

Isolation and Characterization of Human *NBL4*, a Gene Involved in the β -Catenin/Tcf Signaling Pathway

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β -Catenin, a key regulator of cellular proliferation, is often mutated in various types of human cancer. To investigate cellular responses related to the β -catenin signaling pathway, we applied a differential display method using mouse cells transfected with an activated form of mutant β -catenin. This analysis and subsequent northern-blot hybridization confirmed that expression of a murine gene encoding NBL4 (novel band 4.1-like protein 4) was up-regulated by activation of β -catenin. To examine a possible role of NBL4 in cancer, we isolated the human homologue of the murine *NBL4* gene by matching *mNBL4* against the human EST (expressed sequence tag) database followed by 5' rapid amplification of cDNA ends (5'RACE). The cDNA of *hNBL4* encoded a protein of 598 amino acids that shared 87% identity in amino acid sequence with murine NBL4 and 71% with zebrafish NBL4. A 2.2-kb *hNBL4* transcript was expressed in all human tissues examined with high levels of expression in brain, liver, thymus and peripheral blood leukocytes and low levels of expression in heart, kidney, testis and colon. We determined its chromosomal localization at 5q22 by fluorescence *in situ* hybridization. Expression of *hNBL4* was significantly reduced when β -catenin was depleted in SW480 cells, a human cancer cell line that constitutionally accumulates β -catenin. The results support the view that NBL4 is an important component of the β -catenin/Tcf pathway and is probably related to determination of cell polarity or proliferation.

Key words: β -Catenin — Tcf/Lef complex — *hNBL4* — Band 4.1 superfamily — Chromosome 5q22

Recent progress in cancer research has disclosed that β -catenin, which functions in cell-to-cell adhesion and in the wnt-signaling pathway, is a major player in carcinogenesis of various tissues, including colon, liver, ovary, and skin.^{1–6} β -Catenin is normally degraded in the cell through formation of complexes with GSK-3 β (glycogen synthase kinase-3 β), Axin, and APC (adenomatous polyposis coli).^{5,7} However, when mutation of APC or of β -catenin itself occurs, β -catenin accumulates in the cell and forms a complex with Tcf/Lef.^{8,9} That complex translocates into the nucleus and transactivates downstream genes.^{5,8,9} So far, only *c-myc* and *cyclin D1* have been identified as targets of this complex, but the molecular mechanism of the β -catenin-Tcf/Lef signaling pathway is still not well understood.^{10–12}

The wnt/wingless signal is essential for patterning of embryonic segments during development in *Drosophila*.³ This signaling pathway also plays a crucial role in axis

formation of *Xenopus* embryos.¹³ In *Drosophila*, the wingless signal recruits zeste-white-3, the homologue of GSK-3 β , and down-regulates armadillo protein, considered to be a homologue of β -catenin, by phosphorylating its N-terminal region.⁴ Therefore, a failure to recognize cell polarity may be an important factor in tumorigenesis, as it may confer morphological changes that trigger uncontrolled migration of tumor cells.^{5–9}

Members of the band 4.1 protein superfamily are thought to play important roles in regulating interaction between the cytoskeleton and the plasma membrane.^{14–19} This superfamily consists of band 4.1 protein, ezrin, radixin, moesin, merlin, talin, PTPH1 (protein tyrosine phosphatase Hela1), PTPMEG (protein tyrosine phosphatase, megakaryocyte), and NBL1-NBL7.^{19–24} All of these proteins contain a conserved domain in the N-terminal half that is responsible for binding glycoprotein C. Since the N-terminal ends of these proteins appear to be involved in the specific binding of integral membrane proteins, all members are probably located just beneath the membranes.¹⁹

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Here we report isolation, chromosomal assignment, and characterization of human *NBL4*, a molecule involved in the β -catenin/Tcf pathway.

