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RESEARCH PAPER

5-Aza-2′-deoxycytidine suppresses human renal carcinoma cell growth in a xenograft model via up-regulation of the connexin 32 gene

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Background and purpose: The connexin (*Cx*) 32 gene, a member of the gap junction gene family, acts as a tumour suppressor gene in human renal cell carcinoma (RCC) and is down-regulated by the hypermethylation of CpG islands in a promoter region of the *Cx* gene. The current study investigated whether the restoration of Cx32 silenced by hypermethylation in RCC by a DNA demethylating agent could be an effective treatment against RCC.
Experimental approach: Using nude mice bearing Caki-1 cells (a human metastatic RCC cell line), the effects of 5-aza-2′-deoxycytidine (5-aza-CdR), a DNA demethylase inhibitor, on Cx32 mRNA expression and tumour growth were examined by RT-PCR, and by measuring tumour weight and volume. Cx32 expression in Caki-1 tumours was inhibited by Cx32 short interfering (si) RNA, and the effect of siRNA on 5-aza-CdR-dependent suppression of tumour growth in nude mice was evaluated.
Key results: 5-aza-CdR treatment inhibited the growth of Caki-1 cells in nude mice by 70% and increased 7-fold the level of Cx32 mRNA. The intratumour injection of Cx32 siRNA almost totally inhibited the expression of Cx32 mRNA and significantly reduced the suppression of tumour growth in 5-aza-CdR-treated nude mice.
Conclusions and implications: 5-aza-CdR suppressed the growth of Caki-1 tumours in a xenograft model, by restoring Cx32 expression. This finding suggests that treatment with 5-aza-CdR could be a new effective therapy against human metastatic RCC and that Cx32 could be a potential target for the treatment of RCC.
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Keywords: connexin 32; renal cell carcinoma; Caki-1 cells; DNA methylation; 5-aza-2′-deoxycytidine; c-Src; siRNA; vascular endothelial growth factor

Abbreviations: 5-aza-CdR, 5-aza-2′-deoxycytidine; Cx32, connexin 32; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GJIC, gap junction intercellular communication; NSsiRNA, nonspecific short interfering RNA; RCC, renal cell carcinoma; RT-PCR, reverse transcription-PCR; siRNA, short interfering RNA; VBL, vinblastine; VEGF, vascular endothelial growth factor

Introduction

Gap junctions are aqueous channels that connect the cytoplasm of contiguous cells, enabling the cells to directly share small metabolites by a process called gap junction intercellular communication (GJIC) or cell coupling. It has been shown that GJIC is implicated in the regulation of cell growth, mostly by negative growth control of cell proliferation (Mesnil, 2002). Correspondingly, disorders of GJIC are strongly associated with aberrant cell growth diseases, including cancer (Mehta *et al*., 1999; Evans and Martin, 2002; Trosko and Ruch, 2002; Gerido and White, 2004; Nakase and Naus, 2004; Severs *et al*., 2004; Mesnil *et al*., 2005). Owing to their frequent functional alteration in tumours, the genes of gap junction, connexin (*Cx*) genes, have been classified as tumour suppressor genes (Yamasaki and Naus, 1996). Previous reports have found that because of

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cell coupling, individual cancer cells can propagate cell-death signals into adjacent cells, which then also die by apoptosis, and that the bystander effect can enhance the cytotoxicity of anticancer agents in cancer cells (Mesnil *et al*., 1997; Yamasaki and Katoh, 1998; Wilson *et al*., 2000). In addition to this beneficial effect, each *Cx* gene has a negative growth effect on cancer with a particular specificity, and the effect is independent of GJIC (Duflot-Dancer *et al*., 1997; Yano *et al*., 2001); that is, the *Cx* gene preferentially exerts a tumour-suppressive effect on these tumours from normal progenitor cells in which the particular *Cx* gene is naturally expressed. These reports suggest the idea of using a specific *Cx* gene to achieve efficient cancer control by direct effects on cell growth and by exerting a bystander effect on several cancer therapies.

Renal cell carcinoma (RCC) has a very poor prognosis, due in large part to the fact that of nearly 30% of all patients with localized disease, 40% ultimately develop distant metastases following removal of the primary tumour, and available chemotherapeutic agents are ineffective against RCC (Motzer *et al*., 1996). Therefore, management of metastatic RCC may rely on the establishment of new, potential therapies. In our recent studies, we showed that connexin 32 (Cx32) is prominently expressed in normal human renal epithelial cells, a progenitor cell of RCC, whereas, of Cx subtypes, Cx32 is specifically downregulated in several types of human RCC cell lines as well as cancerous regions of human kidneys (Yano *et al*., 2003). On the other hand, several malignant phenotypes of primary and metastatic RCC cells are drastically attenuated by Cx32 transfection (Fujimoto *et al*., 2004, 2005); for instance, Cx32 expression by transfection reduces the growth of primary RCC cells (Caki-2 cells) and metastatic RCC cells (Caki-1 cells) in nude mice, and the metastatic potential of RCC cells in a metastasis model of human RCC using severe combined immune deficiency mice (Fujimoto *et al*., 2005, 2007a). In particular, Cx32 expression drastically suppressed the development of metastatic RCC cells in nude mice (Fujimoto *et al*., 2005).

