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Identification of zinc finger protein of the cerebellum 5 as a survival factor of prostate and colorectal cancer cells

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Key words

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Identification of specific drug targets is very important for cancer therapy. We recently identified zinc finger protein of the cerebellum 5 (ZIC5) as a factor that promotes melanoma aggressiveness by platelet-derived growth factor D (PDGFD) expression. However, its roles in other cancer types remain largely unknown. Here we determined the roles of ZIC5 in prostate cancer (PCa) and colorectal cancer (CRC) cells. Results showed that ZIC5 was highly expressed in CRC and dedifferentiated PCa tissues, whereas little expression was observed in relevant normal tissues. Knockdown of ZIC5 decreased proliferation of several PCa and CRC cell lines with induction of cell death. ZIC5 knockdown significantly suppressed PDGFD expression transcriptionally, and PDGFD suppression also decreased proliferation of PCa and CRC cell lines. In addition, suppression of ZIC5 or PDGFD expression decreased levels of phosphorylated focal adhesion kinase (FAK) and signal transducer and activator of transcription 3 (STAT3) which are associated with PCa and CRC aggressiveness. Furthermore, knockdown of ZIC5 or PDGFD enhanced death of PCa and CRC cells induced by the anti-cancer drugs docetaxel or oxaliplatin, respectively. These results suggest that ZIC5 and PDGFD promote survival of PCa and CRC cells by enhancing FAK and STAT3 activity, and that the roles of ZIC5 are consistent across several cancer types.

Prostate cancer (PCa) and colorectal cancer (CRC) are common causes of cancer-related deaths worldwide. As a result of advances in the diagnosis and treatment of PCa, overall survival rate of patients with this disease has increased; however, metastatic PCa is often incurable and lethal. Patients suffering from metastatic CRC also show poor overall survival despite the development of novel therapeutic strategies including molecular targeted drugs. In cases of chemotherapy treatments, intolerable side-effects and drug resistance limit treatment efficacy.[1–4] Therefore, identification of novel therapeutic molecules to prevent drug resistance and enhance drug efficacy is required.

Previously, to identify therapeutic targets in melanoma, we carried out small interfering RNA (siRNA) screening of genes essential for neural crest development,[5] which led to the discovery of zinc finger protein of the cerebellum 5 (ZIC5) as a factor that promotes melanoma aggressiveness. ZIC5 is a transcription factor that contains C2H2-type zinc finger domains, is highly expressed in human melanoma tissues, and promotes melanoma cell motility, proliferation, viability, and drug resistance. In addition, ZIC5 stimulates platelet-derived growth factor D (PDGFD) expression, inducing activation of the focal adhesion kinase/signal transducer and activator of transcription 3 (FAK/STAT3) axis, which is involved in the aggressive phenotype of many cancers.[6,7] Because ZIC5 expression in normal human tissues is limited to the testis and cerebral cortex (Human Protein Atlas), it appears to be a promising and selective target for melanoma therapy.

Analysis of The Cancer Genome Atlas (TCGA) suggests that ZIC5 is highly expressed in many tumor types such as prostate, colorectal, bladder, esophageal, head and neck squamous, hepatocellular, lung, and stomach carcinoma. However, the roles of ZIC5 in most of these cancers remain unknown, although it is reportedly a growth-promoting factor in non-small cell lung cancer.[8] In the present study, we elucidated the involvement of ZIC5 in the progression of PCa and CRC.

Materials and Methods

Cell culture. DLD-1 and HCT116 CRC and DU145 PCa cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). PC3 PCa cell line was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank (National Institute of Health Sciences, Tokyo, Japan). These cell lines were re-validated by short tandem repeat profiling in 2016 (Promega, Madison, WI, USA). Cells were maintained at 37°C with a 5% CO_2 humidified atmosphere in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS.

