Human homolog of NOTUM, overexpressed in hepatocellular carcinoma, is regulated transcriptionally by β-catenin/TCF

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The *Drosophila Notum* gene, which is regulated by the Wingless pathway, encodes a secreted hydrolase that modifies heparan sulfate proteoglycans. In comparative analysis of the gene expression profiles in primary human hepatocellular carcinomas (HCC) and normal organs, we observed that the human ortholog of *Drosophila Notum* was overexpressed markedly in a subset of HCC, but expressed rarely in adult normal tissues. Immunoblotting confirmed the overexpression of NOTUM protein in 12 of 40 primary HCC cases (30%). High levels of NOTUM protein were significantly associated with intracellular (nuclear or cytoplasmic) accumulation of β-catenin protein: all 10 HCC with high intracellular β-catenin also had high NOTUM expression, whereas only 2 of 30 cases (6.7%) without intracellular β-catenin had high NOTUM expression ($P < 0.00001$). *NOTUM* expression in HepG2 cells was downregulated significantly by induction of a dominant-negative mutant of TCF4, a β-catenin partner. *In vivo* binding of the β-catenin/TCF complex to the *NOTUM* promoter was demonstrated by chromatin immunoprecipitation in HepG2 and SW480 cells, where canonical Wnt signaling is activated constitutively. These findings provide evidence that *NOTUM* is a novel target of β-catenin/TCF4 and is upregulated in Wnt/β-catenin signaling-activated HCC. (*Cancer Sci* 2008; 99: 1139–1146)

Hepatocellular carcinoma (HCC) is one of the leading causes of worldwide cancer mortality; it is estimated that 1 million patients die from HCC each year.[1,2] HCC develops in individuals with chronic liver inflammation and cirrhosis secondary to hepatitis virus infection, excess alcohol intake, and exposure to toxins in food. HCC is highly resistant to available chemotherapeutic agents, which are mostly non-selective toxic molecules. Thus, targeted therapies based on molecular mechanisms of hepatocarcinogenesis are needed urgently. Dysregulation of signaling pathways mediated by insulin-like growth factor, hepatocyte growth factor, epidermal growth factor, transforming growth factor β, and Wnt have been reported to play a role in HCC progression, providing potential targets for new therapeutics.[3]

The Wnt signaling pathway plays a critical role in directing cell fate during embryogenesis, and abberant activation of Wnt signaling drives cell proliferation by turning on genes encoding oncoproteins and cell cycle regulators.[4,5] Aberrant nuclear accumulation of β-catenin has been reported in 17–40% of human HCC.[6–9] Canonical Wnt signaling operates though the cytosolic stabilization of a transcriptional cofactor, β-catenin. Mutations in β-catenin (*CTNNB1*) itself and negative regulators of β-catenin such as the genes encoding adenomatous polyposis coli (*APC*), glycogen synthase kinase-3β (*GSK3B*), and axis inhibition proteins (*AXIN*) are observed frequently in colorectal cancer (CRC) and occasionally in other cancers.[10] Deletion and mutation of *CTNNB1* or loss of function of its negative regulators results in abnormal stabilization of β-catenin and promotes the formation of a complex between TCF and lymphoid enhancer binding factor (LEF). This complex then activates the expression of target genes such as *MYC*, *CCND1*, *Met*, *MDR1*, and *PPARδ*.[10] Although the canonical Wnt signaling pathway has been studied extensively in cancer cells[10,11] there are yet-undiscovered proteins involved in this pathway. Analysis of comprehensive gene expression data has enabled the identification of new downstream targets of Wnt signaling.[12–14]

Here, we report that human *NOTUM*, a novel target of the Wnt signaling pathway, is upregulated in HCC. In gene expression profiling analysis of paired HCC and normal livers, the human ortholog of *Drosophila Notum* (human *NOTUM*) is overexpressed in HCC with high intracellular β-catenin. We showed direct interaction of β-catenin/TCF4 with the *NOTUM* promoter, and also confirmed the responsiveness of the *NOTUM* promoter to Wnt signaling.

Materials and Methods

Patients and tissue samples. Thirty-six patients with HCC who had undergone hepatectomy at the Hepato-Biliary-Pancreatic Surgery Division, The University of Tokyo Hospital (Tokyo, Japan), were included in the present study. The use of human tissues was approved by the local committee and all patients gave informed consent. According to the definition of multicentric HCC,[15] four patients were diagnosed as having two multicentric nodules; thus, 40 tumors and 36 non-cancerous liver tissues were obtained. Among the 36 HCC patients, nine patients were positive for hepatitis B surface antigen, 25 for hepatitis C virus antibody, and two for neither. The clinical parameters and tumor status based on histological findings of resected specimens are summarized in Suppl. Table S1. Surgical specimens were immediately cut into small pieces after resection, snap-frozen in liquid nitrogen, and stored at –80°C.

Cell lines. HepG2 cells from liver cancer, parental L cells, and Wnt3a stably expressing L (Wnt3a-L) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). MKN1 cells from gastric cancer and HeLa cells from

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cervix cancer cells were obtained from RIKEN Bioresource Center (Tsukuba, Japan). HepG2, COS7, HeLa, and parental L cells were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). MKN1 cells were cultured in RPMI-1640 supplemented with 10% FBS. Wnt3a-L cells was cultured in Dulbecco's modified Eagle's medium with 10% FBS and 400 μg/mL G418. A stable cell lines of HEK-293 exhibiting tetracycline-inducible expression of either FLAG-tagged NOTUM or the oncogenic and constitutive active form of β-catenin (S33Y) were generated using the Flp-In/T-Rex system (Invitrogen, Carlsbad, CA, USA). NOTUM or β-catenin (S33Y) protein was induced by the addition of 1 μg/mL doxycycline for 72 h.