The strong suppressive effect of Cx32 *in vivo* on metastatic RCC cells mainly depends on the inhibition of vascular endothelial growth factor (VEGF) production from cells (Fujimoto *et al*., 2005). All tumours must undergo angiogenesis or neovascularization to acquire nutrients for continued growth and metastatic spread (Folkman, 1995), and VEGF is the most important inducer of angiogenesis (Folkman, 1995; Grunstein *et al*., 1999). The main target molecule of Cx32 in RCC cells is one that stimulates VEGF production. In our previous studies, we showed that c-Src is a key target in the regulation of VEGF production in metastatic RCC cells and that Cx32 actually reduces the production of VEGF by cells via inactivation of c-Src (Fujimoto *et al*., 2005; Yonezawa *et al*., 2005). c-Src is a member of the Src family of kinases, intracellular non-receptor tyrosine kinases, widely expressed in tissues and shown to play an important role in the regulation of cell adhesion, cell growth and differentiation (Stein *et al*., 1994; Brown and Cooper, 1996). In a range of human cancers, including RCC, the activation or overexpression of c-Src has been observed (Rosen *et al*., 1986; Yonezawa *et al*., 2005), and activated forms of c-Src are capable of transforming many different cell types, partly because of the inhibition of GJIC (Chang *et al*., 1985; Hirai and Varmus, 1990). In recent studies, there has been increasing evidence that Src plays an important role in tumour cell invasion, angiogenesis and chemoresistance by upregulating signalling molecules related to these events (Turkson *et al*., 1998; Sinbaldi *et al*., 2000). From these reports, it is suggested that c-Src is a potential therapeutic target in various types of cancers, including RCC. As mentioned, in addition to general GJIC-dependent growth control of cancer cells, Cx32 effectively suppresses the *in vivo* growth of metastatic RCC cells through inactivation of c-Src, so it seems that restoration of Cx32 expression by pharmacological treatment is a promising approach to establish a new effective therapy against metastatic RCC.

Methylation of CpG islands in the 5′ regions of tumour suppressor genes is known to inhibit transcription, leading to silencing of the corresponding genes. We have reported that Cx32 is downregulated in RCC because of hypermethylation of CpG islands in the promoter region (Hirai *et al*., 2003; Yano *et al*., 2004). Also, we have confirmed that zebularine, a DNA-demethylating agent, induced re-expression of the Cx32 gene in RCC cells *in vitro* (Shirai *et al*., 2005). These reports led us to speculate that treatment with a DNA-demethylating agent would induce the restoration of Cx32-dependent tumour-suppressive effects in RCC *in vivo*. If so, this treatment approach may be an effective way to establish a new metastatic RCC therapy. To address this issue, we examined whether a DNA-demethylating agent, 5-aza-2′-deoxycytidine (5-aza-CdR), which is frequently utilized in human clinical trials (Mack, 2006), could restore Cx32-driven tumour-suppressive effects in nude mice bearing tumours from Caki-1 cells, a representative human metastatic RCC cell line.

Materials and methods

Cell culture and treatment

A representative human metastatic RCC cell line (Caki-1), obtained from ATCC (Manassas, VA, USA), was routinely maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and penicillin–streptomycin at 37 °C in an atmosphere of 5% CO_2. The cells were plated and treated with 5-aza-CdR or zebularine at the indicated doses for 72 h. For combination treatment with short interfering RNA (siRNA) and 5-aza-CdR, the cells were pretreated with the latter for 48 h before starting the former treatment. The treatment period for PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrrazolo[3,4-d]pyrimidine) was 48 h.

Cell viability assay

The cells were cultured on microtitre plates and treated with each chemical at the indicated doses for the indicated treatment periods. Cell viability was then determined using the Cell Proliferation Assay kit with WST-1 reagent (Sigma, Ishikari, Japan), according to the manufacturer's protocol.

Reverse transcription-PCR and analysis

Total RNA was isolated using QIAshredder (Qiagen, Valencia, CA, USA) and the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. cDNA was synthesized as previously described (Yano *et al*., 2002). The primers were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), accession number (BC023632), sense (nucleotides 737–756), antisense (nucleotides 916–897); Cx32, accession number (NM_000166), sense (nucleotides 194–213), antisense (nucleotides 433–452); VEGF, accession number (NM_001025368), sense (nucleotides 1163–1182), antisense (nucleotides 1370–1390). PCR was carried out by initial denaturation at 94 °C for 2 min, 28 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension for 5 min at 72 °C. The PCR products were electrophoresed on 2% agarose gels and stained with GelStar Nucleic Acid Gel Stain (Takara, Shiga, Japan). The Wide-Range DNA ladder was used as a marker for sizing the reverse transcription-PCR (RT-PCR) products. To estimate each mRNA level semiquantitatively, the ratio of each molecule to GAPDH was calculated by densitometric analysis using Atto Image Analysis Soft (Atto, Osaka, Japan).

Methylation-specific PCR

The chemical modification of cytosine to uracil by bisulphite treatment is a useful method to study DNA methylation. In this reaction, all cytosines are converted to uracil, but 5′-methylcytosines are resistant to modification and remain as cytosine. Gen Elute Mammalian Genomic DNA kit (Sigma) was used to extract genomic DNA from cell lines, as per the manufacturer's protocol. The bisulphite reaction was carried out on genomic DNA as follows (Herman *et al*., 1996): the bisulphite conversion reaction was carried out by incubating DNA with a 5-M bisulphite solution and 100 mM hydroquinone, pH 5.0, at 50 °C for 4 h. Surplus bisulphite was removed using the QIAEX II Gel Extraction kit (Qiagen, Tokyo, Japan). To estimate the methylation status of the promoter region in the *Cx* gene, bisulphite-treated genomic DNA was amplified using methylated-specific primers for Cx32 and unmethylated-specific primers for Cx32, as previously reported (Hirai *et al*., 2003). The PCR product (245 bp) was electrophoresed on 2% agarose gels and stained with GelStar Nucleic Acid Gel Stain (Takara).

