGAA-3′ and 5′-CCACAGATACGACGTGA-3′; for Wnt-5a, 5′-ATGGATATAGATCAGGCG-3′ and 5′-GTGACCAATGGCGATG-3′; for β-actin, 5′-AGGCGAACCCGGAGATG-3′ and 5′-GAAGTCCGCGGGACGATGAC-3′; for Axin, 5′-GAAGTTGTTCCCTTGGGAC-3′ and 5′-GGCTACAAATGGCGAGGT-3′; and for ICAT, 5′-GAATCTGGATACGGCCCGAGAC-3′ and 5′-CTCCAGCTAATGGCTCGGACATG-3′.

Western Blot—Cells were lysed in ice-cold PBS in buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 10% glycerol, 100 mM sodium orthovanadate, and protease inhibitor mixture (1 mM EDTA, 1 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Brains of 13.5 dpc Balb/c mouse embryos and 1-month-old postnatal mice were dissected and homogenized in lysis buffer (20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 100 mM glycerol, 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 2 mM Na3VO4, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin). Homogenates were clarified by centrifugation at 14,000 × g for 15 min at 4 °C, and total extracts were obtained in the supernatant. Protein concentration was measured using Bradford reagent (Bio-Rad). About 20 μg of lyastes was subjected to SDS-PAGE, and Western blot using following antibodies. Mouse monoclonal antibodies against myc and 12C (for the detection of myc and mβ-catenin, Oncogene), βIII-tubulin (BAbCO), MAP2, actin, α-tubulin (Sigma), GFP (Clontech), GSK-3β, β-catenin antibodies (Transduction Laboratories), and a rabbit polyclonal antibody for Axin (kind gift from Dr. Paul Polakis, Genentech Inc.) were used to detect the corresponding proteins. Peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit secondary antibodies (Sigma) were used, and then the proteins were detected by using an enhanced chemiluminescence (ECL) reagent (Santa Cruz Biotechnology, Inc.).

Preparation of Cytoplastic Fraction—Cells were washed twice in PBS and scraped in physiological buffer containing 10 mM Tris-HCl (pH 7.4), 140 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 0.5 mM PMSF, 2 μg/ml leupeptin, and 1 μg/ml aprotinin. Cells were lysed by a chilled Potter-Elvehjem homogenizer (Wheaton) at 4 °C. The lysates were centrifuged at 500 × g for 10 min to remove unbroken cells and nuclei. The cleared lysates were subject to ultracentrifugation at 100,000 × g for 90 min at 4 °C. The supernatants were collected as the cytoplastic fraction. Protein concentration of the cytoplastic fraction was measured using Bradford reagent (Bio-Rad). The protein concentration was used for Western blot using following antibodies.

Construction of Plasmids—To construct pBl-EFGP-Axin, pcS2-MT-mAxin (125–956) was digested with ClaI and NotI, and both ends were filled-in with Klenow fragment (Invitrogen). The 3-kb fragment was inserted into pBl-EFGP (Clontech), which was digested with NheI and blunted with Klenow fragment. The SpeI and HindIII digested fragment was radiolabeled with Klenow fragment of the pUHDTA2-M2 (kind gift from Dr. Wolfgang Hillen, Erlangen University (52)) to construct CMV–rTA2-M2, which has a neomycin resistance gene and improved version of rTA. Mouse ICAT cDNA was cloned by RT-PCR using total mouse RNA as template from
Neuronal Differentiation of P19 EC Cells