MATERIALS AND METHODS

RNA extraction and RT-PCR We previously established a mouse fibroblast cell line, LMT, by introducing into murine L cells a β -catenin transgene which had been activated by deletion of exon 3.²⁵⁾ Total RNAs were extracted from this cell line with TRIZOL reagent (Life Technologies, Inc., Tokyo) according to the manufacturer's protocol. After treatment with DNase I (Boehringer Mannheim, Tokyo), a 0.2- μ g aliquot of poly(A) RNA was purified using OligoTex (TaKaRa, Tokyo) and reversely transcribed to single-stranded cDNAs using oligo(dT)₁₅ primer with Superscript II reverse transcriptase (Life Technologies, Inc.). Each single-stranded cDNA was diluted for subsequent PCR amplification by monitoring *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) as a quantitative control. The primers utilized for amplification were GAPDHF (5'-ACAACAGCCTCAAGATCATCAG-3'), GAPDHR (5'-GGTCCACCACTGACACGTTG-3'), NBL4F (5'-CTCTAATGGGTCAGATTCCT-3'), NBL4R (5'-ATGAGGGATTTGCTTCTTTGG-3'). Each PCR was carried out in a 20 μ l volume of 1 \times PCR buffer for 4 min at 94°C for initial denaturing, followed by 25 (for *GAPDH*) or 28 (for *NBL4*) cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s, in the Gene Amp PCR system 9600 (Perkin-Elmer Applied Biosystems, Foster City, CA).

Fluorescent differential display The fluorescent differential display procedure was performed as described elsewhere.²⁶⁾ PCR products were resuspended in formamide sequencing dye, and run on a sequencing gel containing 4% acrylamide (19:1) with 7 M urea for 3 h at 1800 V. Analysis of the gel images was performed using an FMBIO II Multi-View fluorimage analyzer (TaKaRa). Bands that showed differential expression between L cells and LMT cells were excised from the gels, and each DNA was extracted by boiling the gel fragment in Tris-EDTA buffer. Each sample was reamplified for 30 cycles with the same primer sets that were utilized for the first amplification. The re-amplified products were extracted from the gel, cloned into pBluescript II SK(-) vector (Stratagene, La Jolla, CA) and sequenced using T3, T7 primers and an ABI PRISM Dye Terminator Cycle Sequencing FS Ready Kit (Perkin-Elmer Applied Biosystems) according to the protocol provided by the supplier. Among several differently expressed bands, one up-regulated fragment in LMT cells contained a sequence homologous to the murine *NBL4* gene.

Isolation and DNA sequencing of the human *NBL4* cDNA By searching the database of human expressed sequence tags (hEST) using the BLAST program, we

found several hESTs that showed homology to murine *NBL4*. However, since none of the hESTs contained the 5' part of the coding sequence of *hNBL4*, we performed 5' rapid amplification of cDNA ends (5'RACE) using the Marathon cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. The cDNA template was synthesized from human testis mRNA. For the amplification of 5' part of *hNBL4* cDNA, a specific reverse primer (5'-TCATCCAGGAGCAAA-ACTTCGCAG-3') and AP1 primer supplied in the kit were utilized. The nucleotide sequences of the PCR products were determined directly using the reverse primer with an ABI PRISM 377 DNA sequencer (Perkin-Elmer Applied Biosystems) according to the manufacturer's instructions.

Northern-blot analysis Human multiple-tissue blots (Clontech) were hybridized with a partial cDNA fragment of *hNBL4*, labeled by the random oligonucleotide priming method. Pre-hybridization, hybridization and washing were performed according to the supplier's recommendations. The blots were autoradiographed with intensifying screens at -80°C for 24 h.

Down-regulation of *NBL4* expression by depletion of β -catenin Since expression of the region containing 20 amino acid repeats of wild-type *APC* was reported to deplete β -catenin,²⁷⁾ adenovirus containing this region (Ad-*APC*) was constructed. A 2.5-kb *Hind*III fragment of *APC* cDNA was inserted into the *Hind*III site of the pAd-Bgl II vector²⁸⁾ and recombinant adenoviruses were constructed as described previously.²⁹⁾ Viruses were propagated in the human embryonic kidney 293 cell line and purified by two rounds of CsCl density centrifugation. Viral titers were measured in a limiting dilution bioassay using the 293 cells. Cell monolayers were infected with the viral solutions and incubated at 37°C for 1 h with brief agitation every 15 min. Culture medium was added, and the infected cells were returned to the 37°C incubator for 48 h.