Immunoblot analysis

After each treatment, the cells were lysed in Cell Lysis/Extraction Reagent with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails 1 and 2 (Sigma), and 15 μg protein extract from each sample was loaded onto 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred onto nitrocellulose membranes. The blots were incubated with each primary antibody. Each immunoreactive band was detected with an ECL system (Amersham, Buckinghamshire, UK) and the cooled CCD camera-linked Cool Saver System (Atto). Molecular sizing was performed with the Rainbow MW marker (Amersham). Protein concentration was determined by the DC Protein Assay System (Bio-Rad, Hercules, CA, USA).

Preparation and transfection of siRNA

Short interfering RNAs targeting human Cx32 and nonspecific siRNA (NSsiRNA) with the following sense and antisense sequences were used: Cx32, 5′-GCCTAGAATGTTA CACATTAA-3′ (target sequence), 5′-CUGAAAUGUUACAC AUUAA-dtdt-3′ (sense), 5′-UUAAUGUGUAACAUUUCAG-d GdC-3′ (antisense); NSsiRNA, 5′-AATTCTCCGAACGTGTCA CGT-3′ (target sequence), 5′-UUCUCCGAACGUGUCACG-dtdt-3′ (sense), 5′-AUGUGACACGUUCGGAGAA-dTdT-3′ (antisense). These siRNAs were designed and synthesized by Qiagen. The *Cx* gene was downregulated by the siRNA for Cx32 as described previously (Fujimoto *et al*., 2005). After the cells were pretreated with siRNA for 48 h, combination treatment of 5-aza-CdR and the siRNA was continued for a further 72 h. Then, deletion of Cx32 mRNA expression by siRNA was confirmed by RT-PCR, and the activated status of c-Src was evaluated by immunoblot analysis.

Antitumour effect of 5-aza-CdR on a mouse xenograft model

All animal experiments were conducted under approval from the Institutional Animal Care and Use Committee. Small fragments of Caki-1 tumour ($2 \times 2 \times 2$ mm) were transplanted subcutaneously into the right flank region of 4- to 5-week-old female athymic CAnN.Cg-Foxn1^{nu}/CrlCrlj mice (Charles River Laboratories Japan Inc., Yokohama, Japan). After 10 days, the mice received an i.p. injection of 5-aza-CdR (2.5 mg per kg body weight), dissolved in phosphate-buffered saline (PBS) or PBS only (control), once a week for 4 consecutive weeks. The mice were weighed and the tumour was measured every 3 or 4 days. Tumour volume was calculated as $(\pi/6) \times$ large diameter $\times$ (small diameter)^2. On day 36, tumours were carefully removed after death and stocked at −80 °C for isolation of RNA or ELISA. Part of the tumours were immediately fixed in 20% formalin and embedded in paraffin for histopathological examination.

Additional studies were performed using Cx32 siRNA. Nine days after tumour transplantation, Caki-1 tumour-bearing mice were treated with intratumoral injection of Cx32 siRNA or NSsiRNA at a dose of 10 μg in a total volume of 50 μl PBS twice a week for 3 consecutive weeks, in addition to 5-aza-CdR.

Protein extraction and ELISA

The tumours were lysed in CelLytic-MT (Sigma), and the lysate was cleared by centrifugation. The tumour contents of VEGF were determined with a Quantikine Human VEGF Immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The tumour protein content was measured using the DC RC protein assay system (Bio-Rad).

Histopathological analysis

The tumour samples were subjected to histopathological examination. Paraffin sections (4 μm thick) were routinely stained with haematoxylin and eosin. To assess tumour cell proliferation and death, immunohistochemical staining for Ki67 antigen (DakoCytomation, Kyoto, Japan) was

performed. Briefly, after deparaffinization and rehydration, the sections were immersed in 0.3% hydrogen peroxide to quench intrinsic peroxidase activity. The diluted antibodies were then added to the sections and incubated at 37 °C for 1 h. The labelled antigen was visualized with the Histofine kit (Nichirei Pharmaceutical, Tokyo, Japan), followed by reaction with 3,3′-diaminobenzidine. Finally, the sections were counterstained with haematoxylin. Ki67-positive cells were determined by counting about 500 nuclei in randomly selected microscopic fields, and the Ki67 labelling index was expressed as the ratio of Ki67-positive cells to total cells.

Statistical analysis

Data are expressed as the mean ± s.e. and analysed by one-way ANOVA followed by Dunnett's *t*-test or Student's *t*-test only. $P<0.05$ was considered as showing a significant difference between means.

Materials

All cultures and chemical reagents were purchased from Invitrogen (Carlsbad, CA, USA) and Sigma (St Louis, MO, USA), respectively. PP2 (Src inhibitor) and PP3 (4-amino-7-phenylpyrazol[3,4-d]pyrimidine), (negative control of PP2) were obtained from Calbiochem–Novabiochem (La Jolla, CA, USA). Antibody towards Ki67 antigen was obtained from DakoCytomation. All other antibodies were purchased from BD Biosciences (San Jose, CA, USA).

