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DNA methylation-dependent regulation of TrkA, TrkB, and TrkC genes in human hepatocellular carcinoma

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ABSTRACT

The tropomyosin-related kinase (Trk) family of neurotrophin receptors, TrkA, TrkB and TrkC, has been implicated in the growth and survival of human cancers. Here we report that Trks are frequently overexpressed in hepatocellular carcinoma (HCC) from patients and human liver cancer cell lines. To unravel the underlying molecular mechanism(s) for this phenomenon, DNA methylation patterns of CpG islands in TrkA, TrkB, and TrkC genes were examined in normal and cancer cell lines derived from liver. A good correlation was observed between promoter hypermethylation and lower expression of TrkA, TrkB, and TrkC genes, which was supported by the data that inhibiting DNA methylation with 5-azacytidine restored expression of those genes in normal liver cell lines. Furthermore, Trks promoted the proliferation of HepG2 and induced expression of the metastatic regulator, Twist. These results suggest that Trks may contribute to growth and metastasis of liver cancer.

1. Introduction

DNA methylation, a covalent chemical modification occurring at cytosine residues of CpG dinucleotides, plays important roles in chromatin structure modulation, transcriptional regulation, and genomic stability, and is essential for the development of mammals [1]. Almost all CpG dinucleotides are methylated in normal cells, except those located in CpG islands, which are to a large extent constitutively unmodified and span the 5′ end of the regulatory region of many genes. Alterations of DNA methylation are one of the most commonly occurring epigenetic events in human cancers [2,3]. Human cancers generally show global DNA hypomethylation [4] accompanied by hypermethylation of tumor-suppressor genes [5–8] and inactivation of microRNA genes by DNA methylation [9,10]. DNA hypomethylation may result in chromosomal instability, reactivation of transposable elements, and loss of imprinting [11]. Hypermethylation of CpG islands in the promoter regions generally leads to inactivation of gene expression, and numerous tumor-suppressor genes have been found to be silenced by this mechanism, such as those involved in cell cycle control, DNA repair, and apoptosis [12,13].

Neurotrophins were first identified as promoters of neuronal survival; however, it is now appreciated that they regulate many aspects of neuronal development and function, including synapse formation and synaptic plasticity. In mammals, the neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4/5 [14]. Neurotrophins are ligands for receptor protein tyrosine kinases of the tropomyosin-related kinase (Trk) family. The three most common types of Trk family receptors are TrkA, TrkB, and TrkC, each of which can be activated by one or more of neurotrophins. TrkA preferentially binds NGF, TrkB binds BDNF, and neurotrophin-4/5, and TrkC binds neurotrophin-3. The binding of neurotrophins to the extracellular domain of Trks causes autophosphorylation at several intracellular tyrosine sites and triggers downstream signal transduction pathways. Trk family receptors have been reported to mediate neuronal growth and survival. For example, TrkA and TrkB have been shown to be integrally involved in neuroblastoma biology [15,16], and TrkC is highly expressed in neuroblastomas and medulloblastomas with good-prognosis [17–19]. In addition, neurotrophins and their corresponding receptors have been shown to induce a variety of pleiotropic responses in malignant cells, including enhanced tumor invasiveness and chemotaxis [20–22]. They are also important in the regulation of angiogenesis and mitogenic

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signals that facilitate tumor growth, the prevention of apoptosis, and the spreading of cells and metastasis [23–26]. Trk was initially discovered in a colon cancer–derived oncogene in which tropomyosin was fused to a novel tyrosine kinase domain [27,28]. Recently, there have been a number of new findings in other cancer settings. For example, constitutively active TrkA fusions occurred in a subset of papillary thyroid cancers and colon carcinomas [22]. Also, TrkB was reported to be overexpressed in pancreatic cancer [29] and malignant keratinocytes [30]. Furthermore, a variety of non-neuronal tissues have been shown to express TrkC, and the TrkC gene in breast, lung and pancreatic cancers was mutated within its kinase-encoding domain compared with normal tissues [31,32]. However, relatively little is known about the expression of Trk family receptor genes and their roles in maintaining the malignant properties in hepatocellular carcinoma (HCC).