Differential Expression of Wnt Signaling Components during Neuronal Differentiation of P19 EC Cells—P19 embryonal carcinoma (EC) cells can be differentiated into neuronal cells upon retinoic acid (RA) treatment during aggregation followed by plating on tissue culture plates (47, 48). This process can be detected with the neuron-specific marker proteins, βIII-tubulin and MAP2 (microtubule-associated protein 2) (Fig. 1A). Two days after plating, neurite-like structures began to appear, and after 6 days these structures were obvious and clearly detected by immunohistochemistry staining with βIII-tubulin and MAP2 antibodies. It has been shown that several Wnts were differentially expressed during neuronal differentiation of P19 EC cells (49). We observed a similar pattern of Wnt expression during neuronal differentiation (Fig. 1B). Wnt-1 mRNA is transiently induced. In contrast to either Wnt-1 or Wnt-3a, the level of Wnt-5a mRNA is transiently induced upon RA treatment during aggregation and the early stages of neuronal differentiation have occurred. Interestingly, during the later stages of differentiation (4 and 6 days after plating), the level of Wnt-1 mRNA expression rapidly diminished, whereas the expression of Wnt-3a was induced. In contrast to either Wnt-1 or Wnt-3a, the level of Wnt-5a increases steadily during differentiation. These observations suggest that Wnt-1, Wnt-3a, and Wnt-5a may have different roles in P19 EC cell differentiation. The occurrence of proper neuronal differentiation was confirmed by Western blot analysis using anti-βIII-tubulin and MAP2 antibodies (Fig. 1C).

It was shown that Axin is down-regulated upon Wnt-3a treatment of C57MG and L cells (41, 54). Because the endogenous Wnt-3a level increased during neuronal differentiation of P19 EC cells, we examined the levels of Axin mRNA (Fig. 2A) and protein (Fig. 2B), and found that they were both down-
regulated during neuronal differentiation (3.2 ± 0.9-fold reduction in protein level for the 6-day-differentiated compared with undifferentiated samples \((n = 3)\)). We predicted that down-regulation of Axin might lead to stabilization of cytoplasmic \(\beta\)-catenin. As shown in Fig. 2C, cytoplasmic \(\beta\)-catenin is steadily accumulated during neuronal differentiation (4.1 ± 1.2-fold induction in undifferentiated versus in 6-day-differentiated samples \((n = 3)\)), whereas the actin and total \(\beta\)-catenin levels are unchanged. It is well accepted that accumulated cytoplasmic \(\beta\)-catenin is translocated into nuclei to regulate the expression of downstream target genes. Immunochemical staining with anti \(\beta\)-catenin antibody revealed that \(\beta\)-catenin is mainly localized in the nuclei of differentiated cells, in contrast to its membrane localization in undifferentiated cells (Fig. 2D). These data suggest that down-regulation of Axin and induction of \(\beta\)-catenin signaling is important for neuronal differentiation. To test whether the down-regulation of Axin occurs \textit{in vivo} as well as during P19 EC cell differentiation, the Axin level was compared in the brains of 13.5 dpc mouse embryos (which contain many undifferentiated neuronal precursor cells) versus brains of 1-month-old postnatal mice (which contain more differentiated neurons). The level of the neuron-specific marker \(\beta\)-III tubulin was increased during that period, as shown by others (55), whereas the Axin level was reduced (Fig. 2E). RA treatment of F9 EC, or treatment of P19 EC cells with a low concentration of RA (10 nM), can lead to endodermal differentiation (48). During endodermal differentiation of both cell lines, as monitored by morphological changes (data not shown) the Axin level was not
changed (Fig. 2F). These in vivo and in vitro data imply that the down-regulation of Axin occurs specifically during neuronal differentiation but not endodermal differentiation.

**Inducible, Ectopic Expression of Axin in RA-treated P19 Cells**

**Blocks Formation of Neurite-like Structures**—To test whether the reduction in Axin levels is important for neuronal differentiation, we used the Tet-On inducible system to force the continued expression of Axin in RA-treated P19 cells. Axin was cloned into the bi-directional pBI-EGFP vector allowing EGFP to be used to infer the level of Axin expression in the transfected cells (Fig. 3A). Cells transiently cotransfected with CMV-rtTA and pBI-EGFP-Axin showed clear induction of Axin and EGFP after Dox treatment (Fig. 3B), Axin was clearly induced in pBI-EGFP-Axin-transfected clones after 1 day of Dox treatment and disappeared in a day after removal of Dox, whereas the EGFP level was maintained (Fig. 3C), possibly reflecting an intrinsic difference in the stability of the two proteins in these cells. The rapid reduction in Axin levels after withdrawal of Dox was useful, in that it allowed us to induce Axin expression in transient manner.