Results

Effects of 5-aza-CdR on cell growth, Cx32 expression and Src activation in vitro

To estimate whether a representative DNA-demethylating agent (5-aza-CdR) could suppress the *in vitro* growth of Caki-1 cells based on restoration of Cx32 expression, the cells were treated with 5-aza-CdR. Treatment with 5-aza-CdR significantly inhibited cell proliferation by 40% compared with the control (Figure 1a). Similarly, the treatment increased Cx32 mRNA levels in a dose-dependent manner (Figure 1b), and methylation-specific PCR analysis showed clearly that the treatment induced demethylation of a promoter region in the *Cx32* gene (Figure 1c).

As previously reported (Fujimoto *et al*., 2005), we confirmed that enforced expression of Cx32 induced the inactivation of c-Src in Caki-1 cells and that the inactivation of c-Src led to negative growth control of RCC cells (Figures 2a and b). Similarly, 5-aza-CdR induced the inactivation of c-Src in cells, whereas Cx32 siRNA reversed 5-aza-CdR-induced Cx32 expression and activated c-Src (Figures 2a and c). These results indicate that 5-aza-CdR exerted negative growth control of cells via the inactivation of c-Src by restoration of Cx32 expression.

Effects of 5-aza-CdR in a murine xenograft model

We next examined whether 5-aza-CdR treatment could restore Cx32 expression *in vivo*. On day 36 after tumour implantation, tumour volume and weight in the 5-aza-CdR treatment group were significantly reduced to 29 and 27% compared with the control, respectively (Figures 3a–c). 5-Aza-CdR treatment had no influence on body weight compared with the control group (Figure 3d). In addition, none of the mice died from the treatment during the course of the experiment (data not shown). Cx32 mRNA expression in tumour tissues from the 5-aza-CdR-treated group was drastically increased compared with that from the control group (Figure 4a), and, in densitometric analysis, the level of Cx32 mRNA showed about a sevenfold increase by the treatment. In contrast, 5-aza-CdR treatment in tumour tissues reduced mRNA and protein levels of VEGF (the major molecule necessary for angiogenesis) (Figures 4b and c). Levels of mRNA and protein in the treatment group were reduced to about 10 and 25% of those in the control group,

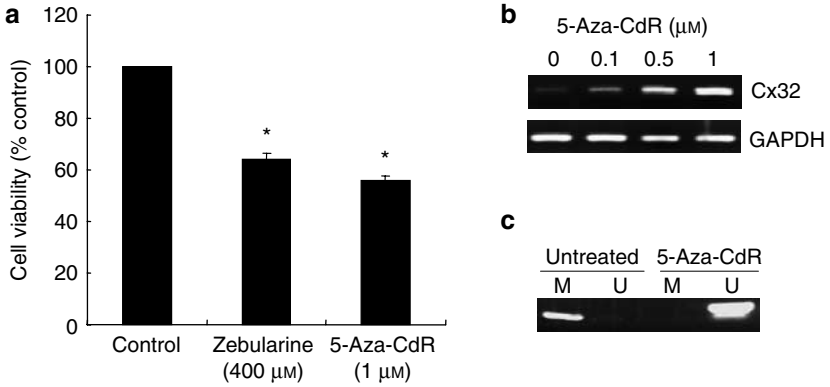

Figure 1 Effects of 5-aza-CdR treatment on cell proliferation and Cx32 expression in Caki-1 cells. (**a**) Cells were treated with 5-aza-CdR or zebularine for 72 h, and subsequently cell proliferation was measured using WST-1 reagent. Zebularine was used as a positive control to confirm the effect of DNA demethylation. Each value is the mean of four determinations; vertical lines indicate s.e.mean. *$P<0.05$ compared to control. (**b**) Restoration of Cx32 mRNA expression in Caki-1 cells after treatment with 5-aza-CdR for 72 h. GAPDH was used as an internal control. This result is representative of two independent experiments. (**c**) Methylation-specific PCR of Cx32 in Caki-1 cells. After 5-aza-CdR treatment (1 μM) for 72 h, methylation-specific PCR analysis was performed as described in Materials and methods. The presence of a PCR product in M indicates the presence of the methylated *Cx32* gene, and the presence of a PCR product in U indicates the presence of the unmethylated *Cx32* gene. This result is representative of two independent experiments. 5-Aza-CdR, 5-aza-2′-deoxycytidine; Cx32, connexin 32; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

