Loss of Function of the Candidate Tumor Suppressor *prox1* by RNA Mutation in Human Cancer Cells[1]

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Abstract

The importance of genetic mutations in carcinogenesis has been recognized, and it has been proposed that aberrant mutation of mRNA may represent a novel oncogenic principle. Here we report that the mRNA of a homeobox gene *prox1*, a candidate tumor suppressor, suffers adenosine-to-inosine nucleotide conversion and loses tumor-suppressive functions in a subset of human cancers. Expression of Prox1 was reduced in pancreatic cancers, and the extent of reduction correlated with progression of tumor differentiation. A-to-G base change was found in *prox1* cDNA taken from human cancer cells, but not in corresponding genomic DNA. We mapped four common mutation sites in *prox1* gene, and the same four sites were mutated in human clinical samples from several cancers. Tetracycline-induced wild-type (wt) Prox1 in tumor cells inhibited transforming activity and cellular proliferation. However, mutant Prox1 with the four common sites altered from A to G lost these inhibitory functions. In mice, xenografts of tumor cells with tetracycline-induced wt-Prox1 formed tumor masses significantly more slowly than control tumors, whereas mutated Prox1 had no effect. These findings may point to a pivotal role of the RNA mutation of *prox1* gene in the pathogenesis of human cancer progression.

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Introduction

Cancer is essentially a genetic disease of somatic cells [1]. According to the currently accepted model of carcinogenesis, a series of mutations in coding regions of oncogenes or tumor-suppressor genes is required for cancer development. In the model, genomic DNA sequence with mutations is transcribed to mRNA that is finally translated into a functionally aberrant protein, leading to deregulation of fundamental cellular processes. Besides genetic mutations, several epigenetic events, such as methylation of promoters and histone acetylation, are known to affect targeted gene expression and, thus, to quantitatively modify the level of functional proteins [2]. In all these scenarios, mRNA is viewed as an intermediate between DNA and protein [3]. However, it has been recently revealed that mRNA is actively regulated by a variety of machineries playing roles at the level of mRNA processing, mRNA stabilization, and gene transcription, such as nonsense-mediated decay (NMD), RNA interference, and RNA mutation, as well as alternative splicing. Recent reports suggest that functional deregulation of these RNA-based mechanisms may be involved in carcinogenesis. NMD is shown to degrade BRCA1 mRNA bearing nonsense mutations [4]. *Ras* genes contain multiple binding sites for the let-7 family of microRNA, and the expression of let-7 and Ras is inversely correlated in human lung cancers [5].

The homeobox gene *prox1* is related to the *Drosophila prospero* gene, which mediates cell fate decisions of neuroblasts [6]. Analysis of Prox1-deficient mice indicated its various role in lens fiber elongation, hepatocyte migration, development of lymphatic vessels, retinal cell differentiation, and pancreatic development. In pancreatic development, lack of Prox1 activity disrupts epithelial pancreatic morphology, hinders pancreatic growth before E11.5, and decreases the formation of islet cell precursors after E13.5 [7]. Prox1 is considered to contribute to the allocation of an adequate supply of islet cells throughout pancreatic ontogeny by preventing exocrine cell differentiation of multipotent pancreatic progenitors, whereas the role of Prox1 in the pancreas of adults remains unknown. Prox1 is also required for the proliferation and migration of hepatoblasts in liver development [8]. However, cells lacking Prox1 are less likely to stop dividing, and ectopic expression of Prox1 forces progenitor cells to exit the cell cycle in the retina [9] and causes abnormal cellular proliferation by a downregulated expression of cell cycle inhibitors in lens fibers [10]. The role of Prox1 is very

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multifunctional, and its physiological functions may change according to developmental stage, organ, or type of cancer.

We have recently reported that there is a significant correlation between Prox1 expression and the differentiation scores of hepatocellular carcinoma (HCC) [11]. Low expression of Prox1 in tumors is closely associated with poor prognosis. Specific knockdown of *prox1* by RNA interference strongly accelerates *in vitro* cell growth, whereas overexpression of Prox1 greatly suppresses growth. These results suggest that Prox1 is involved in the differentiation and progression of HCC and, thus, may be a candidate tumor-suppressor gene for HCC.

In this study, we report our finding that *prox1* suffers adenosine-to-inosine (A-to-I) RNA mutation without genomic mutation in a subset of human cancer cells. RNA mutation is a mechanism capable of changing genetic information without affecting genomic DNA. A-to-I nucleotide conversion might change the coding potential of mRNA and might result in the synthesis of an isoform of the protein, which is not predictable from unaffected genomic DNA. Analyzing the effect of RNA mutation on protein function, we found that wild-type (wt) Prox1 suppressed tumor cell proliferation *in vitro* and *in vivo*, and that the mutant form of Prox1 lost its suppressive effect. *prox1* genomic mutation and methylation have been identified in hematologic cell lines [12], and our study suggests that RNA mutation, as a new mechanism of tumorigenesis, may induce or alter tumor progression.