HCC is one of the most common cancers in the world, showing a rapid progressive clinical course, poor response to pharmacological treatment, and poor prognosis. The lack of predictive markers for HCC may also contribute to the late diagnosis of HCC, its progression, and poor prognosis. TrkA is reported to be overexpressed in HCC tissue, mainly localized in hepatocytes, endothelial and some Kupffer cells [33]. However, the expression patterns of the TrkA, TrkB, and TrkC genes have not been well characterized in human liver cancer tissues and also relatively little is known about whether TrkA, TrkB, and TrkC activation is essential for their contribution to the malignant properties. In this study, we investigated the expression profile and methylation pattern of the TrkA, TrkB, and TrkC genes in HCC obtained from patients and human liver cancer cell lines. Moreover, we examined whether Trks can drive malignant pathophysiology in HCC.

2. Materials and methods

2.1. Cell culture and reagents

Chang liver normal cell lines and human hepatocellular carcinoma cell lines (HepG2, SNU-182, SNU-368, and SNU387) were maintained in DMEM high glucose (Gibco) supplemented with 10% heat-inactivated fetal bovine serum at 37 °C in a humidified 5% CO_2 incubator. 5-azacytidine was purchased from Sigma. Antibodies against V5 tag were purchased from Invitrogen. Antibodies against β-actin were purchased from Sigma–Aldrich.

2.2. Human tumor samples

Normal and matched HCC tissues were obtained from the Kangbuk Samsung Hospital after approval of the institutional review board and ethics committee of the hospital. Clinicopathological characteristics of HCC tissues were described in Supplemental Table 1.

2.3. Quantitative RT-PCR

Total RNA was isolated using RNeasy Mini Kits (Qiagen) according to the manufacturer's instructions and reverse transcribed with hexa-nucleotide Mix (Roche). The resulting cDNAs were used for PCR using SYBR-Green Master PCR mix and Taqman Master PCR mix (Applied Biosystems) in triplicates. PCR and data collection were performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). All quantitations were normalized to an endogenous control 18S. The relative quantitation value for each target gene compared to the calibrator for that target is expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to 18S). Specific primers and probes for TrkA (Hs01021011_m1), TrkB (Hs00178811_m1), TrkC (Hs00176797_m1), and 18S (Hs99999901_s1) were obtained from Applied Biosystems. Primers used to detect human Twist-1 mRNA were as follows: 5′-GGAGTCCGCAGTCTTACGAG-3′ (forward primer), 5′-TCTGGAGGACCTGGTAGAGG-3′ (reverse primer).

2.4. DNA methylation analysis using bisulfite sequencing

Genomic DNAs were isolated from the culturing cells by using Wizard genomic DNA purification kit (Promega). DNA (1 μg) was treated with sodium bisulfite and purified using an EpiTect Bisulfite kit (Qiagen) according to the manufacturer's instructions. Bisulfite-modified DNA was used as a template in the first round of PCR using outer primer sets to amplify the promoter regions of the TrkA, TrkB, and TrkC genes. The PCR products were then diluted from 1:10 to 1:100 depending on the amplification efficiency, and the diluted products were used as templates for the second round of PCR using inner primer sets. PCR primers for the amplification of specific targets in bisulfite-treated DNA were as follows: 5′-TAAAAATGTAGTTGTTTTAAGTTGAGAGAA-3′ (TrkA-outer-FW), 5′-AATATCAACCAAACCAACAAACTACC-3′ (TrkA-outer-RV), 5′-TTTTTAATAGGGGAGGGGGTAGAGG-3′ (TrkA-inner-FW), 5′-CCCAACTATACCAACCAAACTACCC-3′ (TrkA-inner-RV), 5′-GATAGTGGTGAGATAGATTTGTTGGTTTTG-3′ (TrkB-outer-FW), 5′-ACATCCCAAACCCTTACTCCCCTA-3′ (TrkB-outer-RV), 5′-TTTTGATTTTTTTTATTTTTAAATTTTG-3′ (TrkB-inner-FW), 5′-CATTTACAAACCTTATCTAAAAATCC-3′ (TrkB-inner-RV), 5′-TTGTTTAGTAGGTGGGATTTGGTGATTT-3′ (TrkC-outer-FW), 5′-TCTCAATCTTACTACAAACACAATTTACAA-3′ (TrkC-outer-RV), 5′-GGGATTTGGTGATTTTAGTATTATTTTT-3′ (TrkC-inner-FW), 5′-AACCCACATAATCCAACCAAAC-3′ (TrkC-inner-RV). Dr.Taq DNA master mix (BioRnD) was used for all PCRs, and the PCR conditions were as follows: 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 30 s, and then 72 °C for 5 min. The resulted PCR products were subcloned into the T&A cloning vector (RBC biosciences) and transformed into DH5α. Plasmids from at least eight individual bacterial colonies were isolated and sequenced. Analysis of bisulfite data was performed using QUMA software [34].