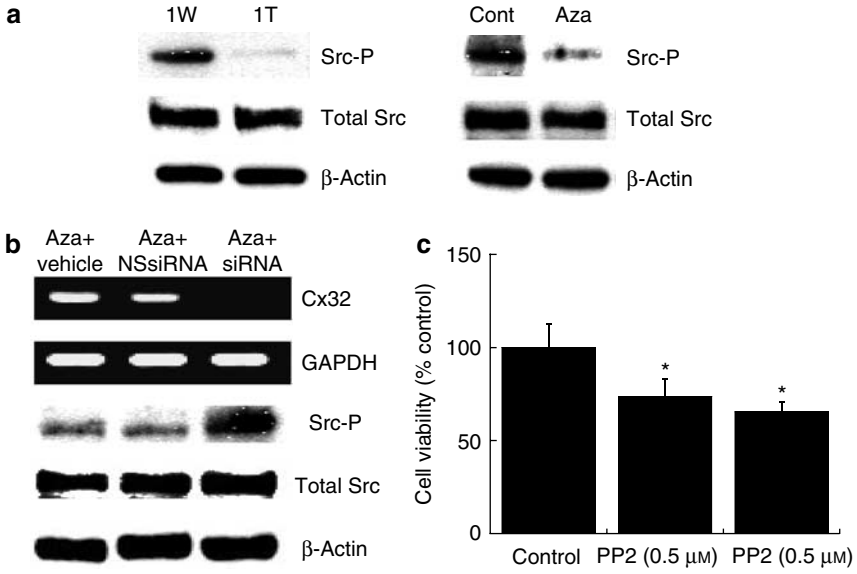

Figure 2 Relation between Src activation and Cx32 expression in Caki-1 cells treated with 5-aza-CdR. (**a**) Effects of forced Cx32 expression or 5-aza-CdR treatment on the activation of c-Src. Cells were treated with 5-aza-CdR (1 μM) for 72 h, and subsequently total c-Src and phosphorylated c-Src (active form) levels were determined by immunoblot analysis as described in Materials and methods. β-Actin was used to confirm the equal loading of protein between the two groups. 1W, empty vector-transfected Caki-1 cells; 1T, Cx32-transfected Caki-1 cells; Cont, vehicle treatment; Aza, 5-aza-CdR treatment. This result is representative of two independent experiments. (**b**) Effect of Cx32 siRNA on c-Src in Caki-1 cells treated with 5-aza-CdR. Cells were treated with Cx32 siRNA and 5-aza-CdR as described in Materials and methods. After treatment, the level of Cx32 mRNA was determined by RT-PCR, and the activation of c-Src was evaluated by immunoblot analysis. Aza + vehicle, 5-aza-CdR and vehicle treatment; Aza + NSsiRNA, 5-aza-CdR and NSsiRNA treatment; Aza + siRNA, 5-aza-CdR and Cx32 siRNA treatment. This result is representative of two independent experiments. (**c**) Effect of PP2 on cell proliferation in Caki-1 cells. Cells were treated with PP2 for 48 h at the indicated doses. After treatment, cell viability was determined as above. We also confirmed that PP2 selectively inhibited Src activity in Caki-1 cells under this treatment condition, using PP3 (negative control of PP2). Each value is the mean of five determinations; vertical lines indicate s.e.mean. $*P<0.05$ compared to control. 5-Aza-CdR, 5-aza-2′-deoxycytidine; Cx32, connexin 32; NSsiRNA, nonspecific short interfering RNA; RT, reverse transcription; siRNA, short interfering RNA.

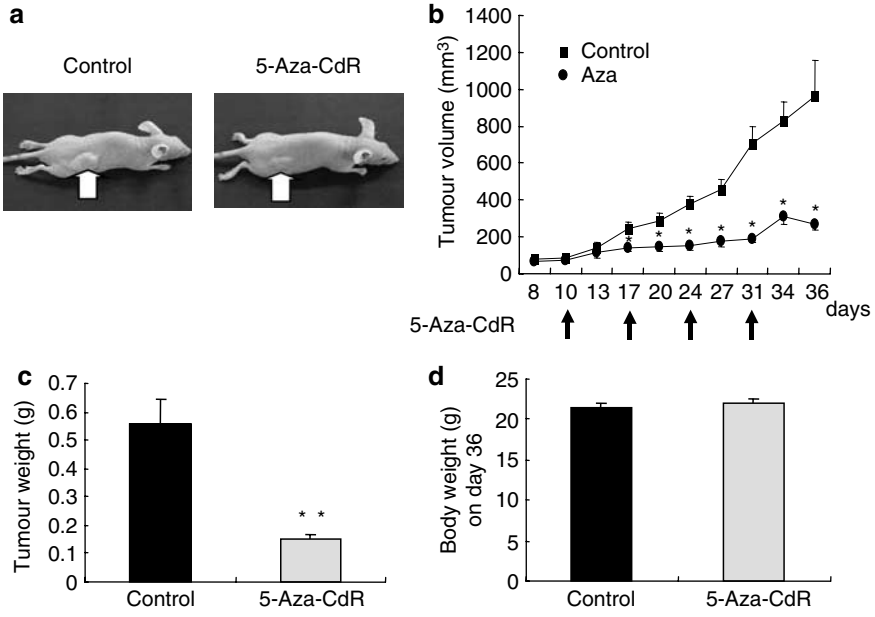

Figure 3 Effects of 5-aza-CdR treatment on the development of Caki-1 tumours in nude mice. (**a**) Representative photos of nude mice bearing Caki-1 tumours each day after cell inoculation. Arrows indicate tumour tissues in each group. (**b**) Tumour volume was measured in each indicated period as described in Materials and methods. 5-Aza-CdR (2.5 mg per kg body weight) was administered by i.p. injection on days 10, 17, 24 and 31 after cell inoculation. Each value is the mean of four determinations; vertical lines indicate s.e.mean. $*P<0.05$ compared to control. (**c** and **d**) Tumour weight and body weight were measured on day 36 after inoculation. Each value is the mean of four determinants; vertical lines indicate s.e.mean. $*P<0.05$ compared to control. 5-Aza-CdR, 5-aza-2′-deoxycytidine.