Materials and Methods

Cells and cDNA Libraries

Miapaca2 and Panc1 are human pancreatic cancer cell lines with relatively poor differentiation, HCT116 is a human colon cancer cell line, and human embryonic kidney 293 cells are used as controls. All four cell lines were cultured in DMEM supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS). Human cDNA libraries of the brain, cerebellum, lung, heart, esophagus, small intestine, colon, liver, pancreas, spleen, kidney, testis, and ovary were purchased from Biochain Institute (Hayward, CA).

Patient Samples

Sixty paraffin-embedded formalin-fixed tissues of pancreatic cancers were retrieved from the 2000 to 2004 surgical pathology files of Kyoto University Hospital (Kyoto, Japan). Frozen surgical tissue samples from 9 cases of esophageal cancer, 24 cases of pancreatic cancer, and 5 cases of colon cancer were obtained for the preparation of total RNA and genomic DNA. Total RNA and genomic DNA were isolated sequentially using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. All samples were obtained with informed consent, and their use was approved by the ethics committee of the hospital.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized and hydrated by standard methods. After antigen retrieval with microwave (600 W for 5 minutes; 200 W for 10 minutes) in sodium citrate, endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 10 minutes. The sections were placed in TNB buffer (TSA Biotin System kit/NEL700; Perkin Elmer Life Science, Wellesley, MA) for 30 minutes to block nonspecific hybridization, after which they were exposed to 1:100 rabbit anti-Prox1 antibody (ReliaTech GmbH, Braunschweig, Germany) in TNB for 16 hours at 4°C, washed with TNT buffer, and incubated for 30 minutes at room temperature with biotinylated secondary antibodies (Dako, Carpinteria, CA) diluted in TNB. Tyramide signal amplification was used to enhance staining. Peroxidase activity was developed with EnVision kit/HRP (DAB; Dako), and sections were counterstained with hematoxylin. In negative control stains, primary antibodies were omitted. Staining intensity was estimated for each cell type on a three-step scale (−, +, and ++) by three investigators.

Genomic DNA and RNA Isolation, and Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

Genomic DNA were isolated using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA from cultured cell lines was isolated with TRIzol Reagent (Gibco BRL, Grand Island, NY), subsequently digested with RNase-free DNase I (Roche, Indianapolis, IN), and tested for integrity. Ten micrograms of total RNA was used to synthesize cDNA by using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and oligo-dT primers. Four sets of PCR primers intended to cover the entire coding region of *prox1* gene were used for sequencing. Primer sequences were as follows: set 1, 5′-ATGCCTGACCATGACAGCAC-3′ (forward) and 5′-TTTCATTGCCCCTTAATGCC-3′ (reverse); set 2, 5′-TAATTCGGGGTATGAGCCAT-3′ (forward) and 5′-TCTGGCCTGGGGGATCTG-3′ (reverse); set 3, 5′-CAGGTTCCTCAGGTCTTC-3′ (forward) and 5′-CTTCCTGCATTGCACTTCC-3′ (reverse); set 4, 5′-CATCTCACCACCTGAGCC-3′ (forward) and 5′-CTACTCATGAAGCAGCTCTTG-3′ (reverse). PCR conditions were 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute; and a final extension step of 72°C for 7 minutes. For analysis of Prox1 expression, its 3′ region was amplified with primers 5′-CAGATGGAGAAGTACGCAC-3′ (forward) and 5′-CTACTCATGAAGCAGCTCTTG-3′ (reverse) and, as control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified with primers 5′-GACAACAGCCTCAAGATCATCA-3′ (forward) and 5′-GGTCCACCACTGACACGTTG-3′ (reverse). Both reactions involved initial denaturation at 94°C for 3 minutes, followed by 25 to 30 cycles at 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds (for *prox1*); or 18 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds (for GAPDH), with a final extension step of 72°C for 7 minutes.