2.5. Viral Production and Infection of Target Cells

The open reading frame of human TrkA (AA 1–799), TrkB (AA 1–821), and TrkC (AA 1–825) were amplified by PCR and subcloned into pLNCX2 (Clontech), which contains a V5 Tag at the C terminus of the protein. The 293T cells were transiently transfected with each retroviral vector by using calcium phosphate. Supernatants were collected 48 h after transfection, and filtered through a 0.45-μM filter. HepG2 cells were spin-infected with retroviruses in the presence of 6 μg/ml of polybrene. Infection with retroviral supernatant for 24 h was followed by G418 (800 μg/mL) selection of transduced cells for 15 days.

3. Results

3.1. Expression of TrkA, TrkB, and TrkC in human hepatocellular carcinoma samples

To determine the expression patterns of the TrkA, TrkB, and TrkC genes in human liver cancer, we measured TrkA, TrkB, and TrkC mRNA expression in 5 normal and 5 invasive human hepatocellular carcinoma and compared these findings to histopathological phenotypes. The mRNA expression of TrkA, TrkB, and TrkC was elevated in 5 of 5 tumors (100%) compared with corresponding patient-matched normal tissue samples (Fig. 1A, B, and C). These results show that, in the majority of human hepatocellular

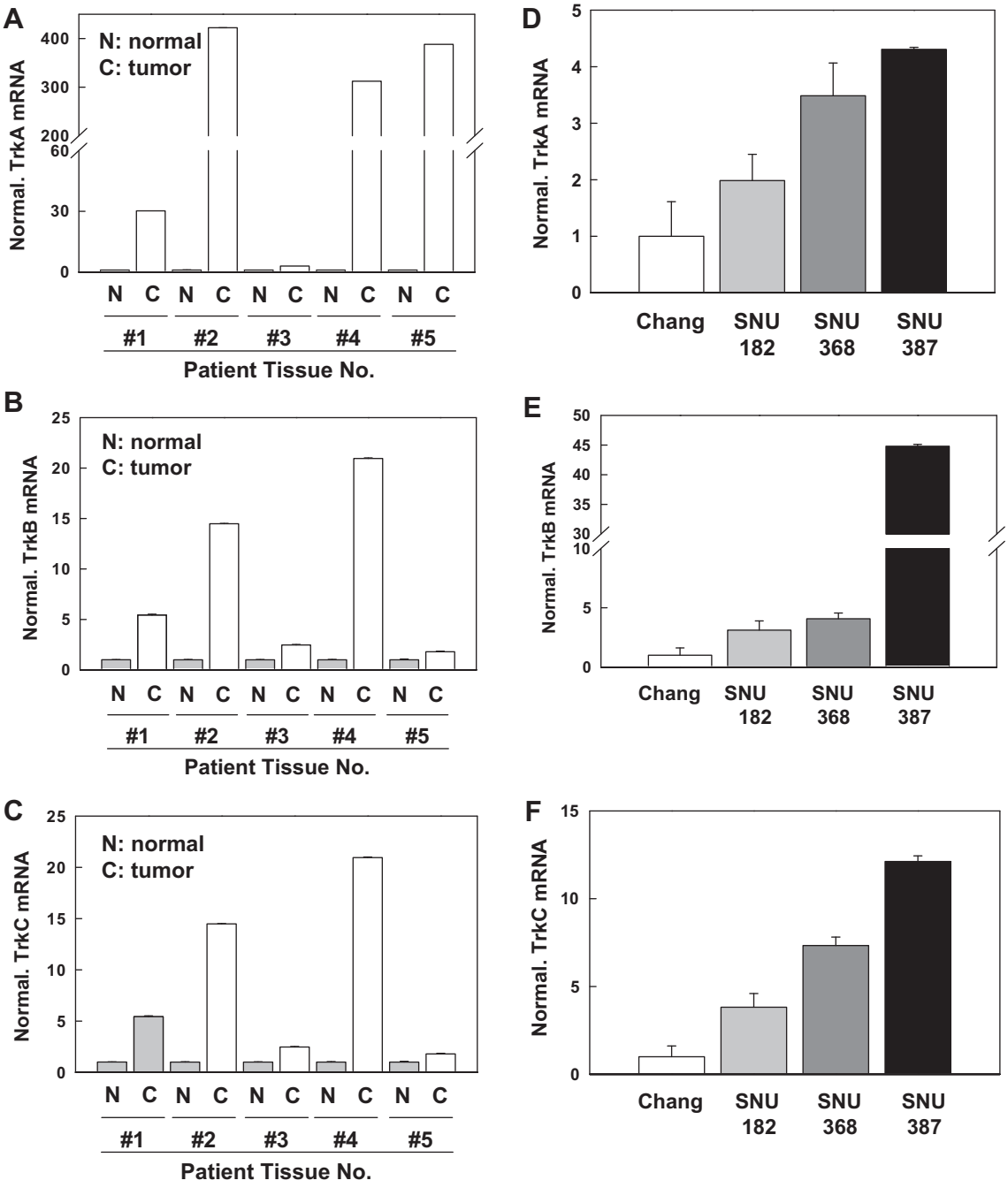

Fig. 1. TrkA, TrkB, and TrkC are overexpressed in human liver carcinoma and human hepatocellular carcinoma cell lines. (A-C) The relative expression levels of TrkA, TrkB, and TrkC in 5 individual normal and matched HCC samples were assessed by quantitative real-time PCR (N, normal; C, cancer). The endogenous 18S mRNA level was measured as the internal control. Error