RNA extraction and oligonucleotide microarray analysis. Tissues or cells were lysed directly in TRIzol reagent (Invitrogen) and homogenized. Total RNA was extracted according to the manufacturer's instructions. RNA representing 28 different normal adult tissues and five different fetal tissues (Takara, Osaka, Japan),[16] 42 cancer cell lines and cancerous tissues; glioblastoma, a pooled sample made up of 12 lung adenocarcinomas, a pooled sample made up of three moderately differentiated HCC, pancreas cancer, stomach cancer, renal cell carcinoma, and a pooled sample made up of three colorectal cancer tissues were analyzed on GeneChip HG U133 oligonucleotide arrays (Affymetrix, Santa Clara, CA, USA). Microarray analysis was carried out as described previously.[17] For global normalization, the average signal in an array was given a value of 100. Further information on other RNA analyzed here is available at our website (http://www.lsbm.org/db/index.html). We selected genes upregulated in HCC, defined as those whose expression level was upregulated in HCC but was minimal in normal vital organs including brain, lung, kidney, spleen, bone marrow, pancreas, stomach, small intestine, and colon. Genes with a signal score >1500 in HCC and <250 in normal vital organs were selected and were evaluated individually.

Vector construction, transfection, and virus infection. cDNA from HepG2 cells was used as a template for reverse transcription (RT)–polymerase chain reaction (PCR) using KOD PLUS (Toyobo, Osaka, Japan). Human *NOTUM* cDNA was subcloned into pENTR/D-TOPO (Invitrogen) using the TOPO Cloning reaction. The following primers were used: sense primer, 5′-ATGGGCCGAGGGGTGCGCGTG-3′; and antisense primer, 5′-GCTTCCGTTGCTCAGCATCCCCAG-3′. After sequence confirmation, the resultant subcloned DNA was moved to the expression vector pcDNA3.2/V5-DEST (Invitrogen), which adds an in-frame COOH-terminal V5 epitope tag. Plasmids were transfected into COS-7 using FuGENE 6 Transfection Reagent (Roche Diagnostics, Basel, Switzerland). The construction of recombinant adenovirus has been described previously.[18] Cells were infected at multiplicity of infection (m.o.i.) 40 with an adenovirus construct expressing the dominant-negative mutant *TCF4E* (Ad-*TCF4E*-ΔN) or *LacZ* (Ad-*LacZ*) as a negative control.[18] RNA from HepG2 cells was isolated at 24 h after infection.

Quantitative RT-PCR. *NOTUM* expression was quantified by quantitative RT-PCR. Reactions contained SYBR Green I nucleic acid gel stain (BMA, Rockland, ME, USA) and the following primers: 5′-ACTCGCACAGGCACAGGGA-3′ and 5′-GCCCCGCTCCAAACATCACT-3′ for *NOTUM*; 5′-AGA-AGGAGATCACTGCCCTGGCACC-3′ and 5′-CCTGCTTGCT-GATCCACATCTGCTG-3′ for *ACTB*; or 5′-TGGATGGGTA-TTCCAGAAGAACCAC-3′ and 5′-CCATGAGAGCCTTTTC-TCCTATGC-3′ for *DKK1*. PCR conditions were: one cycle of 94°C for 3 min, followed by 40 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s. All samples were run in triplicate, and the results were averaged. Non-amplification of primer dimers was verified by checking the melting curve after PCR and gel electrophoresis. The level of *NOTUM* expression was normalized relative to that of *ACTB*.

Sequencing analysis of *CTNNB1*. PCR amplification of exon 3 of *CTNNB1*, which contains four potential sites for GSK-3β phosphorylation, was carried out on the cDNA from each sample using *CTNNB1* exon 2 forward primer, 5′-GAAGGTCTG-AGGAGCAGCTTCAGTCC-3′, and *CTNNB1* exon 4 reverse primer, 5′-CAGCATCTGTGATGGTTCAGCC-3′. After confirmation of the PCR products by agarose gel electrophoresis, we carried out a sequencing reaction using DYEnamic ET (GE Healthcare Bio-sciences, Piscataway, NJ, USA) read in both directions with the primers used for PCR.

Generation of antibodies. Monoclonal antibodies against human NOTUM were generated by immunization of glutathione S-transterase (GST)-fused NOTUM protein (143–496 amino acids) or secreted NOTUM protein tagged with the FLAG peptide. GST–NOTUM was produced in the BL-21 codon PLUS pLys *Escherichia coli*. strain.[19] Full-length secreted NOTUM–FLAG protein was purified from the culture media of a stable *NOTUM* cell line by immunoprecipitation with an anti-FLAG antibody. Monoclonal antibodies against NOTUM were purified from ascites fluid of hybridoma-injected mice by ammonium sulfate precipitation. Antisera against TCF4 was raised in a rabbit by immunization of a keyhole limpet hemocyanin (KLH)-conjugated 3–21-amino acid peptide of TCF4, NH_2-GCHSSLAGTQPQPLSLVTKSLK-COOH.[20]

Immunoblot analysis. Immunoblotting of NOTUM and β-actin was carried out according to a previous report.[19] Briefly, the membrane was incubated with anti-V5 (1:5000; Invitrogen), anti-NOTUM (H9558; 5 μg/mL), or anti-β-actin (1:20 000; Sigma, St Louis, MO, USA) antibody as primary antibody, followed by incubation with horseradish peroxidase-conjugated antimouse IgG antibody (1:5000; GE Healthcare Bio-sciences) as a secondary antibody. ECL-PLUS Detection System (GE Healthcare Bio-sciences) was used as the substrate for chemiluminescent detection.