P19 cell clones stably transfected with pBI-EGFP-Axin/CMV-rtTA2S-M2 or pBI-EGFP/CMV-rtTA2S-M2 were treated with Dox. This resulted in clear induction of myc-tagged Axin in pBI-EGFP-Axin/CMV-rtTA2S-M2 (Fig. 4A, lanes 2 and 4). In the absence of RA treatment (i.e. in undifferentiated cells), there was no apparent change in the level of β-catenin following Axin induction (Fig. 4B, lanes 1 and 2), which might be due to a very low level of cytoplasmic β-catenin in undifferentiated P19 cells. When the P19 cells were induced to differentiate with RA, there was an increase in cytoplasmic β-catenin (Fig. 4B, lanes 1 and 3), consistent with the data of Fig. 2C. However, this accumulation of cytoplasmic β-catenin was reduced by the Dox-induced expression of Axin (Fig. 4B, lanes 3 and 4). Interestingly, the expression of Axin in these cells also blocked the induction of the neuron-specific marker βIII-tubulin (5.2 ± 1.4-fold reduction (n = 4), Fig. 4A, lanes 3 and 4). We took advantage of the bi-directional inducible vector system to examine morphological changes in pBI-EGFP-Axin/CMV-rtTA2S-M2- or pBI-EGFP/CMV-rtTA2S-M2-expressing stable cell clones. When pBI-EGFP/CMV-rtTA2S-M2-expressing stable cell clones were aggregated and differentiated by RA treatment, neurite-like structures formed (arrows in Fig. 4C, top panel, 76.3 ± 5.5% of EGFP-positive cells). However, the Dox-induced expression of Axin in pBI-EGFP-Axin/CMV-rtTA2S-M2-expressing stable cell clones significantly reduced the formation of those structures (Fig. 4C, bottom panel, 34.0 ± 13.5% of EGFP-positive cells).

Previously we and others have shown that overexpression of Axin caused apoptosis in transgenic mice and certain cell lines (56, 57). However, we could not see any obvious cell death after transient induction of Axin in undifferentiated P19 cells (data not shown). We also tested whether the failure of Dox-induced pBI-EGFP-Axin/CMV-rtTA2S-M2 cells to form neurite-like structures upon RA treatment was due to excessive cell death. pBI-EGFP-Axin/CMV-rtTA2S-M2- or pBI-EGFP/CMV-rtTA2S-M2-expressing stable cell clones were aggregated and differentiated in the presence of RA and different concentrations of Dox. The XTT assay revealed no clear difference in viable cell numbers between pBI-EGFP-Axin/CMV-rtTA2S-M2 and pBI-EGFP/CMV-rtTA2S-M2.
clones upon ectopic Axin induction (Fig. 4D). Therefore, the effect of Axin on neuronal differentiation of P19 cells is not due to cell death.

Ectopic Induction of a β-Catenin Signaling Inhibitor, ICAT, Inhibited Expression of βIII-tubulin during Neuronal Differentiation of P19 Cells—To determine whether reduction of β-catenin signaling is the main reason for the inhibition of neuronal differentiation when Axin is induced, we used a different approach to block β-catenin signaling. ICAT is known to inhibit Wnt/β-catenin signaling by blocking the interaction between β-catenin and Tcf/LEF factors (53, 58). We tested whether ectopic induction of ICAT acted similarly to Axin. Western analysis showed that the ICAT level was maintained consistently throughout neuronal differentiation (data not shown). Luciferase reporter assay by transient transfection of ICAT plasmid suggested that our myc-tagged ICAT works as an inhibitor of Wnt/β-catenin signaling (Fig. 5, A–C). Myc-tagged ICAT was cloned into pBI-EGFP vector (Fig. 5A), and P19 cell clones stably transfected with pBI-EGFP-ICAT/CMV-rtTA2S-M2 were treated with Dox for 4 days (lane 2) without RA. Lanes 3 and 4, P19 EC cells were aggregated in the presence of RA and re-plated on tissue culture dishes without (lane 3) or with Dox (lane 4) for 4 days. The induction of the neuronal marker βIII-tubulin (lane 4 in D) was clearly reduced upon induction of myc-ICAT.