bars represent the mean ± SD of triplicate experiments. (D–F) The relative expression levels of TrkA, TrkB, and TrkC in a panel of human normal or metastatic liver cancer cell lines were examined by quantitative real-time PCR. The endogenous 18S mRNA level was measured as the internal control. Error bars represent the mean ± SD of triplicate experiments.

carcinomas, the expression of neurotrophin receptors (TrkA, TrkB, and TrkC) is significantly elevated above normal levels. We also examined whether expression of TrkA, TrkB, and TrkC correlated with certain pathological phenotypes in a panel of established normal and metastatic human tumor cell lines. All three metastatic cell lines overexpressed TrkA, TrkB, and TrkC. In contrast, the expression of TrkA, TrkB, and TrkC was significantly lower in chang liver normal cell lines (Fig. 1D, E, and F). These results suggested that the expression of TrkA, TrkB, and TrkC was often induced during the course of tumor progression and that it might play a causal role in enabling metastatic dissemination.

3.2. Analysis of promoter hypermethylation in the TrkA, TrkB, and TrkC genes

To determine whether elevated expression of Trk receptors in human hepatocellular carcinoma cell lines resulted from DNA hypomethylation, we analyzed the methylation status of the CpG islands associated with the TrkA, TrkB, and TrkC genes by bisulfite genomic sequencing. The methylation target for TrkA spanned the promoter region (-130 to + 76) of the gene, including a total of 25 CpGs; for TrkB, the target was a CpG island spanning the promoter region (-362 to + 4), including a total of 40 CpGs; and for TrkC, the