Immunohistochemistry. Immunostaining of frozen sections for β-catenin was carried out according to standard procedures. In brief, antigens were retrieved by microwaving the slides in citrate buffer (pH 6.0) for 15 min. Sections were incubated with anti-β-catenin monoclonal antibody (1:500; BD Biosciences, San Diego, CA, USA) overnight at 4°C and washed in Tris-buffered saline (TBS). Envision+ (Dako, Denmark) was used as a secondary antibody and substrate. Slides were counterstained lightly with hematoxylin.

Promoter reporter assays and site-directed mutagenesis. Reporter gene assays were carried out using the dual luciferase system (Promega, Madison, WI, USA). The 2.0-kb *NOTUM* promoter with a 500-bp 5′-untranslated region (UTR) region was amplified by PCR from genomic DNA of HepG2 cells with the primers 5′-CTGCGTCTGAAGCTACTGCCCTTG-3′ and 5′-CGTTGCAG-GTCACCGAGGTGTTG-3′, followed by nested PCR with the primers 5′-GGTACCCTGCGTCTGAAGCTACTGCCC-3′ and 57-CTCGAGGCCAGGCTCTTGACTTGCGC-3′. PCR was done using KOD Plus polymerase (**T**o**Y**o**B**o, Osaka, Japan) in the presence of 10% dimethyl sulfoxide (DMSO). The PCR product was then cloned into pCR-Blunt II-TOPO (Invitrogen). The plasmid containing the *NOTUM* promoter region was then digested with *Kpn*I and *Xho*I and the fragment was cloned into the pGL4.10 [luc2] vector (Promega). The mutant construct at the TCF–LEF binding site (CTTTGAT to CTTTGGC) of the *NOTUM* promoter was generated by site-directed mutagenesis using the KOD Plus mutagenesis kit (Toyobo) with forward (5′-ctgtgtccgccgccgctttgGCcccgggggccctgctggat-3′) and reverse (5′-tccagcagggccccccgggGCcaaagcggcggcggacacagggc-3′) primers. HepG2 cells seeded into 96-well plates were transfected with 0.2 μg NOTUM-pGL4 plasmid using FuGENE 6 reagent. As controls for normalization, 20 ng of the Renilla luciferase plasmid phRL (Promega) was cotransfected, and dual luciferase activities were measured using the Dual-Glo Luciferase Assay

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System (Promega) after 30 h. All transfections were done in triplicate. To measure the response to Wnt signaling, control or Wnt3a-expressing conditioned medium was added at 6 h prior to the measurements.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were carried out in accordance with a previous report.[12] Immunoprecipitation was carried out with precleaned samples and Protein A/G beads bound with 5 µg polyclonal anti-β-catenin antibody (sc-7199; Santa Cruz Biotechnology), or 5 µL of antisera for TCF4. The primer pair used to amplify the *NOTUM* and *CCND1* promoter sequences containing the putative TCF-binding element, or the *GAPDH* promoter sequence were as follows: *NOTUM*, 5′-TCGTAGCTGCTTCTCTGACCCCAG-3′ and 5′-GGAGCCGCCAGGGACTTTTATC-3′; *CCND1*, 5′-TGAAGTTGCAAAGTCCTGGAGCC-3′ and 5′-CTCCCTCGCGCTCTTCTGCC-3′; and *GAPDH*, 5′-GTATTCCCCCAGGTTTACAT-3′ and 5′-TTCTGTCTTCCACTCACTCC-3′.

Statistical analysis. We used the χ^2-test to analyze the association between NOTUM expression and β-catenin expression, and Student's *t*-test to examine differences in *NOTUM* expression levels between Ad-*TCF4E*-ΔN-infected cells and Ad-*LacZ*-infected cells. The WST-8 assay was also evaluated using Student's *t*-test. Differences were considered significant at $P < 0.05$.

Results

Identification of NOTUM overexpression by oligonucleotide array analysis. To identify genes expressed selectively at high levels in HCC we used oligonucleotide microarrays to analyze global gene expression in a panel of HCC, normal livers, and other tissues. Among the approximately 39 000 transcripts analyzed, the human homolog of *Drosophila Notum* displayed highly specific expression for HCC. NOTUM mRNA levels were low in all of the normal tissues analyzed except in placenta (Fig. 1). We found an alternative initiator methionine codon 66 amino

