

# Protein kinase C $\delta$ in tumorigenesis of human malignant fibrous histiocytoma

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Received June 3, 2011; Accepted July 13, 2011

DOI: 10.3892/or.2011.1415

**Abstract.** Protein kinase C $\delta$  (PKC $\delta$ ), an isoform of PKC, has been shown to act as a critical mediator of tumor progression and apoptosis; however, its role in musculoskeletal tumors is still unknown. In the current study, we examined the expression of PKC $\delta$  in human musculoskeletal tumor tissue samples, and investigated the effects of siRNA downregulation of PKC $\delta$  on human malignant fibrous histiocytoma (MFH) cell proliferation, migration, and apoptosis, to elucidate its functional roles in musculoskeletal tumorigenesis. Of note, real-time PCR analysis revealed that mRNA expression of PKC $\delta$  in high-grade musculoskeletal MFH tumors was significantly lower than that in benign schwannomas. siRNA downregulation of PKC $\delta$  significantly increased human MFH cell proliferation and migration, and markedly suppressed apoptosis. These findings suggest that PKC $\delta$  has a negative effect on tumorigenesis and/or acts as a pro-apoptotic kinase in human MFH cells. The data presented here could be applied in the development of new therapeutic avenues, with the elevation of PKC $\delta$  expression being one potential strategy to prevent MFH progression. Thus, PKC $\delta$  may be a potent therapeutic target for human MFH.

## Introduction

Malignant fibrous histiocytoma (MFH) is the most common high-grade soft tissue sarcoma to occur in late adult life. Advances in the treatment of MFH have led to multidisciplinary treatment, including surgery, chemotherapy, and radiation therapy, resulting in great improvements in the quality of life for patients with MFH. However, the current chemotherapeutic strategies for MFH are not as effective as those for other malignancies (1,2). The prognosis of patients

with the disease can be poor due to local recurrence and metastases, therefore, new therapeutic strategies against MFH need to be established.

Over the last two decades, protein kinase C (PKC) has attracted attention in the cancer research field as a regulator of tumor development (3,4). PKC is a family of serine/threonine kinases, which consists of at least 12 isoforms classified into three subgroups depending on their requirement for calcium and diacylglycerol for activation; the classical PKCs ( $\alpha$ ,  $\beta$ ,  $\gamma$ ), the novel PKCs ( $\delta$ ,  $\epsilon$ ,  $\eta$ ,  $\theta$ ) and the atypical PKCs ( $\mu$ ,  $\xi$ ,  $\iota$ ) (4). Each PKC isoform is considered to have a distinct role in the regulation of cell proliferation, survival, migration or apoptosis in a cell type-dependent manner (5-7). PKC $\delta$  belongs to the calcium-independent group, and is widely expressed in mammalian cells, including cancer cells (8). Unlike other PKC isoforms, PKC $\delta$  has been regarded as a negative regulator of cell growth and is pro-apoptotic (9-11), whereas recent studies revealed that PKC $\delta$  can act as a positive regulator of tumor progression in some cancer types (12,13). These apparently conflicting data suggest that PKC $\delta$  may have either negative or positive functions, depending on the cellular context (14). However, the expression of PKC $\delta$  in human musculoskeletal tumors has not been studied, and its functional role in the regulation of malignant musculoskeletal tumor cell proliferation and apoptosis are still unknown.

In the current study, we examined the expression of PKC $\delta$  in human musculoskeletal tumor tissue samples and correlated it to the stage of malignancy. Furthermore, focusing on its functional role in MFH, we evaluated the effect of siRNA downregulation of PKC $\delta$  on cell proliferation, cell migration and apoptosis in human MFH cells *in vitro*.

## Materials and methods

**Human musculoskeletal tumor tissue samples.** Tumor samples, including 7 malignant fibrous histiocytomas (MFH) and 10 schwannomas, were obtained by surgery at Kobe University Hospital in accordance with institutional guidelines. All patients participating in this study gave informed consent prior to surgery.