Sequence Analysis

All PCR products were first directly sequenced without cloning using the ABI PRISM Big Dye Terminator Cycle

Sequencing Ready Reaction (Applied Biosystems, Foster City, CA) by ABI PRISM 3100 sequence analyzer (Applied Biosystems). PCR products were run on 2% agarose gels and, if a single clear band of the correct approximate size was obtained, it was excised or purified by QIAquick PCR purification kit (Qiagen). When PCR products were directly sequenced, the extent of mutation at each site was determined with electropherograms from sequencing reactions to estimate the relative amounts of A and G semiquantitatively: (−) no trace of G; (+) presence of a trace of G but less than A; (++) trace of G more than A; (+++) no trace of A. Subsequently, PCR products were cloned using TOPO TA cloning kits (Invitrogen), and editing efficiency was estimated by evaluating the sequence of at least 10 clones.

Plasmids, Transfection, and Cell Proliferation

cDNA of wt-*prox1* or mutant (mut) *prox1* were subcloned into the mammalian expression vector pcDNA6/TO (tet operator; Invitrogen). Both 293 and Miapaca2 cells expressing tet repressor were transfected with wt-*prox1* or mut-*prox1* construct by using Lipofectamine (Gibco BRL) following the manufacturer's instructions. Stable clones were obtained by selecting for blasticidin and zeocin resistance and were further confirmed to induce wt-*prox1* or mut-*prox1* expression in the presence of tetracycline (0.01 μg/ml for 293 cells; 0.2 μg/ml for Miapaca2 cells). Cell proliferation assays were performed by cell counts and colony formation assay. For colony formation assay, 293 cells were seeded into 10-cm culture plates at 5×10^5 cells/plate, cultured for 10 days in the presence of tet, and stained with Giemsa solution. Tet-inducible 293/LacZ or Miapaca2/LacZ cells were used as negative controls.

Transformation Assay

For cell transformation assay, tet-inducible Miapaca2 cells were seeded into 0.4% top agar layer of 10-cm culture plates with 0.8% base agar layer at 10,000 cells/plate and cultured for 21 days in the presence of 0.2 μg/ml tet. The cells were stained with cell stain solution according to the manufacturer's instructions.

In Vivo *Study*

Mice were injected subcutaneously with tet-inducible Miapaca2 cells at 1×10^7 cells/mouse. They were maintained under a continuous administration of 0.4 mg/ml doxycycline (Dox) in drinking water for 7 weeks. Tumor size was measured weekly using the formula width^2 × length/2 and was finally weighed.

Results

Expression of Prox1 Is Downregulated in Human Pancreatic Cancer and Correlates with Its Differentiation

To test whether Prox1 protein is associated with pancreatic tumor progression, we evaluated Prox1 expression in 60 cancerous pancreatic tissues of various grades by immunostaining. Prox1 was expressed exclusively by ductal cells in normal tissues adjacent to cancerous regions (Figure 1*A*), and staining levels were in proportion to the differentiation grades of cancer cells (Figure 1, *B* and *C*). On screening various types of human cancer cell lines for *prox1* expression by RT-PCR, although the distribution of *prox1* expression reported