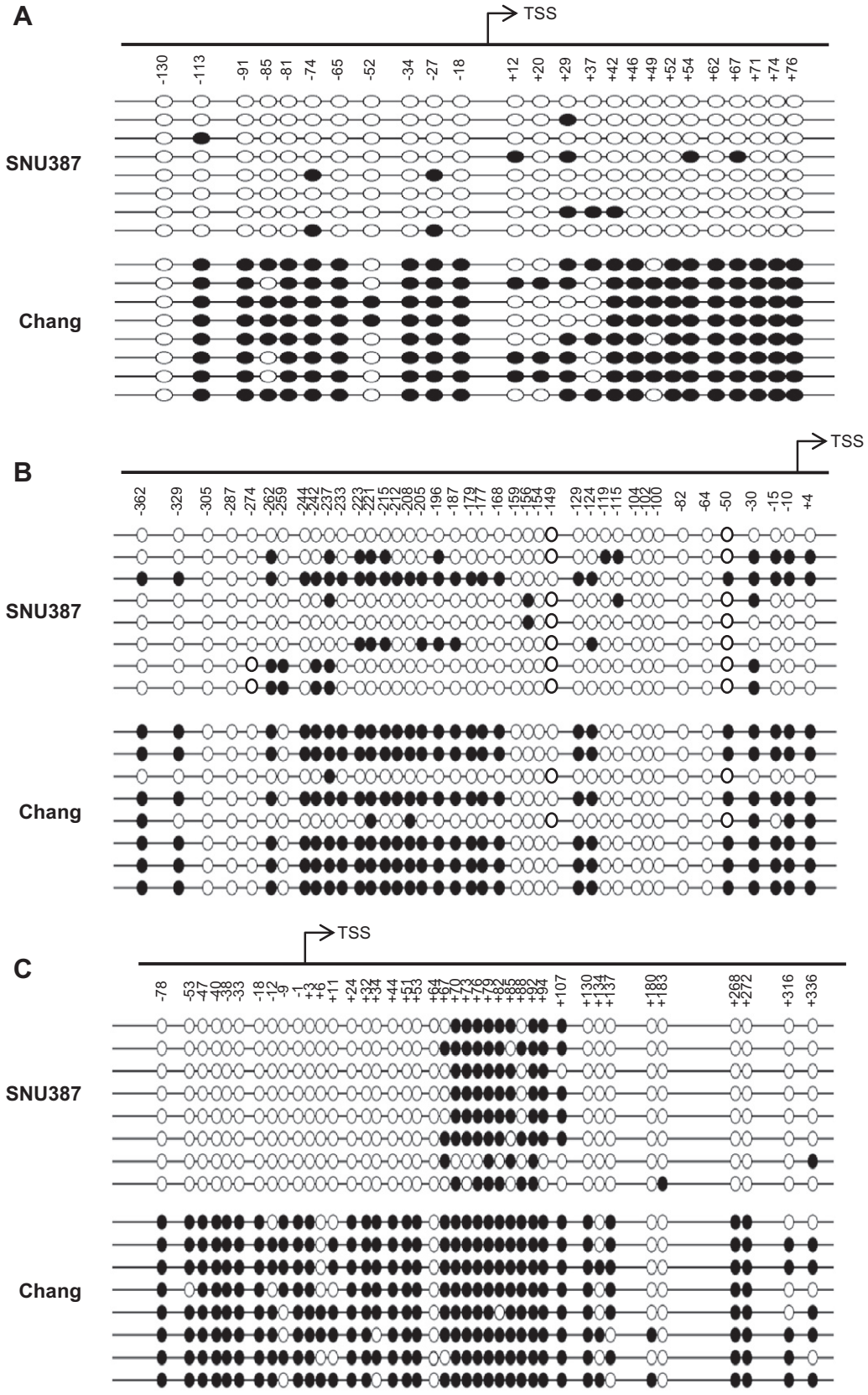

Fig. 2. DNA methylation status of CpG sites in the promoter region of human TrkA, TrkB, and TrkC genes. The DNA methylation status of TrkA (A), TrkB (B), and TrkC (C) promoter regions in human hepatocellular carcinoma cells (SNU387) and normal liver cell (Chang) was determined by bisulfite sequencing analysis. Each line represents one DNA strand; open circle, unmethylated CpGs; filled circle, methylated CpGs. TSS: Transcription start site.