**Human MFH cell lines.** Two human MFH cell lines (Nara-H and TNMY1) were used for *in vitro* studies. Nara-H were

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**Key words:** protein kinase C $\delta$ , malignant fibrous histiocytoma, tumorigenesis, apoptosis

purchased from ScienStuff Co., Nara, Japan (15), and TNMY1 cells were previously established in our laboratory (16). Cells were grown in culture medium consisting of DMEM (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 100 U/ml penicillin/streptomycin solution (Sigma-Aldrich). Cell lines were routinely maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

**RT-PCR analysis.** Total RNA was extracted from all human musculoskeletal tumor tissue samples by selective binding to a silica-gel-based membrane using an RNeasy Mini kit, following the manufacturer's protocol (Qiagen, Valencia, CA, USA). First strand cDNAs were reverse transcribed with 1  $\mu$ g of total RNA and oligo-dT primer by MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA). Equal amounts of cDNAs were subsequently amplified by PCR in a 20  $\mu$ l reaction volume containing 1X PCR buffer, 200  $\mu$ M dNTPs, 0.5  $\mu$ M sense and antisense primer, and 1.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). RT-PCR was carried out as follows: one cycle at 94°C for 10 min, followed by 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min. This was followed by a single cycle at 72°C for 10 min to facilitate final extension. The sequences of the primers for human PKC $\delta$  were 5'-TTTCTACCCACCTCATCTG-3' (forward) and 5'-CGAAGAGTTCATCCTCATCATC-3' (reverse). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for RNA integrity. RT-PCR products were run on 2% agarose gels, stained with ethidium bromide dye, and visualized by UV illumination.

**Quantitative real-time PCR.** The expression of PKC $\delta$  was analyzed by quantitative real-time PCR. We isolated total RNAs from all tumor tissue samples and the two MFH cell lines, and first strand cDNAs were transcribed. Real-time PCR was performed in a 20  $\mu$ l reaction mixture using TaqMan real-time PCR (Applied Biosystems) on the ABI prism 7500 sequence detection system (Applied Biosystems). The PCR conditions were: 1 cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Pre-designed primers specific for human PKC $\delta$  (Hs01090047\_m1) and human GAPDH (Hs99999905\_m1) were purchased from Applied Biosystems. The values were normalized with those for GAPDH, and relative PKC $\delta$  expression levels were calculated using the  $\Delta\Delta C_t$  method.

**siRNA downregulation of PKC $\delta$ .** To evaluate the effect of PKC $\delta$  expression on MFH cells, we transfected the MFH cell lines with small interfering RNA (siRNA) to silence PKC $\delta$  expression. Human PKC $\delta$ -specific siRNA and negative control siRNA (control siRNA) were purchased from Ambion Inc. (Austin, TX, USA). The sequences of PKC $\delta$  siRNA were: 5'-GGAGUGACCGGAAACAUC-3' and 5'-UGAUGUUUCCGGUCACUCC-3'. Briefly, MFH cells were seeded at 2x10<sup>5</sup> cells in a 6-well culture plate, and were transfected one day later with 100 pmol of either PKC $\delta$  siRNA or control siRNA using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, total RNAs and whole cell lysates were collected.

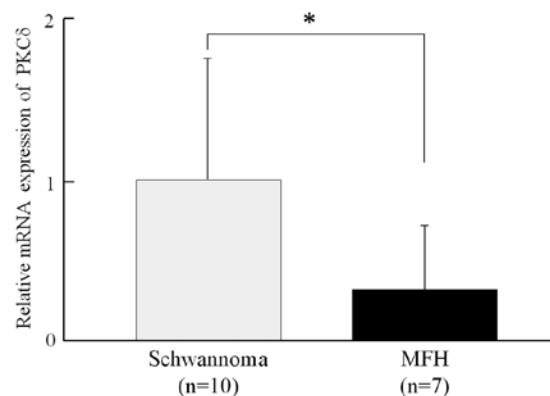


Figure 1. mRNA expression of PKC $\delta$ . mRNA expression of PKC $\delta$  in human musculoskeletal tumor tissue samples was quantified using real-time PCR. PKC $\delta$  expression in high-grade MFH musculoskeletal tumors (n=7) was significantly lower than that in benign schwannomas (n=10) (\*P<0.05).