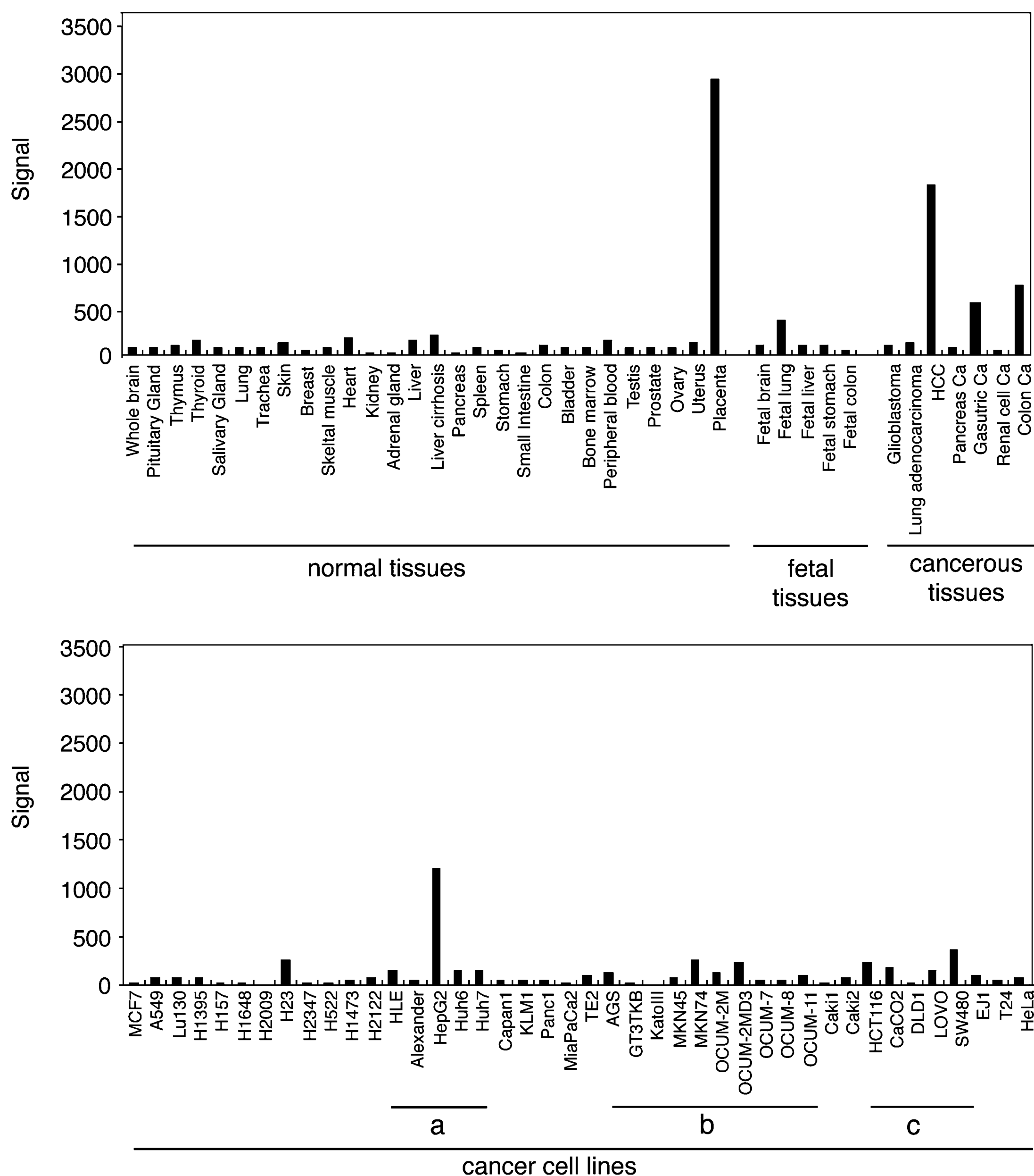

Fig. 1. Gene expression profile of human *NOTUM*. Signals indicate the gene expression level determined by GeneChip analysis of 28 normal tissues, five fetal tissues, cancerous tissues, and 42 cancer cell lines from various cancers. (a) Liver cancer, (b) gastric cancer, and (c) colorectal cancer cell lines.

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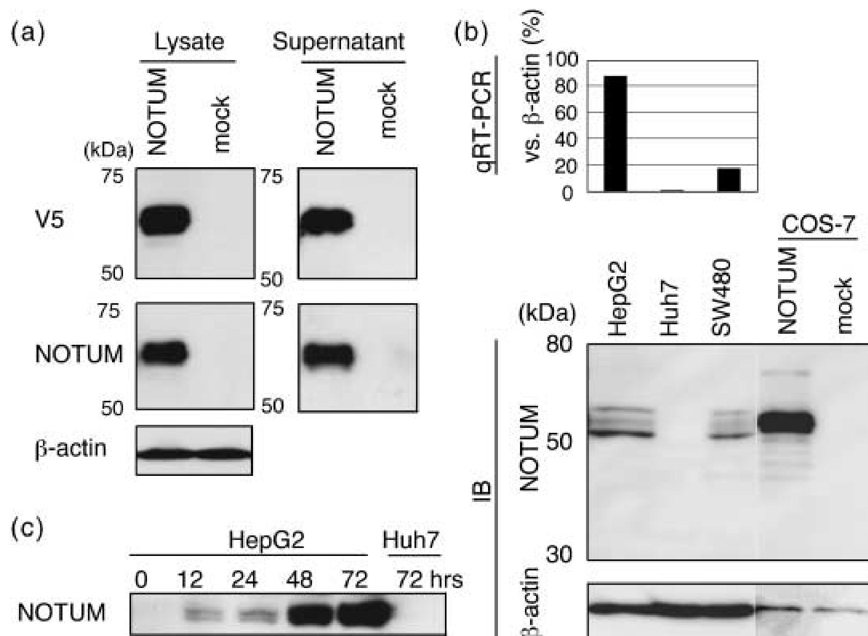

Fig. 2. Generation of the anti-NOTUM antibody. (a) Immunoblot analysis of transiently expressed NOTUM protein. Total lysate and supernatant were subjected to immunoblotting with anti-V5 (top panel), anti-NOTUM (middle panel), and anti-β-actin (loading control; bottom panel). (b) Immunoblot analysis with anti-NOTUM antibody for endogenously expressed NOTUM protein in liver and colorectal cancer cell lines. The upper graph shows *NOTUM* expression measured by quantitative reverse transcription–polymerase chain reaction (qRT-PCR). (c) Detection of NOTUM in the culture medium of HepG2. Huh7 was utilized as a negative control. IB, immunoblotting.

acids upstream of the consensus *NOTUM* ATG (see GenBank accession NP_84858). We cloned the human *NOTUM* gene extending from this upstream ATG through the consensus stop codon from a cDNA library of HepG2 cells. The cloned gene encodes a 496-amino acid protein with 38.4 and 89.7% identity to *Drosophila* and mouse Notum, respectively (Suppl. Fig. S1). The human NOTUM protein has a signal peptide from amino acids 1 to 22 (predicted by SignalP 3.0; http://www.cbs.dtu.dk/services/SignalP/) and, like its *Drosophila* homolog, is a putative secreted protein.