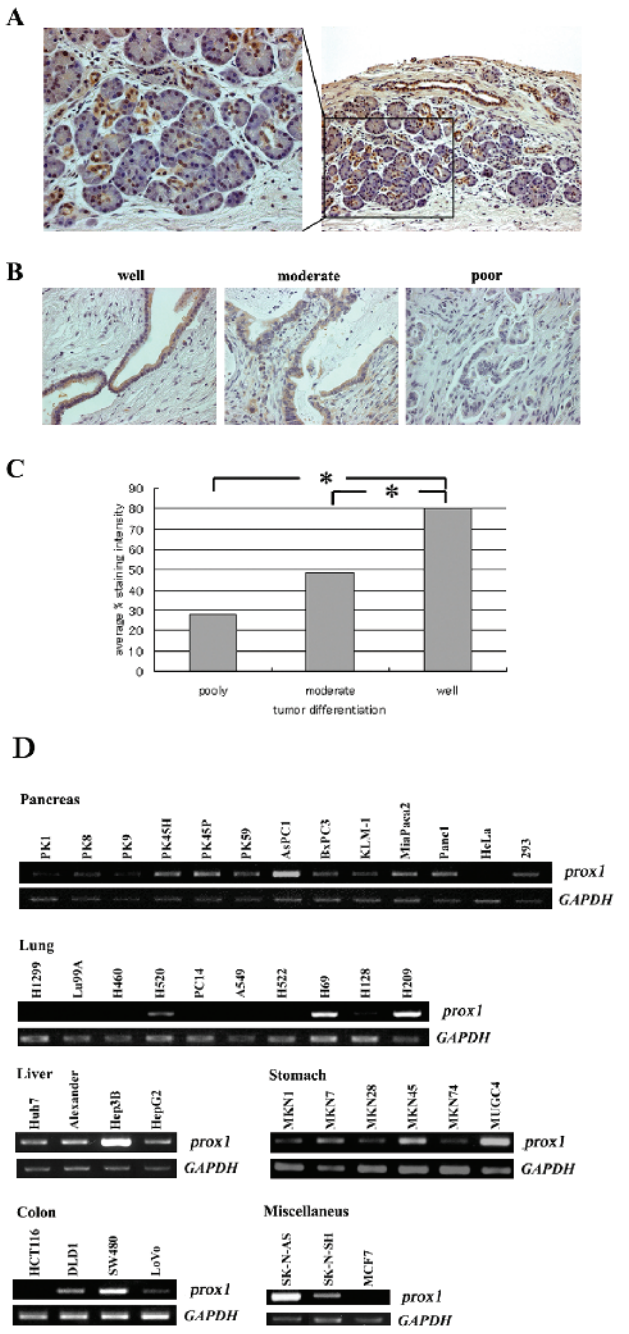

Figure 1. *Expression of Prox1 is downregulated in human pancreatic cancer and correlates with its differentiation. (A) Immunostaining of normal pancreatic tissue. Prox1 expression is localized in ductal cells. (B) Immunostaining of pancreatic cancers of various grades: well-differentiated (well), moderately differentiated (moderate), and poorly differentiated (poor). (C) Staining intensity for Prox1 was estimated for each cell type. Columns show the relationship between tumor differentiation level and the average percent staining intensity for* prox1. **P < .05. (D) Expression and mRNA editing of* prox1 *in diverse types of human cancer cell lines. RT-PCR analyses of* prox1 *expression in human pancreatic cancer cell lines: HeLa and 293 cells (pancreas), human lung cancer cell line (lung), human HCC (liver), human gastric cancer cell lines (stomach), human colon cancer cell lines (colon), and other types of human cancer cell lines (miscellaneous). GAPDH was used as internal control.*

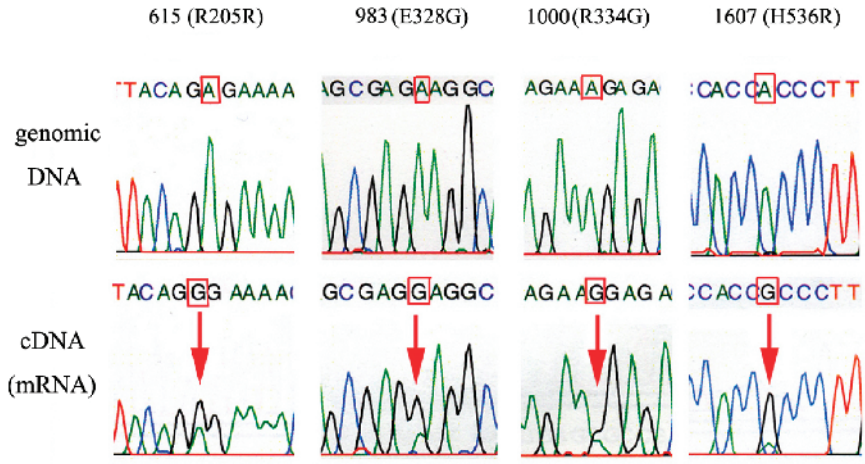

Figure 2. *Matching genomic DNA and cDNA sequences for Miapaca2 cells. The mutated site is characterized by a trace of guanosine in cDNA sequence, where genomic DNA sequence exhibits only adenosine signals (indicated by arrows).*

previously was restricted, detectable levels were found in most types of tumor cell lines in various organs: 11, pancreatic cancer; 3, small cell lung cancer; 4, HCC; 6, gastric cancer; 4, colon cancer; 2, neuroblastoma; 1, breast cancer (Figure 1*D*).

prox1 *mRNA Mutation in Human Cancer Cells*

To investigate mutations in coding regions of *prox1*, cDNA obtained from cancer cell lines were amplified by PCR and sequenced as a population without cloning. We compared the sequences with the published human genomic sequence and found that electropherograms revealed the presence of an unambiguous trace of guanosine in positions for which published data clearly indicated the presence of an adenosine in Panc1, Miapaca2, and HCT116 cells at nucleotide positions 615, 983, 1000, and 1607 (NM002763; CDS 273-2486) (Figure 2). Three of four mutations identified, at nucleotide positions 983 (E328G), 1000 (R334G), and 1607 (H536R), generate amino acid changes in translation products. The four sites did not correspond with known single-nucleotide polymorphisms (SNPs) of genomic origin. We then sequenced matching genomic DNA samples retrieved from the same tumor cell lines and confirmed that genomic DNA sequences exhibit only adenosine signals (Figure 2). The RNA mutations identified are not seen in any cDNA library and publicly available expressed sequence tags covering this gene. Finally, we analyzed human cDNA libraries obtained from the brain, cerebellum, lung, heart, esophagus, small intestine, colon, liver, pancreas, spleen, kidney, testis, and ovary, but found no signs of A-to-G mutation (data not shown).