target spanned the promoter region (-78 to + 336) of the gene, including a total of 40 CpGs. The detailed CpG methylation status of SNU387 and Chang cell lines was shown in Fig. 2. Most CpG sites of the TrkA promoter were not methylated in SNU387 cells that

expressed a relatively high level of the TrkA gene. Although several methylated CpG sites were identified, they were randomly distributed and limited to a few methylated sites per clone. In contrast, TrkA-negative Chang cell line showed dense methylation (> 62.5%) in the TrkA promoter in 20 of 25 CpG sites (Fig. 2A). Similar results were also obtained with the TrkB gene. Most CpG sites of the TrkB promoter showed a lower degree of methylation in SNU387 cells that expressed a high level of the TrkB gene, but 25 of a total of 40 CpG sites analyzed for TrkB were highly methylated (> 62.5%) in Chang cells which do not express the TrkB gene (Fig. 2B). Of 40 CpG sites analyzed for TrkC, 9 showed dense methylation (> 62.5%) in a limited region from + 70 to + 107, but most of the remaining CpG sites were unmethylated in SNU387 cells that expressed a high level of the TrkC gene. In contrast, 35 of a total of 40 CpG sites analyzed for TrkC were highly methylated (> 62.5%) in Chang cells which do not express the TrkC gene (Fig. 2C). These results clearly show good correlations between decreased expressions of Trk family genes and their promoter hypermethylation.

3.3. Azacitidine treatment and reexpression of neurotrophin receptors

Abnormal hypermethylation of the promoters of cancer-related or tumor-suppressor genes is commonly found in primary neoplasms and tumor cell lines [35]. To confirm the correlation between DNA methylation and Trks silencing, Chang normal liver cell lines that showed hypermethylation of TrkA, TrkB, and TrkC promoters were treated with a demethylation agent, 5-azacytidine (azaC) for 72 h. The expression levels of the TrkA, TrkB, and TrkC genes were then quantified by real-time PCR. As shown in Fig. 3, expression of TrkA, TrkB, and TrkC were highly expressed in Chang liver normal cells after treatment with 5-azacytidine. These data strongly suggest that hypermethylation was responsible for silencing of the TrkA, TrkB, and TrkC genes in normal liver cell line.

3.4. Trk-induced cell proliferation and Twist expression

To identify the functional roles of Trk receptors in liver cancer progression, we infected HepG2 cells with a retrovirus expressing the TrkA, TrkB, and TrkC genes and tested whether enforced expression of the TrkA, TrkB, and TrkC genes (Fig. 4A) would affect cell proliferation in HepG2 cells. Compared with control cells transduced with an "empty" virus, HepG2 cells transduced with the retrovirus expressing the TrkA, TrkB, and TrkC genes grew faster, indicating that TrkA, TrkB, and TrkC are required for the proliferation of HepG2 cells in culture (Fig. 4B, C, and D).

The epithelial-mesenchymal transition (EMT) is a key event in the tumor invasion process, and Twist has been identified to play an important role in EMT-mediated metastasis through the regulation of E-cadherin expression [36]. Recently, it was reported that Twist overexpression was correlated with HCC metastasis through induction of an EMT and HCC cell invasiveness [37]. So, we examined whether Trks play a role as upstream regulators of Twist expression in liver cancer cells. Interestingly, the expression of Twist was dramatically higher in TrkA-, TrkB-, and TrkC-expressing HepG2 cells compared with HepG2 control cell (Fig. 4E). These data indicated that TrkA, TrkB, and TrkC could be capable of inducing an EMT by upregulating Twist, and suggested that a Trk-induced EMT could contribute to the phenotypes of tumor invasion and metastasis.

4. Discussion

Recent reports suggest a role for neurotrophin receptors, TrkA, TrkB and TrkC, in malignant behavior of cancer cells [29,38]. In particular, they have been involved in metastasis formation, increased proliferation, and survival of cancer cells, including neuroblastoma, medulloblastoma, thyroid cancer, pancreatic cancer, malignant keratinocytes, breast cancer, and colon carcinoma. Currently, there is great interest in understanding how they function as oncogenic tyrosine kinases in malignant transformation, and these proteins are attractive targets for therapeutic intervention [39]. However, few studies have elucidated their roles in liver cancer. In the present study, we have demonstrated that the expression of Trks are elevated in human hepatocellular carcinoma tissues and liver carcinoma cell lines, and provided evidence that Trks are highly expressed during the course of tumor progression and might play a casual role in metastatic dissemination of liver cancer.