**Immunoblot analysis.** Whole cell lysates were collected from MFH cells that were transfected with either PKC $\delta$  siRNA or control siRNA using a whole cell lysis buffer supplemented with protease and phosphatase inhibitor mix (Roche Applied Science, Indianapolis, IN, USA). After incubation on ice for 20 min, cell lysates were centrifuged at 15,000 rpm for 20 min and supernatants were collected. The protein content of lysates was then quantified using BCA Protein Assay reagent (Bio-Rad, Richmond, CA, USA). Samples containing equal amounts of proteins were electrophoresed through 12% polyacrylamide gels, and transferred to PVDF membranes. After blocking, membranes were incubated overnight at 4°C with the following antibodies in CanGet Signal Solution 1 (Toyobo Co., Ltd., Osaka, Japan): anti-human PKC $\delta$  antibody (1:1000), anti-human caspase-3 antibody (1:1000), anti-human cleaved caspase-3 antibody (1:500), anti-human poly (ADP-ribose) polymerase (PARP) antibody (1:1000), and anti-human cleaved PARP antibody (1:1000) (all antibodies were purchased from Cell Signaling Technology Inc., Danvers, MA, USA). Membranes were re probed with a  $\alpha$ -tubulin antibody (Sigma-Aldrich) to confirm equal protein loading. Following washes, membranes were incubated with the appropriate secondary antibody conjugated to horseradish peroxidase, and were exposed with ECL Plus Western blotting detection system reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Antibody binding was detected by Chemilumino analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan).

**Cell proliferation assay.** To evaluate the involvement of PKC $\delta$  in MFH cell proliferation, we performed WST-8 cell proliferation assays using Cell Counting kit-8 (CCK-8) (Dojindo Inc., Kumamoto, Japan). Two MFH cell lines, Nara-H and TNMY1, were transfected with either PKC $\delta$  siRNA or control siRNA, and after 24 h of transfection, cells were seeded in 96-well culture plates at a density of ~5000 cells/well in 100  $\mu$ l culture medium. At the indicated times, 10  $\mu$ l of the CCK-8 solution was added to each well of the plate and incubated for 2 h. Optical density was measured at a wavelength of 450 nm using a Model 680 Microplate Reader (Bio-Rad). The relative number of viable cells in each well was calculated.