Characterization of Mutation Sites in prox1 *Transcript*

To further analyze mutation sites, PCR products were cloned and at least 10 individual clones were sequenced. Notably, inosine is recognized by translational apparatus as guanosine, so that the change of A-to-G in cDNA implicates A-to-I conversion in mRNA. The frequency of A-to-I mutation was validated for Panc1, Miapaca2, HCT116, and 293 cells. Set 2 primers (see Materials and Methods section) were used to amplify the region covering the first three mutations (*R205R*, *E328G*, and *R334G*). Comparing cDNA sequences with the published genomic sequence revealed a high number of nucleotide discrepancies in three restricted sites (Figure 3*A*, *left*). However, the sequences of cloned genomic fragments harbored nucleotide mismatches at random sites, most probably due to sequencing error (Figure 3*A*, *center*). The number of A-to-G mismatches between the cDNA sequence and the published genomic sequence is higher than all the other 11 types of nucleotide discrepancies (Figure 3*A*, *right*). Excess exclusively in the number of A-to-G discrepancies over other base changes most probably reflects cases of *bona fide* A-to-I RNA mutation. The same results were found in the other mutation (H536R) covered by set 3 primers (data not shown). Consequently, no A-to-G conversion was found in 293 cells, whereas four common mutation sites were found in Panc1, Miapaca2, and HCT116 cells (Figure 3, *B* and *C*). All four sites were within the N-terminal domain of the *prox1* gene, which has not yet been well characterized (Figure 3*B*), but interestingly, adenosine residues at these positions are all conserved in a number of *prox1* homologues from different species (data not shown). Common multiple base changes within a stretch of several hundred nucleotides, all being of the A-to-G type, are not likely accounted for by SNPs or sequencing errors. They are flanked by evolutionarily conserved regions, and these nucleotides may have been passed on to all descendants in the course of evolution because they were somehow important for the function of protein products.

Frequency of the RNA Mutation of prox1 *in Cancer Cell Lines and Clinical Samples*

The overall frequency of mutation in Panc1, Miapaca2, and HCT116 cells was 46%, 56%, and 62%, respectively, as evaluated from cloned sequences, whereas the mutation

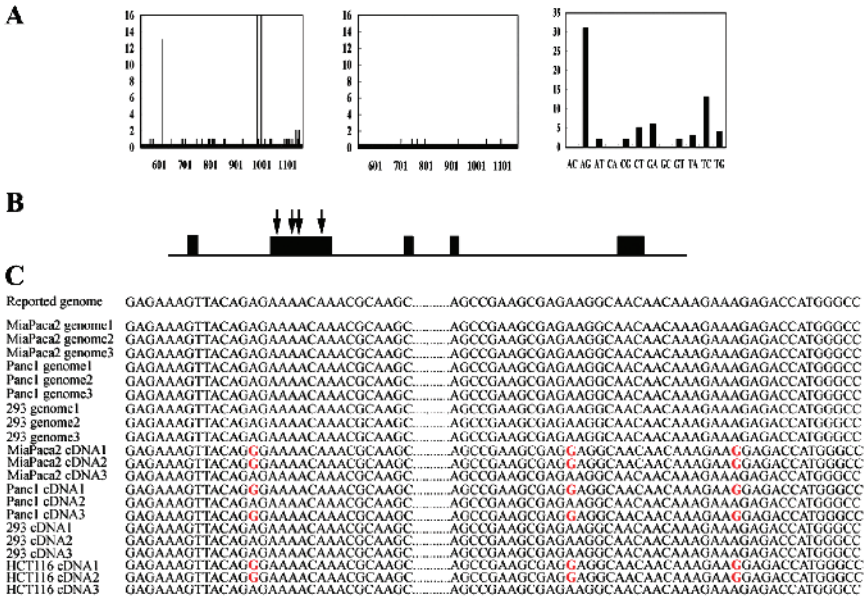

Figure 3. *Characterization of mutation sites in* prox1. *(A) Evaluation of the sequences of cloned PCR products for the* prox1 *gene by set 2 primers (see Materials and Methods section) from Panc1, Miapaca2, and HCT116 cells. The number of nucleotide discrepancies between published human genomic sequences and the sequences of cloned PCR products of cDNA (left) or genomic DNA (center) obtained from Panc1, Miapaca2, and HCT116 cells. Distribution of mismatches between cDNA sequences and published genomic sequences (right). (B) Schematic view of* prox1 *gene with predicted mutation sites (indicated by arrows) in the coding region (box: exon). (C) Sequences of individually cloned fragments were aligned to the published human genomic sequence. Mutations identified are indicated in red.*