Generation of anti-NOTUM antibodies. We generated monoclonal antibodies against NOTUM by immunization of mice with recombinant NOTUM proteins. From 19 clones of hybridomas secreting anti-NOTUM antibodies, we selected clone H9558 because it specifically recognized both ectopically and endogenously expressed NOTUM protein. On immunoblotting of V5-tagged NOTUM protein expressed in COS-7 cells with an anti-V5 antibody, we detected a single approximately 55-kDa protein both from cell lysates and culture medium. The same band was also detected with anti-NOTUM antibody from clone H9558 (Fig. 2a). To detect NOTUM protein expressed endogenously in cancer cells, we examined protein expression in liver and colorectal cell lines, as NOTUM mRNA was expressed at high levels in the liver cancer cell line HepG2 and the CRC cell line SW480, and far less in the liver cancer cell line Huh7 (Fig. 1). Immunoblotting of NOTUM from these cell lines revealed an approximately 55-kDa species in HepG2 and SW480 but not in Huh7 cells (Fig. 2b). The amount of NOTUM protein in cancer cells was roughly proportional to the levels of NOTUM mRNA determined by quantitative RT-PCR. We also detected an approximately 55-kDa species in the supernatant from HepG2 cells (Fig. 2c). Three bands were detected in cancer cell lines, whereas a single band was detected by forced expression of NOTUM in COS-7 cells. To test whether all three species detected in the two cancer cell lines were translated from *NOTUM* or whether they represented crossreacting proteins, we carried out RNA interference (RNAi) experiments. All three bands disappeared upon exposure of cells to RNAi duplexes for *NOTUM* silencing (data not shown). Thus, we concluded that all three ~55 kDa bands recognized by H9558 were forms of NOTUM protein, and treated H9558 as a specific antibody for NOTUM in the following experiments. The presence of three forms of NOTUM protein may indicate post-translational modification. NOTUM has two predicted N-glycation sites at residues 96 and 446 (predicted by NetNGlyc; http://www.cbs.dtu.dk/services/NetNGlyc/).

Overexpression of NOTUM in HCC. We next examined NOTUM protein expression in 40 primary HCC and 36 normal livers by immunoblotting using antibody H9558. NOTUM protein was detectable in 12 of the 40 tumors (30%) but none of the normal livers (Fig. 3a). To assess the potential clinical relevance of NOTUM overexpression, we examined its correlation with a variety of clinicopathological factors, such as patients' background, hepatitis virus infection, histological findings, tumor size, and liver function. No significant differences were observed between the NOTUM-expressing and -non-expressing groups of HCC patients (Table 1; Suppl. Table S1).

Positive correlation of NOTUM overexpression with intracellular β-catenin staining. We investigated the regulation of *NOTUM* expression in HCC, focusing on the status of the Wnt pathway because *Drosophila Notum* is regulated by Armadillo, an ortholog of human β-catenin.[21] Intracellular β-catenin was observed in 10 of 40 HCC by immunohistochemistry (cytoplasmic staining in one case, nuclear staining in nine cases), whereas membrane-bound β-catenin was observed in the remaining 30 HCC. Sequence analysis of *CTNNB1* exon 3 revealed 11 HCC cases with somatic missense mutations (Suppl Table S1). Ten of the 11 mutant cases exhibited intracellular β-catenin staining (nuclear or cytoplasmic), whereas one mutant case exhibited membrane staining. Overexpression of NOTUM was associated with intracellular β-catenin localization (10 of 10 cases), but was observed in only 2 of 30 HCC with membrane staining (6.7%; $P < 0.00001$; Table 1). Immunohistochemistry of NOTUM-overexpressing nodule T2 of case #4 with *CTNNB1* mutation (G34E) indicated intracellular accumulation of β-catenin, whereas NOTUM-negative nodule T1 from the same patient had no mutation in *CTNNB1* and showed membrane staining of β-catenin (Fig. 3a,b).

NOTUM is a direct target for the β-catenin–TCF complex. To demonstrate Wnt pathway regulation of *NOTUM* expression, we examined the sequence of the *NOTUM* promoter region and found conserved consensus TCF/LEF binding sites (CTTTGAT)[22,23]

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Table 1. **Relationship between NOTUM expression, clinicopathological features, and β-catenin aberration in 40 hepatocellular carcinomas (HCC)**

Feature	*n*	NOTUM overexpression† (%)	*P*-value
Age (years)			
≤65	18	7 (38.9)	NS
>65	22	5 (22.7)	
Sex			
Male	29	9 (31)	NS
Female	11	3 (27.3)	
Virus			
HB Ag	9	2 (22.2)	NS
HCV Ab	29	10 (34.5)	
HB Ag (–), HCV Ab (–)	2	0 (0)	
Non-cancerous liver tissue			
NL	2	1 (50)	NS
CH	14	5 (35.7)	
LC	24	6 (25)	
Differentiation			
Well	16	5 (31.3)	
Moderately	15	7 (46.7)	NS
Poorly	9	0 (0)	
Tumor size (cm)			
≤3	16	5 (31.3)	NS
>3	24	7 (29.2)	
Formation of capsule			
Present	23	9 (39.1)	NS
Absent	17	3 (17.6)	
Vascular invasion (and/or IM)			
Present	10	1 (10)	NS
Absent	30	11 (36.7)	
β-Catenin staining in HCC			
Nucleus or cytoplasm	10	10 (100)	<0.00001
Membrane	30	2 (6.7)	

Ab, antibody; Ag, antigen; CH, chronic hepatitis; HB, human hepatitis B; HCV, human hepatitis C; IM, intrahepatic metastasis; LC, liver cirrhosis; NL, normal liver; NS, not significant. †Detection of NOTUM in immunoblotting.