RNA isolation, cDNA synthesis, and quantitative real-time PCR. Total RNA was isolated from cells using the ReliaPrep RNA

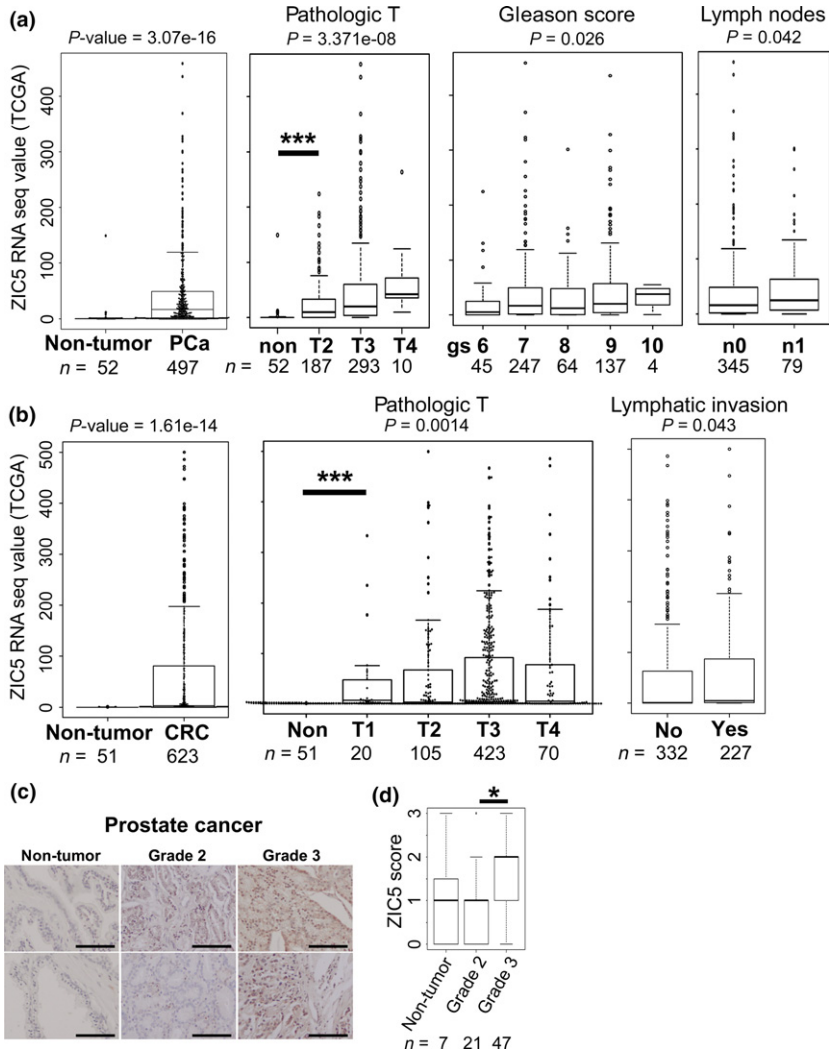

Fig. 1. Zinc finger protein of the cerebellum 5 (ZIC5) expression in prostate and colorectal cancers. (a,b) RNA sequence value of ZIC5 expression in prostate (a; PCa) and colorectal (b; CRC) cancer tissues as well as relevant normal tissues was obtained from The Cancer Genome Atlas (TCGA) database. Statistical difference between the two groups was carried out using the Mann–Whitney *U*-test. Some clinicopathological parameters such as pathological tumor grading of TNM classification (pathologic T), Gleason score, presence of regional lymph node metastasis (n0, no regional lymph node metastasis; n1, metastasis in regional lymph nodes), and presence of lymphatic invasion were assessed in relation to ZIC5 expression. Statistical analysis of more than two groups was carried out by ANOVA. (c) Immunohistochemistry of ZIC5 expression in human PCa tissue arrays containing seven normal and 73 PCa tissues. Two representative images of non-tumor, Grade 2 (moderately differentiated) or Grade 3 (poorly differentiated) PCa tissues are shown. Scale bar, 100 μm. (d) ZIC5 expression in each sample was scored and assessed using the Mann–Whitney *U*-test (***$P < 0.001$, *$P < 0.05$).