levels of other cancer cells were negative, as evaluated semiquantitatively by electropherograms (data not shown). A-to-G base change was not found in corresponding genomic DNA from any of the clones sequenced. Furthermore, we found that the same four sites mutated in human clinical samples taken from pancreatic, esophageal, and colon cancers (Table 1). RNA mutation events were observed in four of nine cases of esophageal cancers (44%), although the frequency of mutation in each case varied. A-to-G conversion was also identified in 2 of 24 cases of pancreatic cancer (8%) and in 2 of 5 cases of colon cancer (40%).

Prox1 Functions as Tumor Suppressor, and Mutated Prox1 Loses Its Function In Vitro

To explore the impact of RNA mutations on tumorigenesis, we expressed wt-Prox1 or mut-Prox1 with the four common sites mutated in 293 and Miapaca2 cells using the tet-on gene expression system and then assessed cellular behavior. We found, by transformation assay, that the suppression of colony formation in soft agar by tetracycline-induced wt-Prox1 was evident in Miapaca2 cells (Figure 4*A*). wt-Prox1 in both 293 and Miapaca2 cells also inhibited cell proliferation (Figure 4*B*). However, overexpression of mut-Prox1 lost these suppressive functions (Figure 4, *A* and *B*).

Prox1, But Not mut-Prox1, Suppresses the Growth of Xenografted Tumors in Mice

To investigate Prox1's suppressive effect on tumor formation and the loss of function by mutation, we grafted tet-inducible derivatives of Miapaca2 cells (wt-Prox1, mut-Prox1, and LacZ) subcutaneously at both sides of the back of nude mice (Figure 5*A*, *left*). wt-Prox1–overexpressing tumors in mice treated with Dox grew more slowly than both LacZ controls in mice with Dox and wt-Prox1 tumors in mice without Dox (Figure 5). However, mut-Prox1 tumors in mice treated with Dox completely lost their suppressive activity on tumorigenesis (Figure 5*B*).

Discussion

Based on discrepancies between mRNA (cDNA) and genomic sequences, we have identified RNA mutations of a novel candidate tumor-suppressor gene *prox1* in a subset of human cancer cells. wt-Prox1 suppressed tumor cell

Table 1. The Frequency of A-to-I RNA Mutation in cDNA and Genomic DNA Obtained from Human Samples of Pancreatic, Esophageal, and Colon Cancers.

Patient Number	cDNA	Genomic DNA
Pancreatic cancer		
5	50% (2/4)	0% (0/4)
24	100% (4/4)	0% (0/4)
Esophageal cancer		
5	29% (4/14)	0% (0/7)
6	30% (10/33)	0% (0/8)
7	7% (1/14)	0% (0/8)
8	75% (3/4)	0% (0/8)
Colon cancer		
1	11% (1/9)	ND
2	11% (1/9)	ND

ND, not determined.

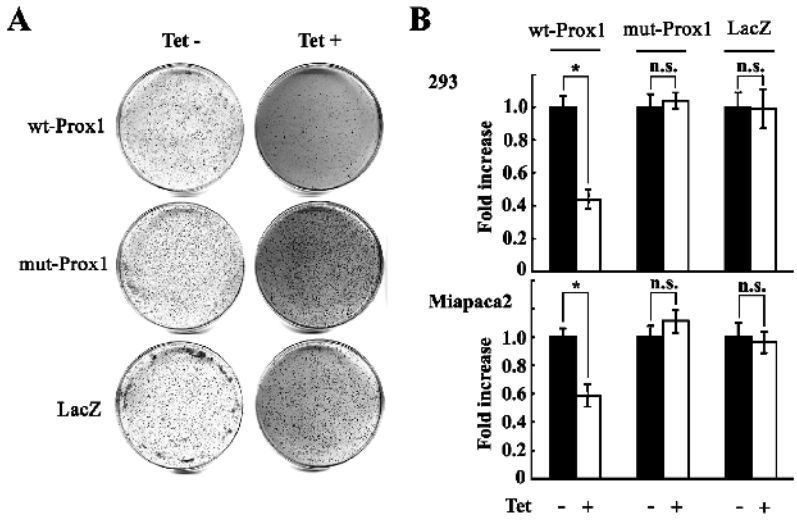

Figure 4. *Prox1 functions as a tumor suppressor, and mutated Prox1 loses its function* in vitro. *(A) Cell transformation assay in Miapaca2 cells with tet-induced wt-Prox1, mut-Prox1, and LacZ. Cells from day 21 after incubation without (left) or with (right) tet are shown. (B) Cell proliferation was suppressed significantly by overexpression of wt-Prox1, but not of mut-Prox1. Graphs show the fold increase of cell numbers (means and SD) for 293 and Miapaca2 cells with tet-induced wt-Prox1, mut-Prox1, and LacZ compared with control cells without tet induction.*