Western blotting analysis of β -catenin Western blotting analysis using mouse anti- β -catenin (Transduction Laboratories Inc., Lexington, KY) and goat anti-actin polyclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) were performed as described elsewhere.³⁰⁾

Fluorescence *in situ* hybridization (FISH) To determine the chromosomal localization of the *hNBL4* gene, we obtained a cosmid clone by screening a human genomic cosmid library and performed a FISH experiment using the cosmid clone as a probe, in a manner described previously.³¹⁾

RESULTS

We previously established a mouse fibroblast cell line, LMT, transfected with an activated form of mutant β -catenin.²⁵⁾ LMT cells showed a multilayer growth pattern and

displayed growth advantage in low serum culture media compared with their parent cells, L cells.²⁵) In the LMT cells, we were able to produce an activated form of β -catenin by withdrawal of doxycycline. Using these cell lines, we aimed to isolate genes regulated by β -catenin. By means of the fluorescent differential-display method and subsequent northern hybridization experiments, we detected a murine gene whose expression was increased in LMT cells as β -catenin accumulated (Fig. 1). A homology search using this partial cDNA sequence revealed that the fragment was identical to a partial 3' sequence of *mNBL4* (mouse novel band 4.1-like protein 4).

Subsequent computer analysis of the DNA database using the BLAST program (National Center for Biotechnology Information, Bethesda, MD) identified five human ESTs (AA406206, AA970609, AA350575, T19528, and T89355) that were highly homologous to murine *NBL4*. Since all of these cDNA sequences matched only 3' partial sequences of murine *NBL4* and the expected size of the *hNBL4* transcript was longer than those ESTs, we carried out 5'RACE to determine the entire coding sequence. Assembly of the sequences of 5'RACE product of the cDNA and the EST sequences yielded the entire coding sequence of the human *NBL4* gene. The cDNA consisted of 2125 nucleotides, including an open reading frame of 1794 nucleotides that encoded a 598 amino acid peptide (DNA sequences are available from GenBank, accession number AB030240).

A comparison of the deduced amino acid sequences of hNBL4 and mNBL4 indicated a high similarity of

the overall structures (Fig. 2). Alignment of amino acid sequences indicated 87% and 71% identity of the human sequence to NBL4s of mouse and zebrafish, respectively (Fig. 3). Moreover, we observed 25–30% identities in amino acids between the hNBL4 product and other members of the band 4.1 protein family including ezrin, moesin, radixin, merlin and talin.

Northern-blot analysis using the *hNBL4* cDNA clone as a probe detected two transcripts of 2.2 kb and 3.0 kb (Fig. 4). The 2.2-kb transcript corresponding to hNBL4 was expressed in all tissues examined; at high levels in brain, liver, thymus and peripheral blood leukocyte, at moderate

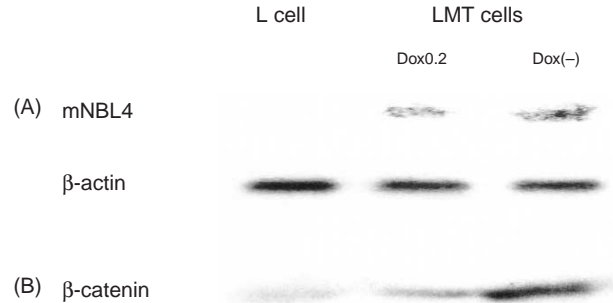


Fig. 1. Northern-blot analysis of mRNAs extracted from murine LMT and parental L cells, using a PCR product of mNBL4 as a probe (A), and western analysis of β -catenin protein in the same cells (B).

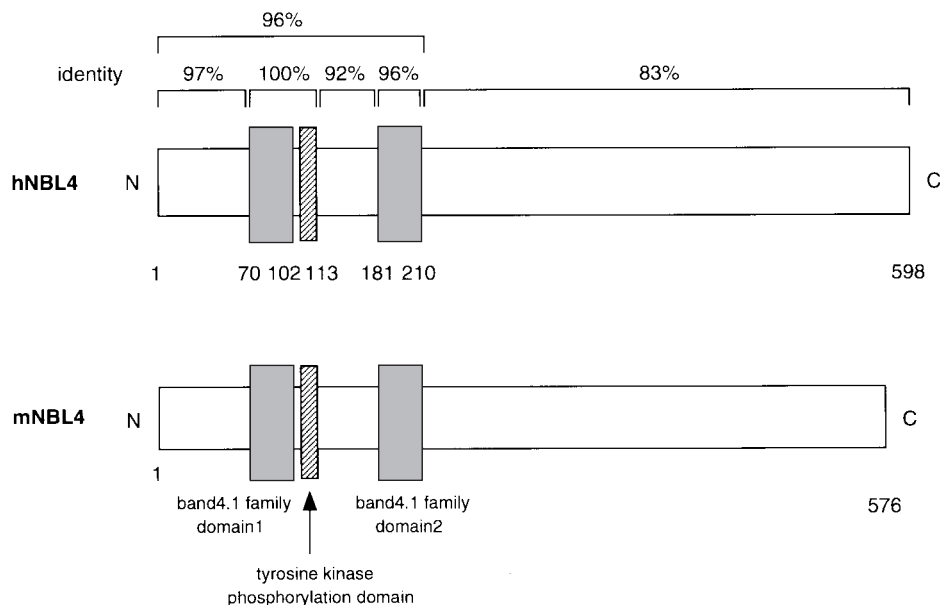


Fig. 2. Structural comparison of hNBL4 and mNBL4 proteins. Positions of the band 4.1-family domain are shown as gray boxes. Each box is drawn to scale to indicate the size of each domain.