at nucleotides –704 to –710 in human and –403 to –409 in mouse *NOTUM* promoters, respectively (Fig. 4a). We hypothesized that *NOTUM* expression might be regulated by the Wnt pathway via a β-catenin/TCF complex. To test this hypothesis, we investigated the effect of a dominant-negative TCF4 mutant (TCF4E-ΔN)[18] on *NOTUM* expression in HepG2 and SW480 cells, in which the Wnt pathway is constitutively activated. Upon induction of TCF4E-ΔN, *NOTUM* expression was reduced approximately 2-fold and 10-fold in HepG2 and SW480, respectively, compared with control levels (Fig. 4b). Expression of *DKK1*, a known TCF/LEF target gene,[18] was monitored as a positive control for effective suppression under the same conditions. We examined whether the putative TCF/LEF binding site was responsible for *NOTUM* expression by Wnt signaling. In HEK-293 cells stably expressing a constitutive active form of β-catenin (S33Y), a mutant of the putative TCF/LEF binding site decreased promoter activity (Fig. 4c). We also confirmed the responsiveness of the *NOTUM* promoter to Wnt signaling. Reporter activity of *NOTUM* promoter–reporter plasmids was increased 2.3-fold by the addition of Wnt3a conditioned medium, whereas the change in reporter activity caused by the addition of control conditioned medium was not significant for the plasmid mutated at the TCF/LEF binding site (Fig. 4c). To test for direct binding of the β-catenin/TCF complex to the *NOTUM* promoter *in vivo*, we carried out ChIP experiments using an antibody against β-catenin or TCF4 followed by PCR for *NOTUM* promoter sequences. In HepG2 and SW480 cells, where *NOTUM* is expressed highly because Wnt signaling is activated constitutively, DNA fragments spanning the TCF binding site of the *NOTUM* promoter were recovered by ChIP with antibodies against β-catenin or TCF4 (Fig. 4d). ChIP also recovered the promoter of *CCND1*, a well characterized β-catenin/TCF target gene, from HepG2 and SW480 cells (data not shown). This binding was not observed in MKN1 and HeLa cells, where the β-catenin/TCF pathway is inactivated (Fig. 4d). These results suggest that the β-catenin/TCF complex binds to the *NOTUM* promoter, as well as to the *CCND1* promoter.

Discussion

Drosophila Notum is an extracellular enzyme induced by high levels of Wingless activity.[21] Wingless plays a role in segmentation, and the development of wings and legs in *Drosophila*. Wingless is secreted by a small number of cells in each of 14 posterior compartments of the parasegments (embryonic segments), and is bound immediately to the surface of these cells by Dally and Dally-like, and heparin sulfate proteoglycans (HSPG) on the cell membrane. Binding of Wingless to HSPG is essential for Wingless signaling and gradient formation.[24,25] When Wingless signaling is activated, Notum removes bound Wingless protein from the cell surface and makes it unavailable for signaling and gradient formation by cleaving the HSPG.[26,27] Gene regulation of *Notum* in the embryogenesis of *Drosophila* has not been examined.

We identified the human ortholog of *Notum* (human *NOTUM*), formerly named *LOC147111*, as a gene overexpressed in a subset of HCC. Overexpression of *NOTUM* was also observed in primary colorectal and gastric cancers and their cell lines (Fig. 1). We confirmed the overexpression of *NOTUM* in CRC by quantitative RT-PCR (data not shown). In addition, the expression database of the International Genomics Consortium (http://www.intgen.org/) shows *NOTUM* overexpression in breast, lung, ovarian, and endometrial cancers. The expression pattern of *NOTUM* in normal organs shows that it is expressed abundantly in the placenta, but not in any other adult organs. However, high *NOTUM* expression was observed in fetal-stage lung and liver. The pattern of *NOTUM* expression in both cancer cells and fetal organs is reminiscent of the expression pattern of oncofetal proteins.

β-Catenin/TCF signaling is activated frequently due to dysfunction of β-catenin degradation in colorectal and liver cancer, and aberrant nuclear accumulation of β-catenin has been reported in 17–40% of human HCC.[6–9] We found nuclear or cytoplasmic β-catenin staining in 10 of 40 (25%) primary HCC, consistent with previous reports. All HCC samples with accumulation of β-catenin in the nucleus or cytoplasm showed NOTUM upregulation (Table 1; Suppl. Table S1), indicating that NOTUM expression is associated with canonical Wnt signaling. The frequency of β-catenin abnormality is correlated with grade of differentiation of liver cancer (i.e. *CTNNB1* mutations are more often observed in well-differentiated liver cancer).[28] Five of 16 well-differentiated HCC (31.3%), 7 of 15 moderately differentiated HCC (46.7%), and 0 of 9 poorly differentiated HCC (0%) exhibited overexpression of NOTUM. Consistent with previous observations regarding aberrant β-catenin expression, upregulation of NOTUM was rare in poorly differentiated HCC in the present study.

The positive correlation of NOTUM expression with aberrant Wnt signaling in HCC prompted us to hypothesize that human *NOTUM* is regulated directly by β-catenin/TCF. TCF binding sites are highly conserved, with the consensus sequence: 5′-CTTTG [A/T] [A/T]-3′.[22,23] By *in silico* analysis of the *NOTUM* promoter, we found a TCF/LEF binding site in the promoters of both human and mouse *NOTUM* (Fig. 4a). Upon induction of a dominant-negative mutant TCF4, *NOTUM* expression was reduced (Fig. 4b). By site-directed mutagenesis and reporter