Cell Miniprep System (Promega) and reverse transcribed to generate cDNA (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was carried out using the THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan) with a CFX96 thermocycler (Bio-Rad, Munich, Germany). The following primer sequences were used: *PDGFD*, F: AAG ATTT CCA ACC CGC AGCA, R: TCC AGA GCA TCC GCA ATCAG; and *GAPDH*, F: AGC CTC CCG CTT CGC TCTCT, R: CCA GGC GCC CAA TAC GACCA.

Plasmids, siRNA, and transfection. The open reading frame of human *ZIC5* cDNA was amplified by PCR and subcloned into the pcDNA3.1 expression vector (Invitrogen). siRNA for *ZIC5* and *PDGFD* were as previously described.[5] Transient transfections were carried out using Lipofectamine 2000 or Lipofectamine RNAiMax (Invitrogen), according to the manufacturer's protocol. In each experiment, the total amount of transfected DNA or siRNA was adjusted with relevant empty vectors or negative control siRNA, respectively. To select cells transfected with the expression vector, cells were treated with 500 μg/mL G418 (Invitrogen) for 10 days.

Cell proliferation assays. Cells were plated in 96-well plates at a density of 1000 cells/well in triplicate. Cell nuclei were stained with Hoechst33342 (Dojindo Kumamoto, Japan), and total cell number was determined with the In Cell Analyzer 2000 (GE Healthcare, Little Chalfont, UK).

Migration assays. Transwell migration assays were carried out as previously described[9] using cell culture inserts with pores 8 μm in size (BD Biosciences, San Jose, CA, USA). Number of migrated cells was normalized to total cell number.

Apoptosis assays. Cells were incubated with FITC-labeled annexin V reagent (MBL, Nagoya, Japan) and Hoechst33342 (Dojindo) for 40 min, after which they were analyzed using the In Cell Analyzer 2000 with a DAPI and FITC filter. Ratio of annexin V-positive cells to Hoechst33342-positive cells was determined with the In Cell Analyzer Workstation 3.7 (GE Healthcare). Experiments were carried out two or three times in triplicate.

Reagents. Oxaliplatin (Sigma Aldrich, St Louis, MO, USA) or docetaxel (Cayman Chemical, Ann Arbor, MI, USA) was used at the indicated concentrations to treat CRC or PCa cells, respectively.

Western blot analysis. Western blotting was carried out as previously described with some modifications.[10] Primary antibodies for STAT3 (BD Biosciences), GAPDH, phospho-STAT3 (Tyr705) (Cell Signaling, Danvers, MA, USA), β-actin (Sigma Aldrich), ZIC5 (Aviva Systems Biology, San Diego, CA, USA), PDGFD (Santa Cruz, Dallas, TX, USA), phospho-

Table 1. Correlation between the expression of ZIC5 and clinicopathological features in prostate cancer

Prostate cancer				
Characteristics	No. of patients	Zic5 low (≤median)	Zic5 high (>median)	*P*-value
Number	497	248	249	
Age (years), *n*				
≤60	223	118	105	0.24
>60	274	130	144	
TNM scores				
Tumor (pathological), *n*				
2	187	111	76	0.0003
3	293	133	160	
4	10	1	9	
NA	7	3	4	
Nodes (pathological), *n*				
0	345	175	170	0.17
1	79	33	46	
NA	73	40	33	
Metastasis (clinical), *n*				
0	455	223	232	0.87
1a	1	1	0	
1b	1	0	1	
1c	1	0	1	
NA	39	24	15	
Gleason score, *n*				
6	45	29	16	0.13
7	247	125	122	
8	64	33	31	
9	137	60	77	
10	4	1	3	

P-values were examined using Fisher's exact test. NA, not available; ZIC5, zinc finger protein of the cerebellum 5.