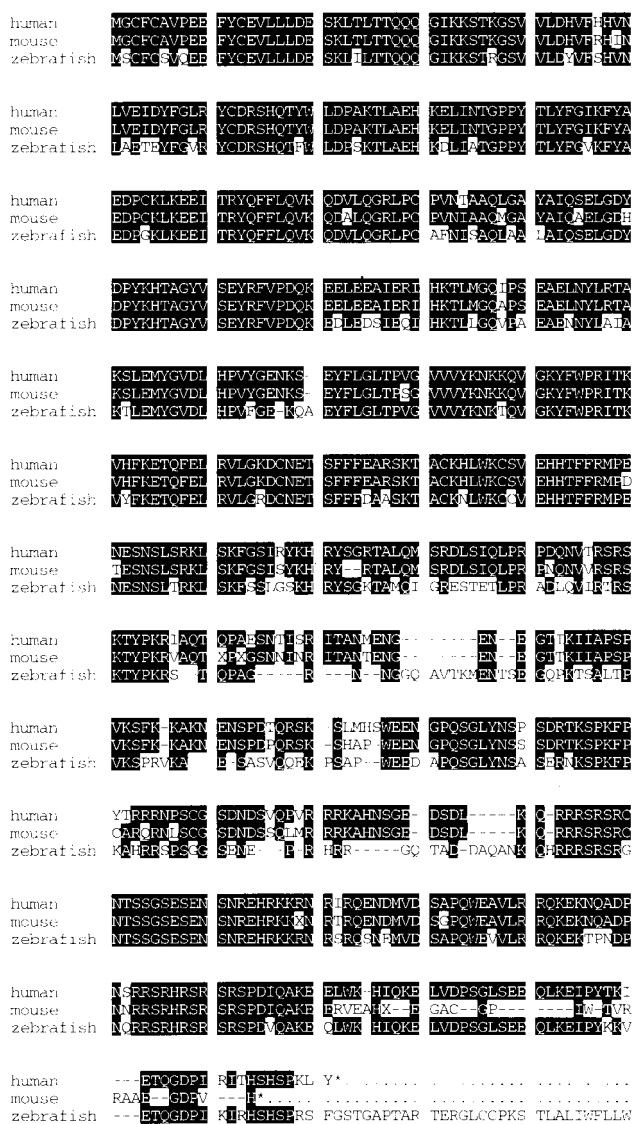


Fig. 3. Alignment of the predicted amino acid sequences of hNBL4, mNBL4 and zebrafish NBL4 (GenBank accession numbers: AB030240, U13673, and D30788, respectively). Shading indicates conserved residues.

levels in placenta, lung, skeletal muscle, pancreas, spleen, prostate, ovary and small intestine, and at low levels in heart, kidney, testis and colon. The 3.0-kb transcript showed a low level of expression in liver, spleen and thymus. To determine whether the 3.0-kb transcript might correspond to the *hNBL4* gene or not, we screened a total of 1×10^6 clones from the thymus cDNA library with the *hNBL4* clone, but we obtained neither cDNA clones corresponding to the 3.0-kb transcript nor homologous clones. All positive clones isolated from the library contained

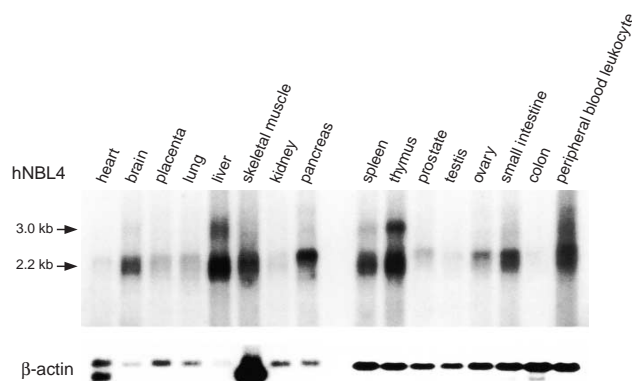


Fig. 4. Northern-blot analysis of hNBL4 in various human tissues. Molecular size is indicated in kb at the left.