Fig. 6. The late stage of neuronal differentiation is sensitive to the inhibitory effects of Axin. Proteins were isolated from pBI-EGFP-Axin/CMV-rtTA-expressing stable cell clones after different periods of Dox treatment and withdrawal. Lane 1, undifferentiated cells; lane 2, cells differentiated for 10 days without Dox; in lanes 3–7, Dox was added during the 4-day aggregation period, and then either continued or withdrawn at different times after plating; lane 3, 4 days Dox; lane 4, 4 days Dox plus 2 days withdrawal; lane 5, 6 days Dox; lane 6, 4 days Dox plus 6 days withdrawal; lane 7, 10 days Dox. The level of β-III tubulin was used to monitor neuronal differentiation. Constitutive induction of Axin during later neuronal differentiation blocked the induction of β-III tubulin (lanes 6 and 7).

Fig. 5. Induction of ICAT down-regulates the expression of the neuronal marker βIII-tubulin. A, diagram of pCS2-ML-ICAT and pBI-EGFP-ICAT constructs. B, expression of myc-GFP and myc-ICAT after transient transfection. C, luciferase reporter assay after transfection of 293T cells with indicated plasmids showed that myc-ICAT blocked β-catenin mediated induction of Tcf signaling. D: lanes 1 and 2, P19 EC cells stably transfected with CMV-rtTA2SM2 and pBI-EGFP-ICAT were treated with Dox for 4 days (lane 2) without RA. Lanes 3 and 4, P19 EC cells were aggregated in the presence of RA and re-plated on tissue culture dishes with RA and/or with Dox (lane 4) for 4 days. The induction of the neuronal marker βIII-tubulin (lane 4 in D) was clearly reduced upon induction of myc-ICAT.
The Later Phase of Neuronal Differentiation by P19 Cells Is the Sensitive Period for Blockage by Axin—The ability to transiently induce the expression of Axin by a short treatment with Dox (Fig. 3C) allowed us to examine the stage of P19 neuronal differentiation that is sensitive to Axin expression. Neuronal differentiation of pB1-EGFP-Axin/CMV-rtTA2s-M2 cells (with no Dox treatment) resulted in induction of βIII-tubulin expression (Fig. 6, lanes 1 and 2), as shown above for P19 cells (Fig. 2). Dox-induced expression of Axin during the entire 10-day culture period caused a reduction in βIII-tubulin induction (Fig. 6, lane 7). However, Dox-induced expression of Axin during the first 4 days (aggregation), followed by withdrawal of Dox for the next 6 days (differentiation), had no effect on βIII-tubulin expression (Fig. 6, compare lanes 2 and 6). Axin expression was eliminated within 2 days after withdrawal of Dox (Fig. 6, lane 4). These data suggest that induction of Axin during the 4-day aggregation period is not sufficient to block neuronal differentiation. Induction of Axin for the first 6 days has no greater effect on βIII-tubulin expression than does induction for the first 4 days (Fig. 6, compare lanes 4 and 5). In Fig. 4 (A and C), neuronal differentiation was blocked when Axin was induced only during the neuronal differentiation period and not during aggregation. Overall, these experiments suggest that the later stages of RA-induced neuronal differentiation by P19 EC cells are sensitive to the level of Axin expression.