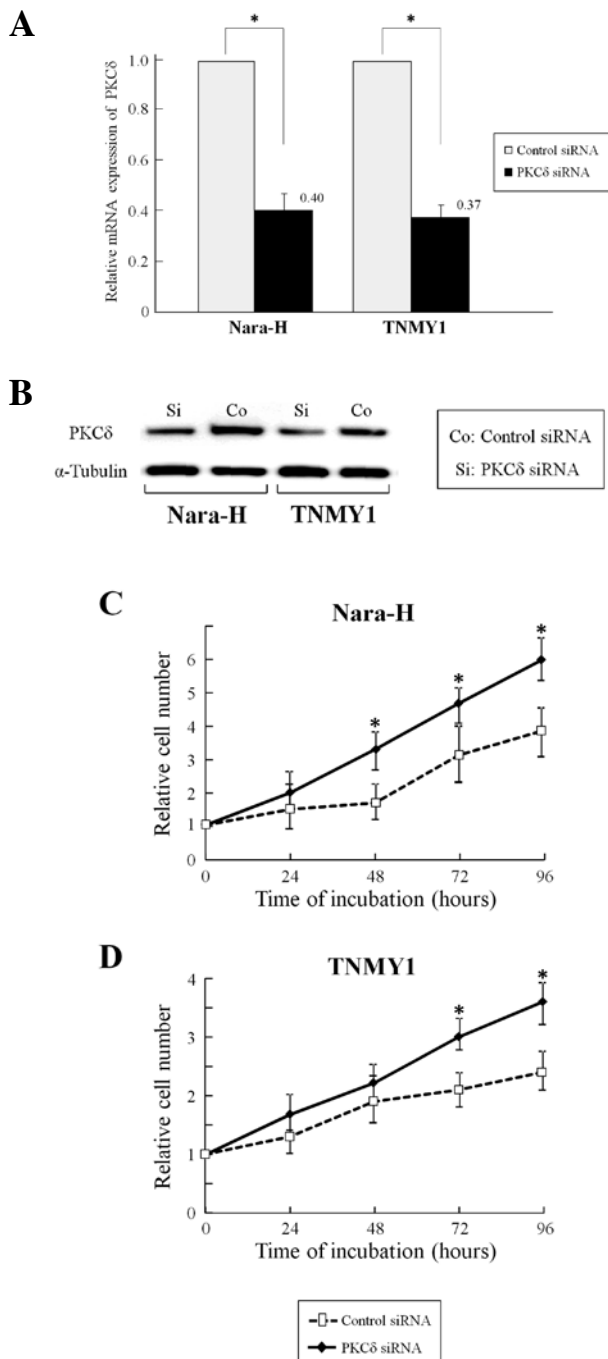


Figure 2. Cell proliferation assays of siRNA-transfected MFH cell lines. (A) Real-time PCR analysis revealed that in both Nara-H and TNMY1 cells mRNA expression of PKCδ was reduced significantly after 48 h of PKCδ siRNA transfection compared with control (\* $P < 0.05$ ). (B) Immunoblot analysis also revealed PKCδ protein expression was greatly reduced after 48 h of PKCδ siRNA (Si) transfection compared with control (Co). Cell proliferation assays (WST-8 assay) were performed with PKCδ siRNA-transfected MFH cell lines [Nara-H (C) and TNMY1 (D)]. Cells were transfected with PKCδ siRNA or control siRNA, and incubated for 48 h. Each cell line was then seeded in 96-well culture plates, and WST-8 assays were performed following 24, 48, 72 and 96 h of incubation. siRNA downregulation of PKCδ significantly increased the proliferation rate of Nara-H cells at 48 h and TNMY1 cells at 72 h (\* $P < 0.05$ ).

**In vitro scratch wound healing assay.** To evaluate whether PKCδ downregulation affects MFH cell migration, we performed *in vitro* scratch wound healing assays (17). Both MFH cell lines were transfected with either PKCδ siRNA or

control siRNA, and then cells were seeded in a 6-well culture plate and allowed to form a confluent monolayer. A denuded area was created by scraping with a 200  $\mu$ l pipette tip, and each well was washed three times with PBS to remove dead cells. Scratch wounds were then inspected microscopically and photographs of each wound were taken at 0, 12 and 24 h after wounding. The distance between the opposing edges of wound was measured at two points on each photograph.

**Apoptosis assay.** To examine the effect of siRNA downregulation of PKCδ on apoptosis of MFH, we performed DNA fragmentation assays using the Cell Death Detection ELISA<sup>plus</sup> system according to the manufacturer's protocol (Roche Applied Science). Cellular extracts from Nara-H cells transfected with either PKCδ-siRNA or control siRNA were incubated in 96-well plates coated with anti-histone antibodies. Following washes, the plate was incubated for an additional 2 h with anti-DNA antibodies conjugated to peroxidase. Substrate solution was added and absorbance was measured at 405 nm using a microplate reader.

**Statistical analysis.** Each experiment was performed independently at least three times, and data are presented as the mean  $\pm$  SD. The statistical significance of the differences between means was evaluated by ANOVA with Fisher's PLSD post hoc test, and all tests were considered significant at  $P < 0.05$ .

## Results

**mRNA expression of PKCδ in MFH samples is significantly lower than that in benign schwannomas.** We evaluated mRNA expression of PKCδ in human musculoskeletal tumor tissue samples by quantitative real-time PCR analyses. Of note, we found a significant decrease ( $P < 0.05$ ) of PKCδ mRNA expression level in MFHs (high-grade malignant tumors) compared with that in schwannomas (benign tumors) (Fig. 1). These results indicate that PKCδ is expressed in both schwannoma and MFH, but the expression level in MFH is lower than that in schwannoma.

**siRNA downregulation of PKCδ significantly increases MFH cell proliferation.** To examine how PKCδ expression affects MFH cell proliferation, we tested the effect of PKCδ downregulation by siRNA transfection on the proliferation rate of MFH cells. In both MFH cell lines, transfection of PKCδ siRNA sufficiently decreased both mRNA (Fig. 2A) and protein expression of PKCδ (Fig. 2B). Cell proliferation of siRNA-transfected MFH cells was assessed using WST-8 assays. Downregulation of PKCδ by siRNA transfection resulted in a significant increase ( $P < 0.05$ ) in the proliferation rate of MFH cells following 48 or 72 h of incubation compared with control cells (Fig. 2C and D). These results indicate that PKCδ may play an inhibitory role in MFH cell proliferation.