the sequences corresponding to the 2.2-kb transcript of *hNBL4*. Moreover, no alternatively spliced transcript was amplified when we used primer sets for various regions of *hNBL4* cDNA in RT-PCR experiments. Although we can not completely exclude the possibility that the 3.0-kb band corresponded either to an alternatively spliced form or an alternative polyadenylation, it is more likely that it represented cross-hybridization to a gene with a relatively low homology. FISH using a cosmid clone as a probe showed clear twin-spot signals specifically on chromosomal band 5q22. No signals could be detected on any other chromosomes among 100 metaphase cells examined (Fig. 5).

To confirm transactivation of *NBL4* by activated β -catenin, we constructed a system in which β -catenin was depleted by transfection of an adenovirus vector containing the 20-amino-acid repeat region of wild-type APC. Transfection of this virus into a colon carcinoma cell line (SW480) in which a large amount of β -catenin accumulates in the nucleus, reduced the accumulation of β -catenin in the nucleus (data not shown). These changes were not observed when we transfected adenovirus containing a *LacZ* gene instead of the *APC* gene fragment. In the cells transfected with the 20-amino-acid repeats of wild-type APC, *hNBL4* expression was reduced significantly, but this was not the case in cells transfected with the control virus (Fig. 6).

DISCUSSION

Band 4.1-like proteins are major components of the undercoat of erythrocyte membranes.¹⁶⁾ The N-terminal halves are highly conserved among the members of this superfamily, and this region is considered to be responsible for binding to glycophorin C.¹⁴⁾ These proteins also contain several consensus sequences for A-kinase and

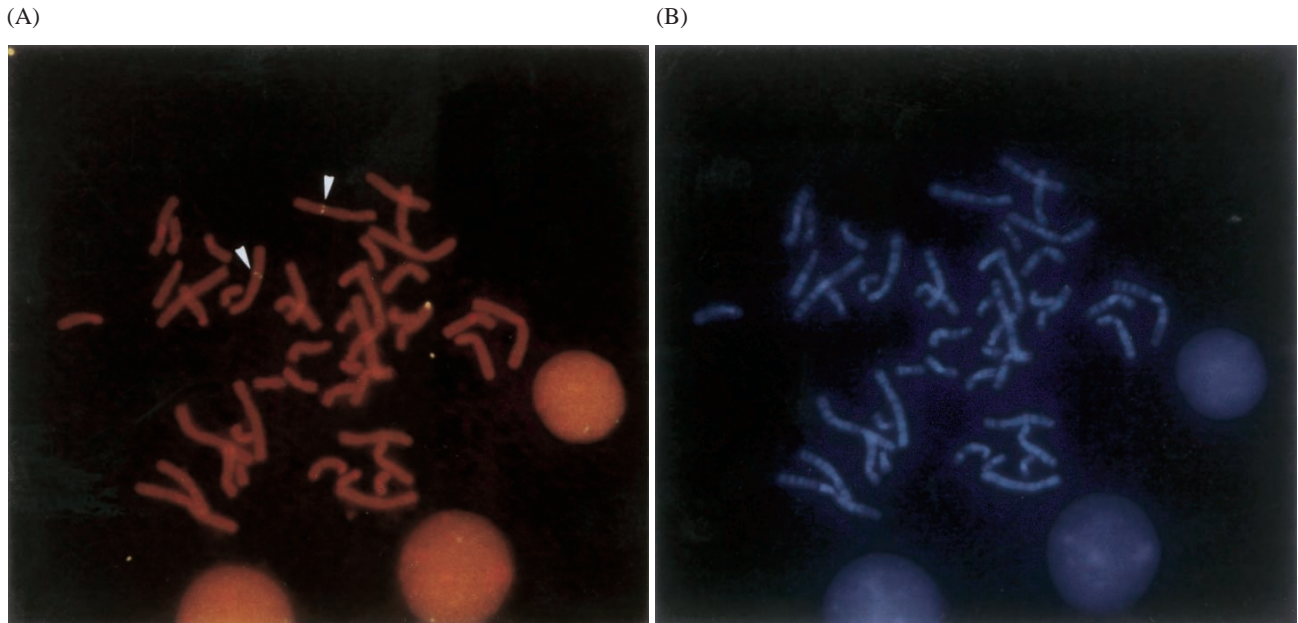


Fig. 5. Chromosomal localization of the *hNBL4* gene. (A) Metaphase chromosomes stained with propidium iodide show spot signals on the long arm of chromosomes 5q22 (indicated by arrows). (B) G-band pattern of the same metaphase chromosomes, delineated through a UV-2A filter (Nikon, Tokyo), indicates that the *hNBL4* clone hybridized to chromosome 5q22.