FAK, (Signalway Antibody, Pearland, TX, USA), and FAK (Acris, Hiddenhausen, Germany) were used. Images were obtained using LuminoGraph I (ATTO, Tokyo, Japan) or ImageQuant LAS 400 image capture software (GE Healthcare). Band intensity was quantified using the CS Analyzer (ATTO).

Immunohistochemistry. Paraffin-embedded human PCa tissue arrays were purchased from US Biomax (Rockville, MD, USA). Immunohistochemical assays for human ZIC5 were carried out using anti-ZIC5 antibody (Aviva Systems Biology) with a Vectastain Elite Rabbit ABC Kit (Vector Laboratories, Burlingame, CA, USA). Images were obtained using a BX51 microscope (Olympus, Tokyo, Japan).

Antibody array. The Proteome Profiler Human Phospho-Kinase Array (R&D Systems, Minneapolis, MN, USA) was carried out according to the manufacturer's protocol. Images were obtained using LuminoGraph I software (ATTO), and spot intensity was quantified using the CS Analyzer (ATTO).

Statistical analysis. Expression value of *ZIC5* in CRC and PCa tissues was obtained from mRNA sequence data in TCGA database (http://cancergenome.nih.gov/), and statistical analysis was carried out using the Mann–Whitney *U*-test or one-way analysis of variance (ANOVA) to compare the means of more than two groups. All statistical analyses were done using the R statistical software package (v. 3.0.1). Mean ± standard deviation (SD) is shown in the bar graphs. Significance of differences was determined by the statistical tests indicated in the individual figure legends. $P < 0.05$ was considered statistically significant.

Table 2. Correlation between the expression of ZIC5 and clinicopathological features in colorectal cancer

Characteristics	No. of patients	Zic5 low (≤median)	Zic5 high (>median)	*P*-value
Number	621	310	311	
Age (years), *n*				
≤60	194	105	89	0.17
>60	427	205	222	
Gender, *n*				
Male	331	175	156	0.13
Female	290	135	155	
TNM scores				
Tumor (pathological), *n*				
is	1	0	1	0.43
1	20	7	13	
2	105	56	49	
3	423	213	210	
4	70	32	38	
NA	2	2	0	
Nodes (pathological), *n*				
0	352	178	174	0.89
1	150	75	75	
2	115	55	60	
nx	2	0	2	
NA	2	2	0	
Metastasis (pathological), *n*				
0	460	221	239	0.24
1	87	48	39	
mx	64	36	28	
NA	10	5	5	
Stage, *n*				
1	105	51	54	0.56
2	229	113	116	
3	179	83	96	
4	88	49	39	
NA	20	14	6	
Lymphaticinvasion, *n*				
No	332	184	148	0.013
Yes	227	101	126	
NA	62	25	37	

P-values were examined using Fisher's exact test. NA, not available; ZIC5, zinc finger protein of the cerebellum 5.

Results

ZIC5 expression was elevated in PCa and CRC. First, we confirmed that ZIC5 was highly expressed in PCa and CRC tissues compared to the relevant normal tissues, as determined by the statistical analysis of RNA sequence data from the TCGA database (Fig. 1a,b). Summaries of clinicopathological parameters and ZIC5 expression are shown in Tables 1 and 2. ZIC5 expression in each pathological tumor stage of TNM classification (pathological T) showed that ZIC5 was significantly enhanced in the early stage of PCa and CRC. In PCa, ZIC5 expression was elevated according to the progression of pathological tumor stage (Spearman's rank correlation, rho = 0.36; $P < 0.001$; Fig. 1a [Pathologic T]). Moreover, high ZIC5 expression tended to predict the presence of lymph node metastasis in PCa or lymphatic invasion of CRC (Fig. 1a,b; Table 1 and 2). Moreover, immunohistochemical analysis of human PCa tissue microarrays, including 73 PCa tissues and seven non-tumor tissues, revealed the elevated protein