**Migration of MFH cells is significantly increased by PKCδ downregulation.** We evaluated the effect of selective PKCδ downregulation on MFH cell migration ability using *in vitro* scratch wound healing assays. In MFH cells (Nara-H and TNMY1) transfected with PKCδ siRNA, cell migratory ability

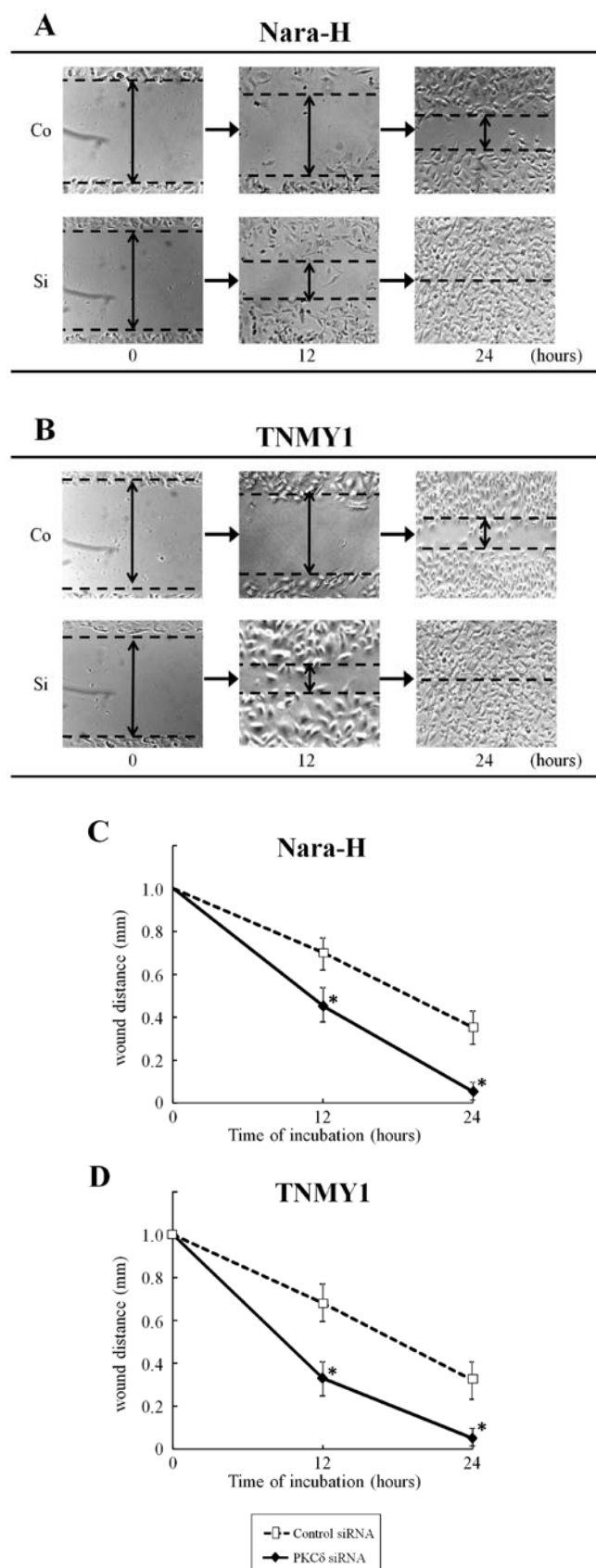


Figure 3. *In vitro* scratch wound healing assays of siRNA-transfected MFH cell lines. *In vitro* scratch wound healing assays were performed with siRNA-transfected MFH cells [Nara-H (A) and TNMY1 (B)]. Dotted lines indicate the margin of migrating cells, and the average wound distance of cell migration into the wound surface was determined under an inverted microscope at 0, 12 and 24 h after wounding. In both cell lines, wound closure occurred significantly earlier in PKC $\delta$  siRNA-transfected cells compared with control cells after 12 h of incubation (C and D) (\* $P < 0.05$ ).