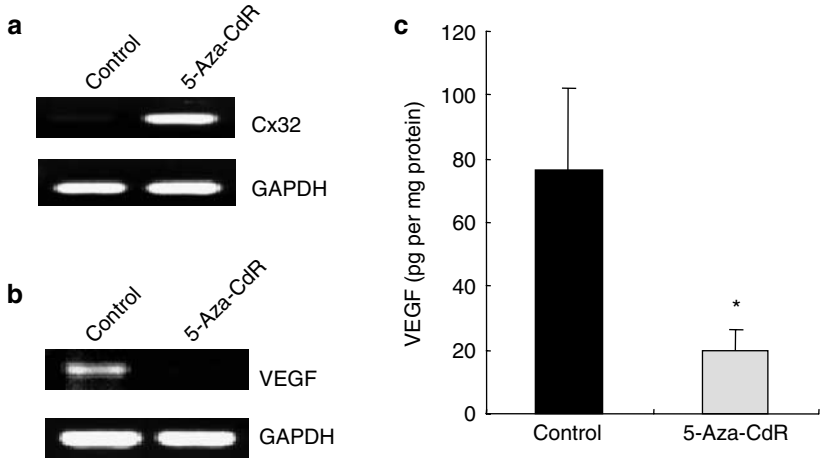

Figure 4 Effects of 5-aza-CdR treatment on Cx32 and VEGF expression in Caki-1 tumours of nude mice. (**a** and **b**) Representative levels of Cx32 (**a**) and VEGF (**b**) mRNA were determined by RT-PCR analysis. GAPDH was used as an internal control. (**c**) Intratumoral VEGF contents were measured by ELISA. The amount of VEGF was normalized to the protein content in each tumour tissue. Each value is the mean of five determinations; vertical lines indicate s.e.mean. *$P<0.05$ vs control. 5-Aza-CdR, 5-aza-2′-deoxycytidine; Cx32, connexin 32; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; VEGF, vascular endothelial growth factor.

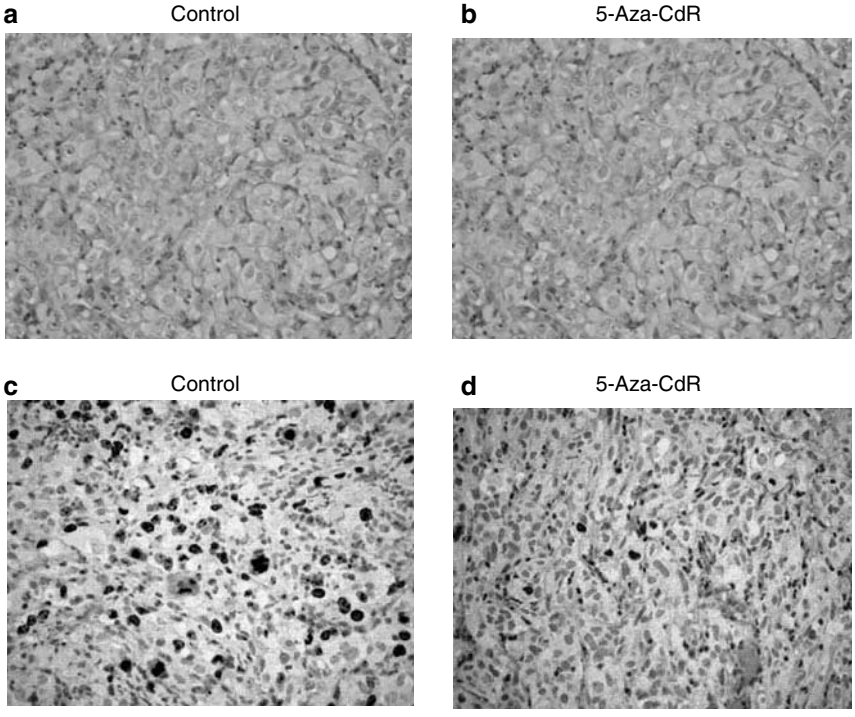

Figure 5 Histopathological examination of Caki-1 tumours in nude nice. (**a** and **b**) Representative H&E-stained 4-μm sections from Caki-1 tumours. (**c** and **d**) Immunohistochemical staining for Ki67 antigen. (**a** and **c**) Sections from mice that received PBS. (**b** and **d**) Sections from mice that received 5-aza-CdR. 5-Aza-CdR, 5-aza-2′-deoxycytidine; H&E, haematoxylin and eosin; PBS, phosphate-buffered saline.

respectively. Histologically, both control (Figure 5a) and 5-aza-CdR-treated (Figure 5b) tumour tissues were basically identical under routine microscopic observation. Immunohistochemical examination showed that the proliferative activity of tumour cells indicated as Ki67-positive cell number was higher in the control than in the 5-aza-CdR-treated samples (Figures 5c and d). The ratio of Ki67-positive cells to total cells in the former and latter samples was 32.5 and 7.3%, respectively. These results demonstrate that 5-aza-CdR-induced expression of Cx32 was closely associated with negative growth control of Caki-1 cells in this xenograft model.