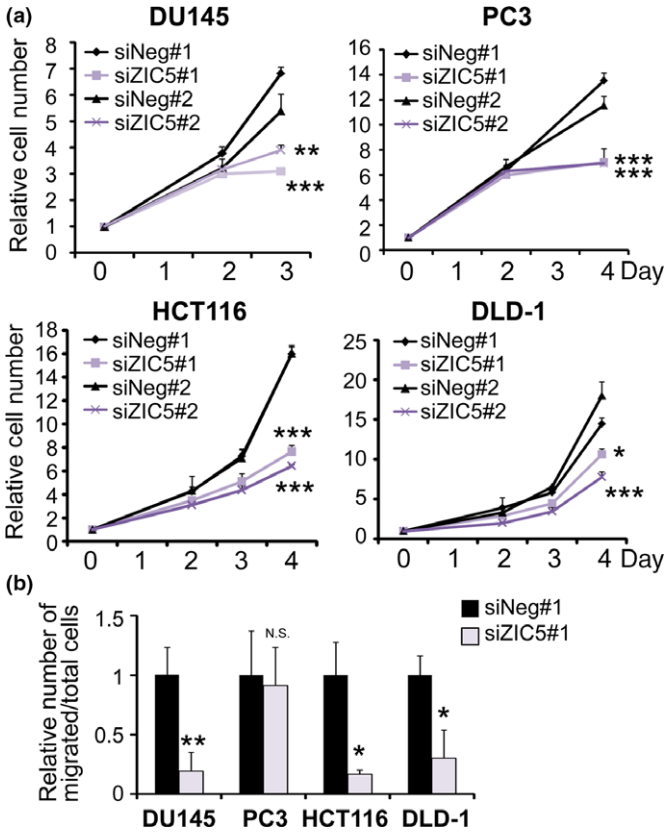

Fig. 2. Zinc finger protein of the cerebellum 5 (ZIC5) positively regulated the proliferation and migration of prostate cancer (PCa) and colorectal cancer (CRC) cells. DU145, PC3 (PCa cell lines), HCT116, and DLD-1 (CRC cell lines) were transfected with negative control (siNeg) or ZIC5 (siZIC5) small interfering RNA (siRNA). Target sequence of siRNA was different between #1 and #2. (a) Cell proliferation was monitored at the indicated times. Statistical analysis was done using Tukey's multiple comparison test. (b) Two days after siRNA transfection, transwell migration assays were carried out. Statistical analysis was carried out using Student's *t*-test (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). N.S., not significant.

expression of ZIC5 in Grade 3 (poorly differentiated) compared to Grade 2 (moderately differentiated) PCa samples (Fig. 1c,d). However, no significant differences between PCa tissues and non-tumor control tissues were observed, possibly because of the small sample size. Thus, increasing sample number might clarify the difference between these tissues. These data are in accordance with a recent report which demonstrated that ZIC5 expression was upregulated in high-grade PCa tissues.[11] The results suggest that ZIC5 expression is elevated in CRC and PCa tissues and correlates with PCa progression.

ZIC5 positively regulated proliferation and migration of PCa and CRC cells. To elucidate the roles of ZIC5 in PCa and CRC cells, we carried out siRNA-mediated knockdown of ZIC5 in several cell lines. Cell proliferation analyses revealed that ZIC5 knockdown reduced the cell numbers of PCa (DU145 and PC3) and CRC (HCT116 and DLD-1) cell lines to about half that of control cells on days 3 or 4 (Fig. 2a). Transwell cell migration assay revealed that ZIC5 knockdown decreased the motility of PCa (DU145) and CRC (HCT116 and DLD-1) cell lines to approximately 10–30% that of control cells (Fig. 2b). These results indicate that ZIC5 promotes the growth and migration of these cancer cells.