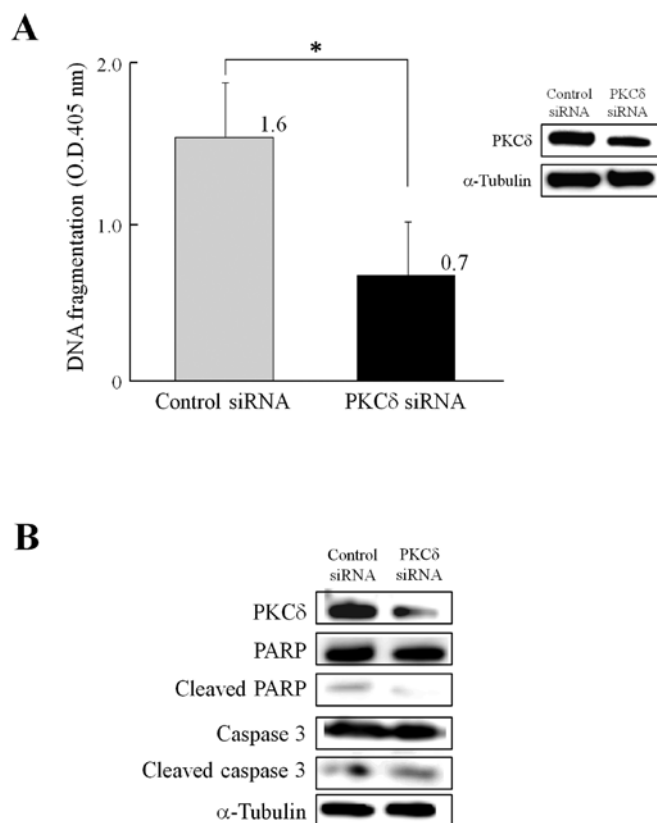


Figure 4. Effect of PKC $\delta$  downregulation on apoptosis of Nara-H cells. We performed DNA fragmentation assays (A) and immunoblot analysis of apoptosis-related proteins, PARP and caspase-3 (B) in Nara-H cells that were transfected with either PKC $\delta$  siRNA or control siRNA. DNA fragmentation was reduced significantly by PKC $\delta$  siRNA transfection (A) (\* $P < 0.05$ ), and protein expression of both cleaved PARP and cleaved caspase-3 was also decreased by PKC $\delta$  downregulation, while the expression of total PARP and total caspase-3 was unchanged (B).

was significantly increased compared with that of control cells (Fig. 3). These results suggest that PKC $\delta$  expression can negatively regulate the migration of human MFH cells.

*PKC $\delta$  downregulation significantly suppresses apoptosis of MFH cells.* We determined the role of PKC $\delta$  on apoptosis in MFH cells by DNA fragmentation assays and immunoblot analysis. As shown in Fig. 4A, PKC $\delta$  downregulation in Nara-H MFH cells resulted in a significant decrease in DNA fragmentation, a hallmark of apoptosis. Immunoblot analysis revealed that siRNA downregulation of PKC $\delta$  significantly decreased the expression of both cleaved PARP and cleaved caspase-3, while the expression of total PARP and total caspase-3 was unchanged (Fig. 4B). The observed decrease in DNA fragmentation and reduced cleaved PARP and cleaved caspase-3 expression following knockdown of PKC $\delta$  indicate that PKC $\delta$  may play a positive role in apoptosis of MFH cells.

## Discussion

Several PKC isoforms have been reported to act as regulators of cell differentiation, growth, survival, migration, invasion, and apoptosis in various human malignancies (7,10,18-33). Therefore, PKC may be an attractive candidate for targeting using novel anticancer therapies. Among the PKC isoforms,

PKC $\alpha$ ,  $\beta$ , and  $\gamma$  are the most abundant isoforms in various tissues (19), and in general, PKC $\alpha$  and PKC $\beta$  have been linked to increased invasion, proliferation, drug resistance and genetic instability in tumor cells (9,19,21,24-32), while PKC $\delta$  is thought to promote apoptosis (7,10,33). Koivunen *et al* theorized that increased proportional activation of PKC $\alpha/\beta$  to PKC $\delta$  is an important factor in the aggressiveness of cancers (9), and other studies demonstrated that PKC $\beta$  plays an important role in the signal transduction pathway for vascular endothelial growth factor-mediated tumor development and angiogenesis in human malignancies (26,27).

Dysregulated PKC activity leads to tumor progression in a variety of human sarcomas, including rhabdomyosarcomas (28), osteosarcomas (29), fibrosarcomas (30) and leiomyosarcomas (31). Keller *et al* reported that the PKC isoforms  $\alpha$ ,  $\gamma$  and  $\beta 1$ , but not  $\beta 2$  or  $\delta$ , are involved in the regulation of cell shape and locomotion of human fibrosarcoma HT1080 cells (30). Zhan *et al* demonstrated that p53 signaling has been linked to the regulation of PKC $\alpha$  expression, and that PKC $\alpha$  activates the multiple drug resistance (MDR) gene in a human leiomyosarcoma cell line (31). We have previously reported that PKC412, a selective inhibitor of classical PKC, has an inhibitory effect on cell proliferation in human osteosarcoma and MFH cell lines by reducing PKC $\alpha$  activity (32). However, the role of PKC $\delta$  in MFH has not previously been described.