As aberrant hypermethylation of CpG islands located in the promoter regions plays an important role in the regulation of gene expression in human cancers, we investigated the methylation pattern of the promoter region and the first exon of Trk receptor genes in liver cell lines. SNU387 cell lines that expressed a high level of Trk receptors did not show methylation in the promoter regions of those genes. In contrast, Chang cell lines with low or undetectable expression level of Trk receptors showed a dramatically increased pattern of methylation. Furthermore, treatment with the demethylation agent (5-azacytidine) resulted in the reexpression of Trk receptors in normal hepatocyte cell line Chang liver cells. The data provide strong evidence that promoter methylation plays an important role in the downregulation of Trk receptors in normal hepatocyte cell line.

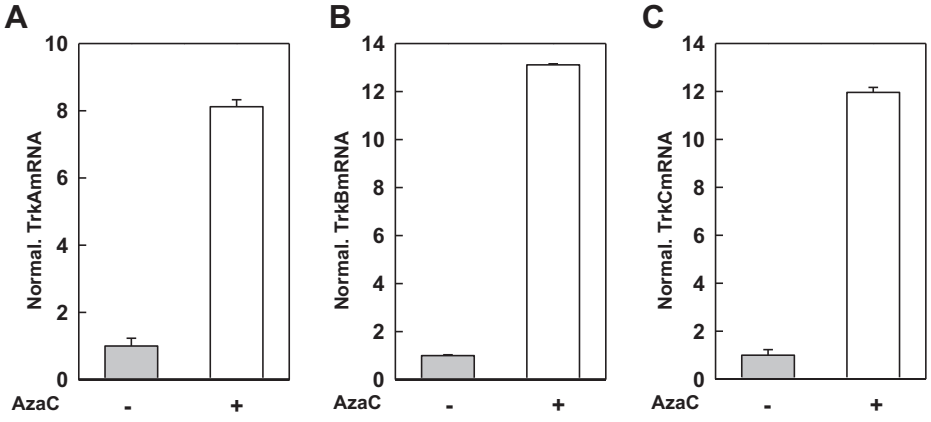

Fig. 3. Azacitidine treatment induces reexpression of neurotrophin receptors. Chang cells were treated with 1 μmol/L 5-azacytidine or vehicle control (DMSO) for 72 h. The relative levels of TrkA (A), TrkB (B), and TrkC (C) expression from Chang cells treated with or without 5-azacytidine were assessed by quantitative real-time PCR. The endogenous 18S mRNA level was measured as the internal control. Error bars represent the mean ± SD of triplicate experiments.