Ectopic Expression of Axin Has No Effect on the Initiation of Differentiation but Blocks the Maturation of Neurite-like Structures—We have shown that ectopic induction of Axin blocks neuronal differentiation, such as the formation of neurite-like structures. Next, we tested whether induction of Axin blocks other differentiation processes. It is known that the level of the embryonic antigen SSEA-1 and E-cadherin are reduced upon RA induction of P19 EC cells (59, 60). Undifferentiated pB1-EGFP-Axin/CMV-rtTA2s-M2 cell clones, and those induced to differentiate in the absence or presence of Dox, were immunochemically stained with antibodies specific for SSEA-1, βIII-tubulin, MAP-2, and E-cadherin. Consistent with the above data, the Dox-induced expression of Axin greatly reduced the expression of the neuronal markers βIII-tubulin and MAP-2 (Fig. 7; compare with Fig. 4). However, this treatment had no effect on the down-regulation of SSEA-1 or E-cadherin (Fig. 7). These data suggest that ectopic induction of Axin does not block the initiation of differentiation by P19 cells but blocks a later step in the neuronal differentiation pathway.

Wnt3a Enhances Neuronal Differentiation of P19 EC Cells—Smolich and Papkoff (49) showed that overexpression of Wnt-1 could not induce normal neuroectodermal differentiation of P19 EC cells, although it could enhance certain aspects of that process. They suggested that correct timing of Wnt expression is necessary for proper neural differentiation. Wnt3a enhanced neuronal pattern formation, as shown by others (49) and in Fig. 1B, suggest that Wnt-3a may have a more important role than Wnt-1 in late neuronal differentiation. Because Wnt-3a is induced during the late neuronal differentiation period, we tested whether Wnt-3a could enhance neuronal differentiation. Consistent with several published reports, incubation of P19 EC cells with Wnt-3a-conditioned media (CM) caused a reduction in the Axin level and an increase in the cytoplasmic β-catenin level (Fig. 8). Incubation of undifferentiated P19 EC cells in the presence of Wnt-3a CM did not induce expression of the neuronal marker
protein βIII-tubulin. These data suggest that Wnt-3a does not have the ability to induce neuronal differentiation. However, when P19 EC cells were aggregated and differentiation was induced by RA in the presence of Wnt-3a-conditioned media, the level of βIII-tubulin was obviously enhanced (Fig. 8). These data suggest that Wnt-3a signaling through the down-regulation of Axin and up-regulation of cytoplasmic β-catenin may play an important role in neuronal differentiation.

**DISCUSSION**

While the evidence for the significance of Wnt signaling in overall neural development was accumulated in many model organisms, such as in mouse, zebrafish, and frog, specific roles of different Wnts in neuronal cell differentiation were not well studied. Only a few studies, which revealed determination of neuronal cell fate by Wnt3a and regulation of presynaptic axon structure by Wnt7a, have been published (10, 46). The involvement of Wnt signaling in the neuronal differentiation of P19 embryonic carcinoma cells has been suggested by the differential expression of various Wnts during that process (49). Here, we provide evidence for the role of Wnt/canonical β-catenin signaling specifically in neurite extension/maturation. We report that Axin, a negative regulator of the canonical Wnt signaling pathway, is down-regulated while the β-catenin level is increased during neuronal differentiation of P19 cells. Furthermore, the forced expression of either Axin or ICAT, a β-catenin signaling inhibitor, during the neuronal differentiation process resulted in blockage of neurite formation and the induction of the neuron-specific marker βIII-tubulin. In addition, the enhanced expression of βIII-tubulin after treatment with Wnt3A-conditioned medium suggests that Wnt/β-catenin signaling has a positive role in neuronal differentiation.