In the current study, we found that the mRNA expression of PKC $\delta$  was detected in both benign and malignant musculoskeletal tumor tissue samples; however, the expression in high-grade MFH tumors was significantly lower than that in benign schwannomas. Consistent with our results, Yadav *et al* reported that loss of PKC $\delta$  was observed in human cutaneous squamous cell carcinoma (SCC), and described PKC $\delta$  as a tumor suppressor which controls apoptosis and tumorigenesis of SCC (33). Thus, we hypothesized that low expression of PKC $\delta$  enhances tumor progression in human MFH, owing to the lack of its anti-tumoral effects, and we tested our hypothesis by exploring the direct effect of selective PKC $\delta$  downregulation on cell proliferation, migration and apoptosis in human MFH cell lines. As a result, we found that siRNA downregulation of PKC $\delta$  significantly increased MFH cell proliferation and migratory ability; furthermore, PKC $\delta$  downregulation markedly suppressed the apoptotic ability of MFH cells. These results indicate that PKC $\delta$  may be an important regulator of the tumorigenesis of human MFH cells, and that uncontrolled cell growth of human MFH might be associated with suppression of apoptosis, possibly through reduced expression of PKC $\delta$ . In agreement with our results, the pro-apoptotic effect of PKC $\delta$  has also been described in human endometrial cancer (7) and human cutaneous SCC (10), however, the mechanism of PKC $\delta$  action in MFH cells is still unknown.

Previous studies elucidated the mechanism of the anti-tumoral effect of PKC $\delta$  (34-36). Fukumoto *et al* reported that PKC $\delta$  inhibits the proliferation of vascular smooth muscle cells by suppressing the expression of cyclins D1 and E, and promoting an increase in p27<sup>Kip1</sup> tumor suppressor gene expression and a decrease in retinoblastoma protein (pRb) phosphorylation (34). Vucenik *et al* also demonstrated that inositol hexaphosphate (IP<sub>6</sub>), which causes G1 arrest of human breast cancer cells, activates PKC $\delta$  to result in upregulated

expression of p27<sup>Kip1</sup>, and reduced pRb phosphorylation (35). Nakagawa *et al* showed that PKC $\delta$  is a selective mediator of G1 arrest by inducing upregulation of the tumor suppressor p21 in lung adenocarcinoma cells (36). Nonetheless, several studies demonstrated that PKC $\delta$  positively regulates tumorigenesis in cancer cells (37-39). In renal cell carcinoma cells, the von Hippel-Lindau (VHL) tumor suppressor gene plays an important inhibitory role in cell proliferation by blocking the association of PKC $\delta$  and insulin-like growth factor-I (IGF-I) receptor (37). Furthermore, transfection of dominant negative PKC $\delta$  resulted a marked decrease in invasive activity (37). Kiley *et al* revealed that PKC $\delta$  levels are upregulated in highly metastatic mammary tumor cells compared with less metastatic cell lines, and that excessive activation of PKC $\delta$  leads to increased anchorage-independent cell growth and is functionally significant in metastasis of mammary carcinoma cell lines *in vivo* (38,39).

There is considerable debate as to whether PKC $\delta$  acts as a promoter or a suppressor of cancer progression, however, our results suggest that PKC $\delta$  may act as a tumor suppressor at least in human MFH. The data presented here could be applied in the development of new therapeutics, since elevation of PKC $\delta$  expression is one potential strategy to inhibit MFH cell progression.

In summary, we show that mRNA expression of PKC $\delta$  is significantly decreased in malignant musculoskeletal tumors. We also demonstrate that siRNA downregulation of PKC $\delta$  increases cell proliferation and the migratory ability of MFH cells, and that apoptosis is significantly suppressed by PKC $\delta$  siRNA transfection. Further characterization of the function of PKC $\delta$  will provide novel insights into the pathogenesis of MFH, and will aid new therapeutic approaches to treating MFH.

## Acknowledgements

We thank Takeshi Ueha, Minako Nagata, Maya Yasuda, and Hiroko Tanaka for expert technical assistance.

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