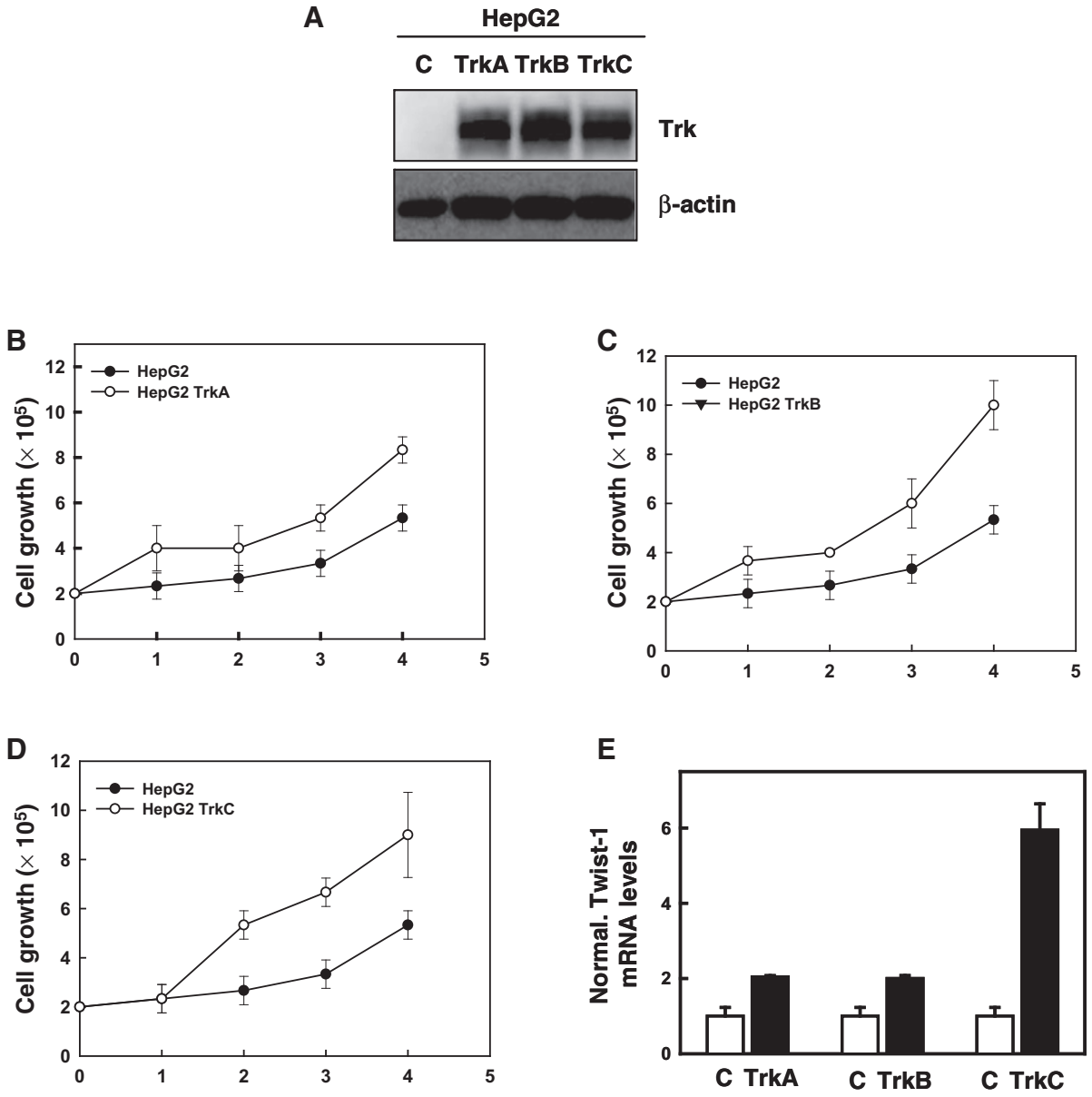

Fig. 4. Overexpression of TrkA, TrkB, and TrkC induces cell proliferation, and Twist expression. HepG2 cells were infected with indicated retroviruses, and G418-resistant cells were selected. (A) Whole cell lysates were separated with SDS/PAGE and subjected to western blotting using antibodies to V5 tag and β-actin. Population doublings of wild HepG2 cells stably expressing control, pLNCX2/TrkA (B), pLNCX2/TrkB (C), and pLNCX2/TrkC (D) were counted. Each data point represents the mean of cells counted in triplicate dishes. (E) Expression of Twist-1 was examined by quantitative real-time PCR in HepG2 cells infected with indicated retroviruses. The endogenous 18S levels were measured as internal controls. Error bars represent the mean ± SD of triplicate experiments.

The EMT refers to a complex molecular and cellular program by which epithelial cells shed their differentiated characteristics, including cell–cell adhesion, planar and apical–basal polarity, and lack of motility. After the transition, epithelial cells acquire mesenchymal features instead, including motility, invasiveness, and a heightened resistance to apoptosis. The EMT is critical for the induction of invasiveness and metastasis of human cancers [40,41], and significance of individual EMT regulators, including Twist in HCC, has been demonstrated [37,42]. Twist has been found to suppress E-cadherin expression and induce an EMT, and its overexpression in a variety of human tumors is associated with increased tumor invasion, metastasis, and poor prognosis [43]. We have shown here that overexpression of Trks promotes the proliferation of HepG2 and induces the expression of Twist. These data indicated that TrkA, TrkB, and TrkC could be capable of inducing an EMT by increasing the expression of a metastatic regulator, Twist, and suggested that a Trk-induced EMT could contribute to the phenotypes of invasion and metastasis. The characterization of regulation mechanism of Twist mediated by Trks is an interesting area for future investigation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.116.

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