Several groups have used P19 cells to test the role of Wnt signaling in neuronal differentiation by simple overexpression of specific Wnts (49, 50). However, the dynamic changes in the expression of diverse Wnts during neuronal differentiation (Fig. 1B) lead us to test the significance of canonical Wnt signaling by blocking it, through the induced expression of Axin, rather than by overexpression of specific Wnts. Although Wnt1 and Wnt3a show similar spatial and temporal expression patterns in vivo and are considered to belong to the same group of Wnts, which signal through β-catenin, the temporal expression of these two Wnts do not seem to overlap during neuronal differentiation of P19 cells (Fig. 1B). These differing expression profiles imply that Wnt1 and Wnt3a may have different roles in the regulation of the neuronal differentiation process, which might account for the failure of Wnt1 to induce normal differentiation when overexpressed in P19 cells (49). Recently emerging data suggest that Ca²⁺ signaling is involved in learning and memory, and a non-canonical protein kinase C/Ca²⁺ pathway is also important in transducing certain Wnt signals (for review, see Refs. 2 and 61). The steady increase of Wnt5a, which is believed to regulate the protein kinase C/Ca²⁺ pathway, during neuronal differentiation (Fig. 1B) suggests a role for non-canonical Wnt signaling in neuronal differentiation of P19 cells (although it was not examined in the current work). We found that the Wnt1 and Wnt3a mRNA levels are increased and β-catenin is accumulated in nuclei during neuronal differentiation (Fig. 2). It is generally considered that Wnt1 and 3a have mitogenic activity rather than a role in differentiation. However, recently it has been shown that Wnt3a/β-catenin signaling is necessary and sufficient for myogenic differentiation in P19 cells, and Wnt1 is also known to have role in melanocyte expansion and differentiation during mouse embryogenesis (62, 63). This raises the interesting question of how the same β-catenin accumulation in nuclei directs two opposite outputs: enhancement of mitogenic activity and differentiation.

To determine whether the blockage of neuronal differentiation by ectopic Axin induction was due to down-regulation of canonical β-catenin signaling, rather than activation other signaling pathways, such as JNK activation, we used two different approaches: down-regulation of β-catenin by Axin (Fig. 4) and inhibition of β-catenin-Tcf complex interaction by ICAT (Fig. 5). When either Axin or ICAT was induced during the period of neuronal differentiation, the expression of the neuron-specific marker βIII-tubulin was clearly reduced, although the effect of ICAT was weaker (5.2-fold reduction in Axin induction versus 2.3-fold reduction in ICAT induction, Figs. 4 and 5). This may be due to the relatively high level of endogenous ICAT (data not shown), which could reduce the impact of induced ICAT expression. In any case, the results support the conclusion that at least part of the activity of Axin is due to its effects on the β-catenin pathway.

In addition to the inhibition experiments, we used Wnt-3A-conditioned media to confirm that Wnt/β-catenin signaling has a positive role in neuronal differentiation. Although the results were consistent with such an effect, we cannot rule out the possibility that the enhanced expression of the neuron-specific marker βIII-tubulin was caused by other molecules secreted by the Wnt-3A-expressing cells rather than Wnt-3a itself.

Although induction of Axin during the neurite extension period (on tissue culture plates after aggregation) blocked the formation of neurite-like structures, induction of Axin during the aggregation period did not (Fig. 6). Furthermore, induction of Axin throughout the aggregation and differentiation periods did not block the initiation of differentiation (i.e. the disappearance of markers of undifferentiated cells) (Fig. 7). One possible explanation is that endogenous Axin is already highly expressed during the aggregation stage, so that induction of exogenous Axin did not further enhance the down-regulation of β-catenin in that period. Another plausible explanation is that Wnt signaling is not involved in the initiation of differentiation. In all our experiments we compared undifferentiated versus differentiated P19 cells after aggregation, as shown in the diagram of Fig. 6. The weak level of Wnt1 expression right after aggregation (Fig. 1B, lane 2) suggests that aggregation in the presence of RA is itself sufficient to initiate differentiation stage without Wnt signaling.

The findings, that the Axin level was lower in the brains of 1-month-old postnatal mice than in 13.5 dpc mouse embryos and that the abnormal induction of Axin blocked the formation of neurite-like structures in P19 cells, suggest that Wnt signaling has a similar function in vivo. We are currently testing that possibility by using a neuron-specific promoter to direct Tet-inducible expression in transgenic mice.

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