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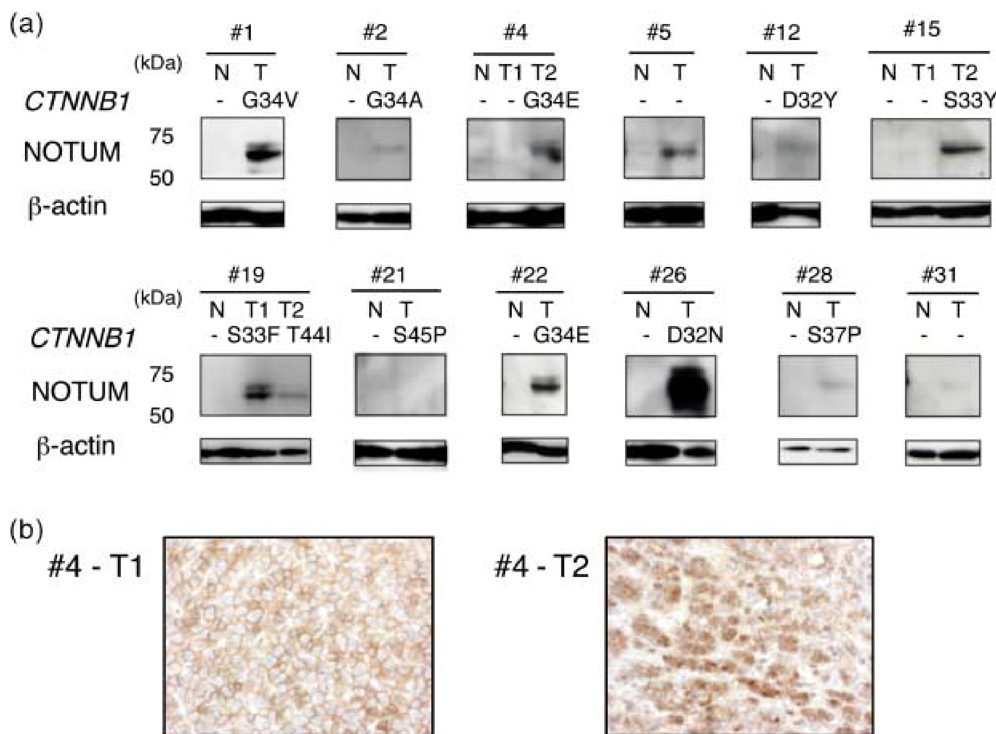

Fig. 3. Protein expression analysis of NOTUM in hepatocellular carcinoma (HCC). (a) Immunoblotting of NOTUM with paired samples from non-cancerous (N) and cancerous (T) regions of the liver. Samples of T1 and T2 indicate distinct cancerous regions from the same individuals. (b) Immunohistochemical staining for β-catenin in HCC. The cancerous T1 nodule of patient #4 had no mutation or deletion in *CTNNB1*, whereas the cancerous T2 nodule from the same individual had a mutation in *CTNNB1* (G34E).

assay, we confirmed that this putative TCF/LEF site was responsible for canonical Wnt signaling-dependent *NOTUM* expression (Fig. 4c). ChIP with anti-β-catenin or TCF4 antibody followed by promoter-specific PCR amplification provided evidence for a direct interaction of TCF4/β-catenin with the *NOTUM* promoter (Fig. 4d). These observations provide further support for the hypothesis that *NOTUM* is a new member of the human Wnt signaling pathway. NOTUM is a secreted protein and therefore could potentially be a serum marker for the diagnosis or monitoring of HCC with aberrant Wnt signaling.

The *Drosophila* glypicans Dally and Dally-like form an extracellular Wingless morphogen gradient in the wing disc.[29] We and others have previously reported that GPC3, the human ortholog of *Drosophila* Dally-like protein, is overexpressed in HCC.[30–32] Very recently, Traister and coworkers demonstrated that mouse Notum has enzymatic activity of glycosylphosphatidylinositol (GPI)-phospholipase D, and induces the release of GPI-anchored proteins such as GPC3 into the extracellular environment.[33] GPC3 is currently being studied as a serological marker of HCC and other cancers[31,32,34–37] and the mechanism of shedding GPC3 into the sera has been of interest. GPC3 is overexpressed in 60–70% of HCC[31,38] whereas NOTUM shows its overexpression in 30% of HCC. Overexpression of GPC3 in HCC was not associated with β-catenin deregulation[30] and no significant association of high GPC3 expression with NOTUM overexpression in primary HCC and liver cancer cell lines was observed by Wilcoxon signed rank test (Suppl. Fig. S2). Although upregulation of both GPC3 and NOTUM in HCC suggests that aberrant Wnt signaling contributes to hepatocarcinogenesis, they may not be expressed coordinately in liver cancer cells.

Drosophila Notum was originally identified as a negative regulator of Wnt signaling. Accumulating evidence indicates that Wnt signaling plays an important role in the differentiation of embryonic and adult stem cells, whereas many reports show that constitutive activation of the canonical Wnt pathway is causally involved in oncogenesis. Overexpression of NOTUM in cancer cells may appear to contradict the currently known functions of negative regulators of the canonical Wnt pathway. However, the luciferase activity of TOP-FLASH, a well-established LEF1 construct, was not changed by *NOTUM* silencing in HepG2 cells (data not shown). Because constitutive activation of Wnt signaling in cancer cells is caused by aberrant nuclear accumulation of β-catenin, extracellular NOTUM protein may no longer inhibit Wnt signaling. Under physiological conditions, such as developmental stage, human NOTUM may modulate Wnt signaling by inhibiting extracellular Wnt ligands. Negative regulation of Wnt signaling by NOTUM may be cell-type specific, and the role of NOTUM in cancerous and non-cancerous cells should be further examined.

In summary, we have demonstrated that human NOTUM is expressed at high levels in a subset of liver cancer; that *NOTUM* is a novel target gene of the canonical Wnt signaling pathway in humans; and that *NOTUM* expression is mediated by TCF/β-catenin complexes.