Effects of Cx32 siRNA on tumour development of 5-aza-CdR-treated mice

To confirm a significant role of Cx32 in 5-aza-CdR-dependent growth control of Caki-1 cells in the xenograft model, we evaluated whether intratumoral injection of Cx32 siRNA effectively abolished the suppressive effect of 5-aza-CdR. As

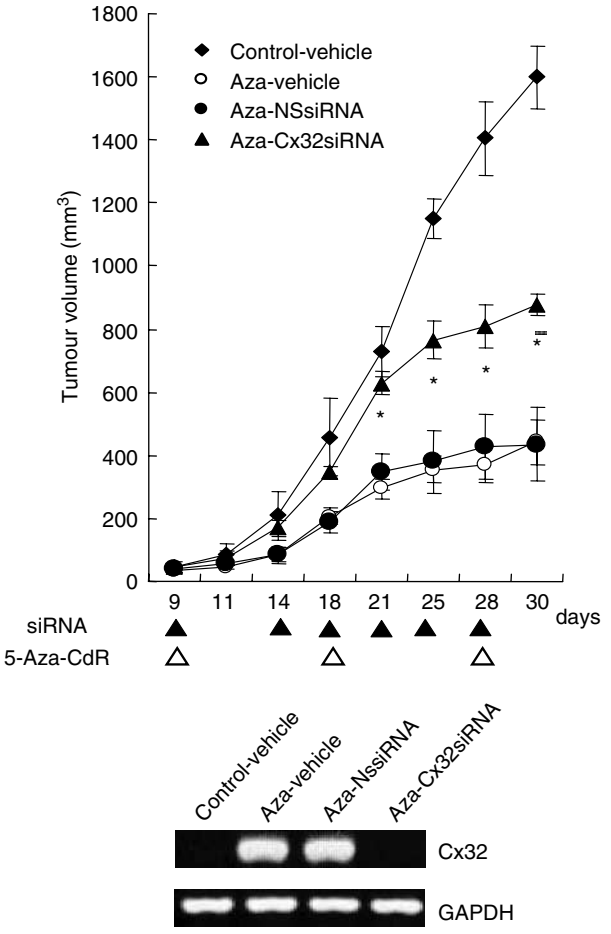

Figure 6 Suppressive effect of Cx32 siRNA on Cx32 mRNA expression and the effect of Cx32 siRNA on tumour growth in 5-aza-CdR-treated nude mice. Mice bearing Caki-1 tumours were treated with intratumoral injection of Cx32 siRNA or NSsiRNA on days 9, 14, 18, 21, 25 and 28 after cell inoculation. 5-Aza-CdR was also administered by i.p. injection on days 9, 18 and 28. Tumour volumes were measured in each indicated period. Knockdown of Cx32 by siRNA was confirmed by RT-PCR. Control-vehicle, PBS treatment; Aza-vehicle, 5-aza-CdR and PBS treatment; Aza-NSsiRNA, 5-aza-CdR and NSsiRNA treatment; Aza-siRNA, 5-aza-CdR and Cx32 siRNA treatment. Each value is the mean of five determinations; vertical lines indicate s.e.mean. $*P<0.05$ compared to Aza-vehicle. 5-Aza-CdR, 5-aza-2′-deoxycytidine; Cx32, connexin 32; NSsiRNA, nonspecific short interfering RNA; PBS, phosphate-buffered saline; RT, reverse transcription; siRNA, short interfering RNA.

shown in Figure 6, about 40–50% of the 5-aza-CdR-dependent suppressive effect in the xenograft model was reversed by Cx32 siRNA treatment. Also, we confirmed silencing of the *Cx32* gene by RT-PCR. This result strongly supports the above observation that restoration of Cx32 expression by 5-aza-CdR treatment plays an important role in suppressing the development of Caki-1 tumours in the xenograft model.

Discussion

Human metastatic RCC is known to show severe chemoresistance against several types of chemotherapeutic agents and has a poor prognosis in most cases. Therefore, it is a priority to establish a new therapy against RCC. In our previous studies, we reported that Cx32 reduced several malignant phenotypes of primary and metastatic RCC cell lines, mainly due to the regulation of c-Src signalling (Yano *et al*., 2006). Also, we have demonstrated that upregulation of c-Src during tumour progression in kidneys contributes to the acquisition of resistance against cancer therapy (Yonezawa *et al*., 2005). From these reports, we hypothesized that restoration of Cx32-dependent tumour suppression by pharmacological treatment could lead to the establishment of a new effective therapeutic strategy for RCC. The present study was undertaken to examine this possibility. Here, we report that 5-aza-CdR significantly suppressed the growth of human metastatic RCC cells (Caki-1 cells) in a xenograft model, mainly because of the restoration of Cx32 gene expression.