proliferation *in vitro* and *in vivo*, and the mutant form of Prox1 lost its function, implicating that RNA mutations, rather than genomic mutations, could induce or alter tumor progression. To our knowledge, this is the first demonstration of RNA mutation of a transcriptional factor that may be involved in oncogenesis. Although RNA mutation found in malignant tissues may be a cause or a consequence of carcinogenesis, mutations of the recoding process of RNA processing, particularly when it targets functionally critical residues in proteins, may be a source of diversity in disease. As an unfortunate side effect of this diversity, aberrant RNA mutation might lead to unpredicted base modifications and might cause epigenetic instability in cancer.

We have recently reported that Prox1 negatively regulates tumor progression in HCC [11]. A transient knockdown of *prox1* by siRNA significantly accelerated the growth of HCC cell lines *in vitro*, and we also demonstrated that overexpression of Prox1 resulted in suppression of cell

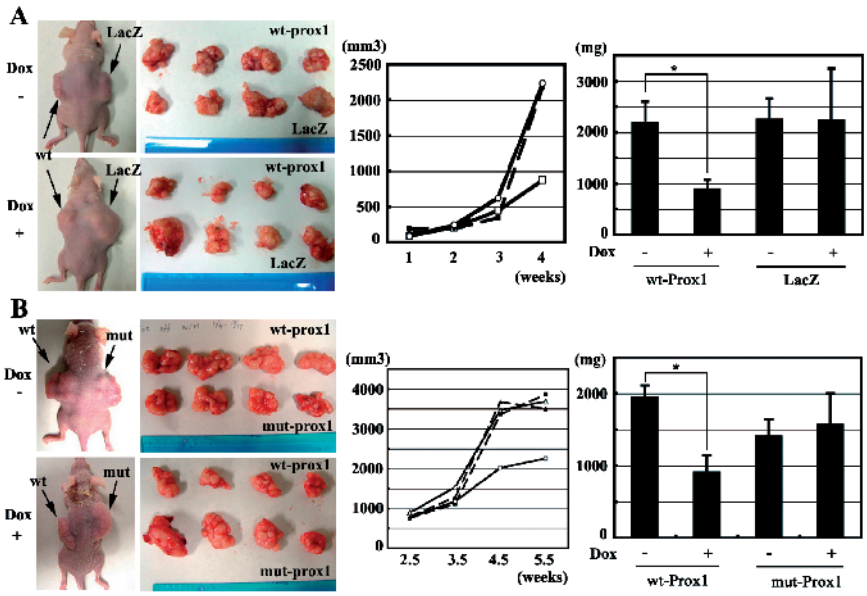

Figure 5. *Prox1, but not mut-Prox1, suppresses the growth of xenografted tumors in mice. (A) Macroscopic views reveal that Miapaca2 cells with tet-induced wt-Prox1 grow more slowly than control tumors (left). Growth dynamics of Miapaca2 cells estimated by the formula: tumor width^2 × length, expressing wt-Prox1 or LacZ (center). (■) wt-Prox1, Dox$^-$; (□) wt-Prox1, Dox$^+$; (●) LacZ, Dox$^-$; (○) LacZ, Dox$^+$. Tumor weight (mg) measured after the sacrifice of mice proves that Dox-induced wt-Prox1 suppresses tumor growth (right). *P < .05. (B) Dox-induced mut-Prox1 loses the suppressive effect of Prox1 on tumorigenesis (left). Growth dynamics of Miapaca2 cells with Dox-induced mut-Prox1 shows loss of the suppressive function of tumor formation (center). (■) wt-Prox1, Dox$^-$; (□) wt-Prox1, Dox$^+$; (▲) mut-Prox1, Dox$^-$; (△) mut-Prox1, Dox$^+$. Tumor weight (mg) shows that mut-Prox1 loses the suppressive function of tumor formation (right). *P < .05.*

proliferation. Clinically, a lower expression level of Prox1 corresponded to poorer differentiation of HCC and to poor prognosis for patients with HCC. In this study, we have demonstrated that Prox1 overexpression using tet-on system conferred a slower growth phenotype to pancreatic cancer cell lines and enabled them to form much smaller tumors in nude mice. Expression of Prox1 is downregulated in human pancreatic cancer and correlates with its differentiation. These data support novel functions for Prox1 as a tumor suppressor or as a factor to regulate the progression of the malignant character of cancer cells.