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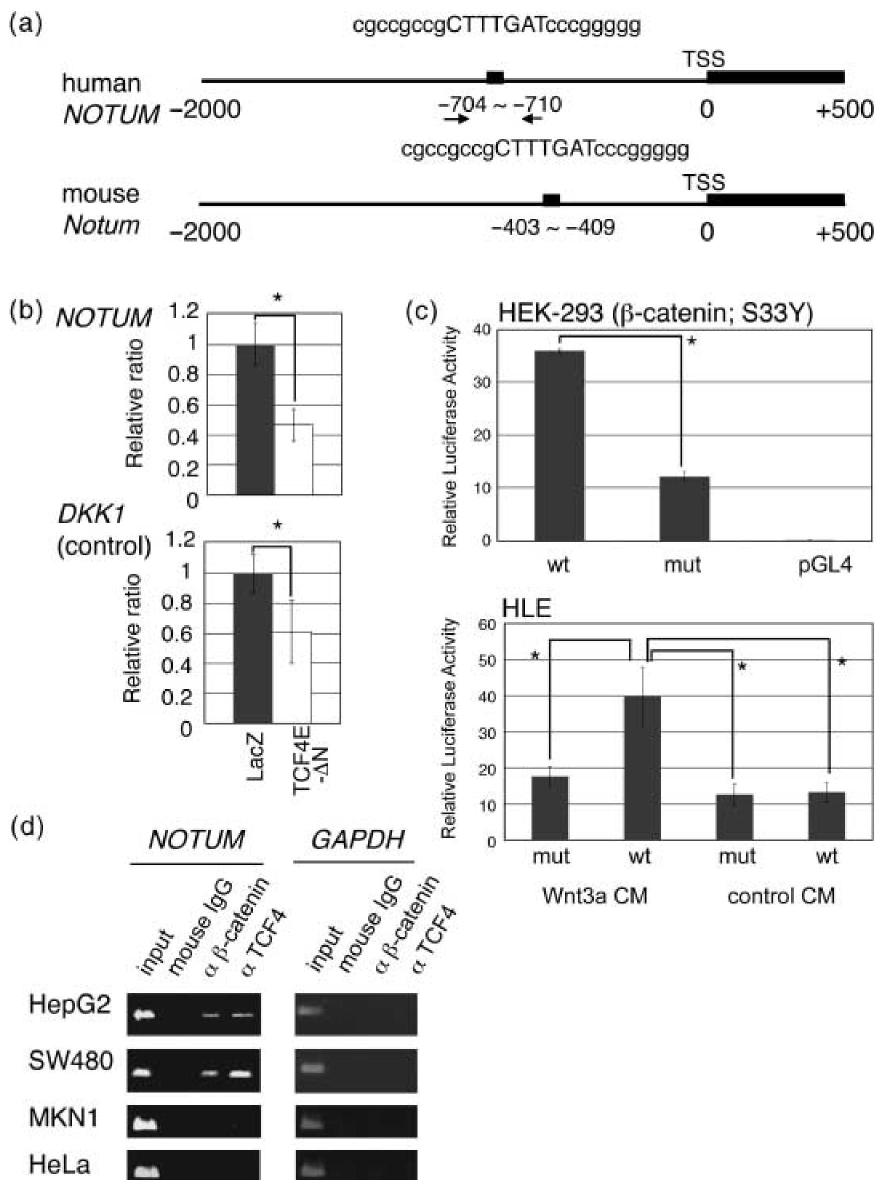

Fig. 4. *NOTUM* is a direct target for β-catenin/TCF transcriptional regulation. (a) Schematic representation of human and mouse *NOTUM* promoters. A TCF-binding element, 5′-CTTTG[T/A][T/A]-3′ was found in the human and mouse *NOTUM* promoters. Two arrows indicate the primers used to amplify the *NOTUM* promoter region in the chromatin immunoprecipitation (ChIP) assays. TSS, transcription start site. (b) Suppression of *NOTUM* expression by expression of *TCF4E*-ΔN. The expression levels of *NOTUM* in HepG2 and SW480 infected with Ad-*TCF4E*-ΔN or Ad-*LacZ* were quantified by reverse transcription–polymerase chain reaction. *DKK1* was used as a positive control of a TCF target gene. Measurements were carried out in triplicate. Error bars represent SD. $*P < 0.05$. (c) Reporter activity assay of the *NOTUM* promoter and responsiveness of *NOTUM* promoter to Wnt signaling. Upper, luciferase activity of the *NOTUM* promoter–luciferase vector (TCF/LEF binding site, wild-type (wt) CTTTGAT, and mutant (mut) CTTTGGC) was observed in HEK-293 stably expressing the constitutive active form of β-catenin (S33Y). Bottom, reporter activity of luciferase regulated by the *NOTUM* promoter was observed with Wnt3a conditioned medium (Wnt3a CM) or control conditioned medium (control CM). HLE is a hepatocellular carcinoma cell line, in which β-catenin status is intact. Measurements were carried out in triplicate. Error bars represent the SD. $*P < 0.05$. (d) Detection of the *NOTUM* promoter by ChIP assay with anti-β-catenin or TCF4 antibody. Chromatin from HepG2 or SW480 cells was subjected to ChIP with antibody to β-catenin, TCF4, or mouse IgG, followed by polymerase chain reaction to amplify the *NOTUM*, *CCND1*, or *GAPDH* promoter region. *CCND1*, a proven β-catenin/TCF target, was used as a positive control, and *GAPDH*, which is not a TCF target gene, was used as a negative control. MKN1 and HeLa cells were used as a negative control cell line, where the β-catenin/TCF pathway was inactivated.

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Supplementary materials

The following supplementary material is available for this article:

Fig. S1. Alignment of *Drosophila*, mouse, and human NOTUM.

Fig. S2. Comparison between NOTUM expression and GPC3 expression.

Table S1. Clinicopathological data according to NOTUM expression

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