tyrosine kinase phosphorylation sites, suggesting that phosphorylation-dependent regulation is important for various cellular events.^{32–34} Inactivation of merlin, one member of the band 4.1 superfamily, is responsible for development of neurofibromatosis 2 (NF2) and is also involved in familial and sporadic schwannomas and meningiomas.^{21, 22, 35} Other studies have indicated that another band 4.1-like protein, moesin, is likely to play an important role in invasiveness and metastasis of estrogen receptor (ER)-negative breast cancers.²⁰ Hence, like other members of the band 4.1 family, NBL4 may be involved in a signal transduction pathway related to carcinogenesis.

Comparison of the predicted amino acid sequence of hNBL4 with those of mouse and zebrafish NBL4s showed 87% and 71% identity, respectively. Although the overall amino-acid identity of hNBL4 to other members of the band 4.1 family of proteins is less than 30%, the N-terminal conserved region that includes a putative glycoporphin C-binding (putative membrane-binding) motif is highly conserved in all members of the family, including ezrin, moesin, radixin and merlin (data not shown). Therefore the N-terminal sequence may be crucial for interactions with other membrane-associated proteins.

Northern-blot analysis revealed several differences in tissue distribution between *hNBL4* and *mNBL4*. The 2.2-kb transcript of *hNBL4* was detected in all tissues examined, with high levels of expression in brain, liver, thymus

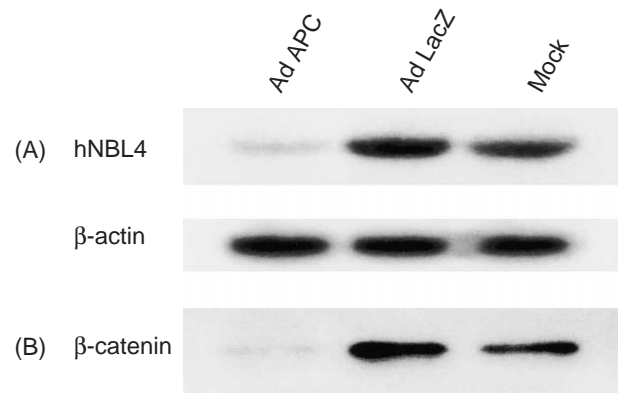


Fig. 6. (A) Northern-blot analysis of the *hNBL4* gene in SW480 cells. Cells were untreated (Mock) or transfected for 72 h with either adenovirus containing LacZ (Ad LacZ) or adenovirus containing the 20 amino acid repeat region of the wild-type APC gene (Ad APC). (B) Western-blot analysis of β -catenin in the same cells.

and peripheral blood leukocytes, and low levels of expression in heart, kidney, testis and colon, while a 2.5-kb *mNBL4* transcript was also expressed in all murine tissues examined, except for thymus and kidney.¹⁹ These results suggest that hNBL4 may play tissue-specific roles.

By RT-PCR and northern blotting, we confirmed that expression of *mNBL4* was up-regulated by activated β -catenin in mouse fibroblasts. Moreover, this association was confirmed in the experiment using human colon-carcinoma cells (SW480) that contain a large amount of β -catenin. Depletion of β -catenin in those cells by adenovirus-mediated *APC* transfer, with consequent reduction of the DNA-binding activity of the β -catenin-Tcf/Lef complex, caused a significant reduction of *hNBL4* expression. The combined results of our experiments using murine and human cells strongly suggest that *NBL4* is an important component of the β -catenin signaling pathway.

Until now, only two genes, *cyclin D1* and *c-myc*, were known to be up-regulated by activation of β -catenin.^{10–12)} Since cyclin D1, in conjunction with retinoblastoma protein, is involved in regulating the cell cycle, its induction

may confer uncontrolled cellular growth.^{11,12)} *NBL4*, a likely membrane associated protein, may be involved in determination of cell polarity, in adhesion, in cell motility and/or in cell-to-cell communication. Further investigation of *NBL4* may elucidate its function in the wnt/wingless signal pathway.

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