Drosophila neuroblasts divide asymmetrically to produce differentiated cells as a result of the asymmetrical localization of cell fate determinants and cortical cell polarity determinants, including the Prox1 homologue prospero, as well as Numb, Partner of Inscuteable (PINS), and aPKC [13]. It is known that the disruption of the machinery regulating asymmetrical cell division in neuroblasts leads to symmetrical cell division and, consequently, to tumor formation. Cells lacking PINS, Numb, or prospero are tumorigenic, and neuron-specific expression of a constitutive active variant of aPKC causes an increase in dividing neuroblasts [14]. Consistent with the tumorigenic potential in *Drosophila*, aPKC has been identified as a tumor suppressor in human lung cancers [15]. In fact, these molecules could protect against tumorigenesis through mechanisms independent of their roles in cell polarity in humans. Although it is not known whether Prox1 regulates asymmetrical division by stem cells in the liver or in the pancreas, it is intriguing that Prox1 has been also identified as a tumor suppressor in human cancers.

Posttranscriptional RNA editing is a process by which the nucleotide sequence of a nuclear mRNA is changed from that encoded in genomic DNA. RNA editing occurs through base modification, by deamination of cytidine (C) to uridine (U) or by deamination of adenosine (A) to inosine (I), in nuclear mRNA. Uridine and inosine are recognized by translational apparatus as thymidine and guanosine, respectively, so the net effects are changes of C-to-T and A-to-G. A-to-I RNA editing is mediated by members of the ADAR (adenosine deaminases acting on RNA) family [16]. Until recently, only a limited number of human editing substrates with an editing site in coding regions were known, including brain-specific glutamate receptor and G-protein–coupled serotonin receptors (5-HT_{2C}R) [17]. Abundant A-to-I changes in mRNA prompted us to validate *prox1* as a potential substrate of A-to-I RNA editing. We investigated whether *prox1* could be a new target for ADARs, but there was no relationship between ADAR1 or ADAR2 expression and A-to-I mutations for *prox1* gene in cancer cell lines (unpublished data). Auxiliary protein may be needed for supporting the full activity of ADARs. Alternatively, a different machinery may be responsible for A-to-I RNA mutation of the *prox1* gene because the cluster of editing sites lacks Alu repeats, where A-to-I editing in humans has been known primarily to occur. Indeed, a recent report suggests that the transcription repressor gene *PRDM1* is a target of RNA mutation, but the type of nucleotide conversions (G→A, U→A) identified in their studies does not involve the deamination process involved in A-to-I conversions mediated by ADARs [18]. Another example of G→A RNA mutation has been reported in hnRNP K protein mRNA in colorectal adenocarcinoma, and the mechanism of RNA mutation is believed to be carried out by yet unknown factors [19]. In human immunovirus-1 mRNA, which undergoes G→A mutation isolated from chronically infected H9 cells, no consensus was detected in the sequence surrounding mutation sites, and there were no consistent secondary structures evident in these regions [20].

Evidence of RNA editing as a potential oncogenic principle has come from limited tumor types, such as malignant neurofibromas in neurofibromatosis type I (NF1) and Wilms tumors [21]. C-to-U RNA editing of the tumor-suppressor *NF1* gene and of the Wilms tumor susceptibility gene *WT1* was found in tumor samples of patients. A-to-I RNA hyperediting of the hematopoietic cell phosphatase (*PTPH6*) gene in acute myeloid leukemia may contribute to the decrease in wt protein levels, suggesting its involvement in leukemogenesis [22]. In transgenic animals of APOBEC-1, the catalytic subunit of the apoB mRNA editing enzyme complex, the hepatic overexpression of APOBEC-1 mediated by the promoter of apolipoprotein E causes hepatocellular dysplasia and carcinoma [23]. NAT1 (novel APOBEC-1 target 1), whose hyperediting was identified in APOBEC-1 transgenic animals, was postulated to contribute to oncogenesis [24]. However, RNA editing of NAT1 mRNA or NF1 has not yet been identified in any natural human carcinoma [25].

In conclusion, the finding of RNA mutation in *prox1* may challenge the currently accepted model of carcinogenesis requiring mutations in DNA. RNA editing (of which cellular mechanisms are currently being discovered) or RNA mutation could be an important prerequisite for potential deregulation during cancer development. Because the genome is intact, RNA mutations may be repaired if the mechanisms underlying mutation are identified, and this report would greatly contribute to the provision of new drugs and novel therapies for cancer patients.

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