In this study, we demonstrated that 5-aza-CdR reinduced Cx32 expression in Caki-1 cells *in vivo* and that restoration of the Cx gene was closely associated with the suppression of RCC cells. Furthermore, to confirm the contribution of Cx32 to the negative growth control of RCC cells *in vivo*, we planned to induce silencing of the Cx32 gene using siRNA in 5-aza-CdR-treated xenograft model mice. As a result, the intratumoral injection of Cx32 siRNA to 5-aza-CdR-treated nude mice clearly demonstrated that Cx32, restored by 5-aza-CdR treatment, dominantly contributed to the suppression of tumour development in nude mice inoculated with Caki-1 cells. From these results, we concluded that restoration of Cx32 by 5-aza-CdR was absolutely required for the *in vivo* negative growth control of RCC cells driven by the agent. However, some parts of 5-aza-CdR-dependent suppressive effects were not affected by Cx32 siRNA, even though siRNA almost totally suppressed the expression of the *Cx32* gene. From this observation, it was assumed that 5-aza-CdR upregulated other tumour suppressor genes related to cell growth control. To determine the tumour suppressor gene(s), we examined mRNA levels of death-associated protein kinase (DAPK), the Ras association domain family 1A (RASSF1A), von Hippel-Lindau (VHL) and $p16^{INK4a}$, which are well known for suppression due to hypermethylation in human RCC (Sanz-Casla *et al*., 2003; Alleman *et al*., 2004; Tokinaga *et al*., 2004; Christoph *et al*., 2006). In Caki-1 tumours, mRNA levels of DAPK, RASSF1A and VHL, determined by RT-PCR, were not changed by 5-aza-CdR treatment, suggesting that the expressions of these genes were not suppressed by hypermethylation in Caki-1 tumours. In contrast, we confirmed that $p16^{INK4a}$ mRNA expression was not observed in Caki-1 tumours and that 5-aza-CdR treatment induced $p16^{INK4a}$ expression (data not shown). As $p16^{INK4a}$ prevents the entry of cells into S phase in the cell cycle by antagonizing the activity of cyclin D-dependent kinases (Sherr and Roberts, 2004), the expression of $p16^{INK4}$ may contribute to some 5-aza-CdR-dependent suppressive effects on the development of Caki-1 tumours in nude mice.

In our previous study, enforced expression of the *Cx32* gene effectively suppressed the growth of primary and metastatic RCC cells *in vivo* as well as *in vitro* because of the inactivation of c-Src (Fujimoto *et al*., 2005). In this study, we

observed that 5-aza-CdR-induced Cx32 expression exerted negative growth control of Caki-1 cells via the inactivation of c-Src, and this observation was confirmed by Cx32 siRNA and Src inhibitor experiments. As mentioned, it is well known that c-Src plays an important role in tumour cell proliferation, survival, invasion and angiogenesis (Turkson *et al*., 1998). Of the Src-regulated signal molecules, the signal transducer and activator 3 is considered key to inducing the Src-dependent appearance of malignancy in several types of cancers (Gray *et al*., 2005). We have observed that the inactivation of signal transducer and activator 3 based on the inhibition of c-Src leads to the suppression of cell growth by the inhibition of cyclin D required for G1/S progression in the cell cycle, survival by decreased Bcl-xL as a representative antiapoptotic molecule and angiogenesis by the down-regulation of VEGF as an inducer of angiogenesis in RCC cells (Yonezawa *et al*., 2005). We also found that 5-aza-CdR significantly reduced the production of VEGF by Caki-1 tumours in nude mice, indicating that the restoration of Cx32 expression by this agent might suppress the development of Caki-1 tumours *in vivo* via the inactivation of c-Src/signal transducer and activator 3 signalling. However, the inhibition of c-Src by Cx32 transfection or Src inhibitors in Caki-1 cells induces a cytostatic effect but not apoptotic cell death, maybe due to higher activity of c-Src (Yonezawa *et al*., 2005; Fujimoto *et al*., 2005, 2006). In contrast, the same treatment by Src inhibitors can cause apoptotic cell death in other RCC cells such as ACHN cells (Yonezawa *et al*., 2005). Of the RCC cells utilized in the study, Caki-1 cells were established from human metastatic RCC and had higher c-Src activity, and other RCC cells were established from human primary RCC cells and had lower c-Src activity (Yonezawa *et al*., 2005). These observations suggest that the increase of c-Src activity during tumour progression in the kidney contributes to the acquisition of resistance against the induction of apoptosis. The available chemotherapeutic agents, such as vinblastine (VBL), are ineffective against metastatic RCC (Torti, 1983); thus, overcoming treatment-resistant malignancy is necessary to establish an effective therapy for metastatic RCC.

The significant advances in understanding the tumour-suppressive effect of Cx32 on metastatic RCC might lead to the development of therapies. We recently reported that transfection of the *Cx32* gene into Caki-1 cells potentiated VBL-induced cytotoxicity *in vivo* as well as *in vitro*. The enhancing effect of Cx32 depends on the bystander killing effect based on Cx-driven GJIC and the inactivation of c-Src in a GJIC-independent manner (Sato *et al*., 1983, 2007b). In particular, Cx32 enhanced VBL accumulation in RCC cells by the suppression of P-glycoprotein, partly because of the inactivation of c-Src. As P-glycoprotein appears to function as an energy-dependent transport pump capable of decreasing the level of VBL in RCC cells (Ambudkar *et al*., 1999), the downregulation of P-glycoprotein by Cx32 expression may directly contribute to the enhancement of VBL-induced cytotoxicity in RCC cells. Collectively, it seems that the combination of the bystander effect and c-Src inhibition exerted by Cx32 was one of the most effective procedures to enhance VBL-induced cytotoxicity in metastatic RCC. If this is confirmed, 5-aza-CdR-induced restoration of Cx32 expression could be an effective procedure to improve chemotherapy against metastatic RCC.

In summary, 5-aza-CdR treatment significantly suppressed the development of Caki-1 tumours in a xenograft model via the restoration of Cx32 expression caused by demethylation in the promoter region. The present data suggest that Cx32 is a potential target for the treatment of human metastatic RCC and that treatment with 5-aza-CdR is a promising therapy against RCC.

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Conflict of interest

The authors state no conflict of interest.

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