ZIC5 positively regulated PDGFD expression in PCa and CRC cells. Our previous study demonstrated that ZIC5 enhances PDGFD expression to promote the proliferation and aggressiveness of melanoma cells.[5] To determine whether the ZIC5/PDGFD axis is conserved across several cancer types, PDGFD expression in ZIC5-suppressed PCa and CRC cells was assessed by qPCR and Western blotting. PDGFD mRNA expression was significantly decreased by ZIC5 knockdown in DU145 cells (Fig. 3a). Moreover, levels of proPDGFD protein, the cellular form of PDGFD, were also decreased by ZIC5 suppression in DU145, PC3, DLD-1, and HCT116 (Fig. 3b), indicating that ZIC5 positively regulates PDGFD expression in PCa and CRC cells.

PDGFD contributed to the proliferation of PCa and CRC cells. The tumor-promoting activity of PDGFD has been reported for some PCa and CRC cell lines.[12,13] To determine the involvement of PDGFD in the proliferation of DU145, PC-3, DLD-1, and HCT116 cells, we carried out siRNA-mediated knockdown of PDGFD. PDGFD suppression decreased the reproductive ratios of all PCa and CRC cell lines tested (Fig. 3c). These results suggest that PDGFD has important roles in the ZIC5-mediated proliferation of these cancer cells.

ZIC5 or PDGFD knockdown reduced FAK and STAT3 activity. It was recently reported that FAK and STAT3 are associated with the aggressive phenotypes of many cancer types.[6,7] In addition, we previously reported that ZIC5 is involved in the activation of FAK and STAT3 in melanoma cells.[5] Therefore, to determine whether ZIC5 contributes to maintaining FAK and STAT3 activation in PCa and CRC cells, their phosphorylation levels were examined by Western blotting. As shown in Figure 4(a), ZIC5 knockdown significantly reduced the amount of phosphorylated FAK (Tyr576/Tyr577) and STAT3 (Tyr705) in DU145, DLD-1, and HCT116 cells. PC3 cells had no STAT3 expression (Fig. 4a,c). Furthermore, PDGFD knockdown also suppressed the levels of phosphorylated FAK and STAT3 in DU145, DLD-1, and HCT116 cells (Fig. 4b,c), indicating that the ZIC5/PDGFD axis regulates FAK and STAT3 activation in various cancer cell lines.

ZIC5 or PDGFD suppression induced PCa and CRC cell death. STAT3 induces the expression of anti-apoptotic factors that promote PCa and CRC cell survival.[14,15] To determine whether ZIC5 and PDGFD are also involved in the survival of these cells, we next carried out apoptosis assays using FITC-labeled annexin V. When the expression levels of ZIC5 and STAT3 among cell lines were compared, DU145 and HCT116 cells had relatively high levels of ZIC5, and significantly high levels of phosphorylated STAT3, whereas the expression of ZIC5 in PC3 and DLD-1 cells was relatively low. DLD-1 cells had low levels of phosphorylated STAT3, and PC3 cells had no STAT3 expression (Fig. 5a). ZIC5 knockdown markedly induced death of DU145, HCT116, and DLD-1 cells, but only slightly induced death of PC3 cells, in which no STAT3 expression was observed (Fig. 5a,b,d). It is worth noting that approximately 60% of ZIC5 knockdown HCT116 cells underwent cell death compared to only 3% of control cells. Knockdown of PDGFD also induced cell death in DU145, HCT116, and DLD-1 cells, but did not induce significant cell death in PC3 cells (Fig. 5c,d). These results clearly demonstrate that ZIC5 and PDGFD contribute to the survival of PCa and CRC cells.

ZIC5 or PDGFD suppression promoted susceptibility of PCa and CRC cells to anti-cancer drugs. Improving drug sensitivity is important for preventing the drug resistance of cancer cells. To understand the role of ZIC5 and PDGFD in the drug sensitivity of PCa and CRC cells, apoptosis assays were carried out using

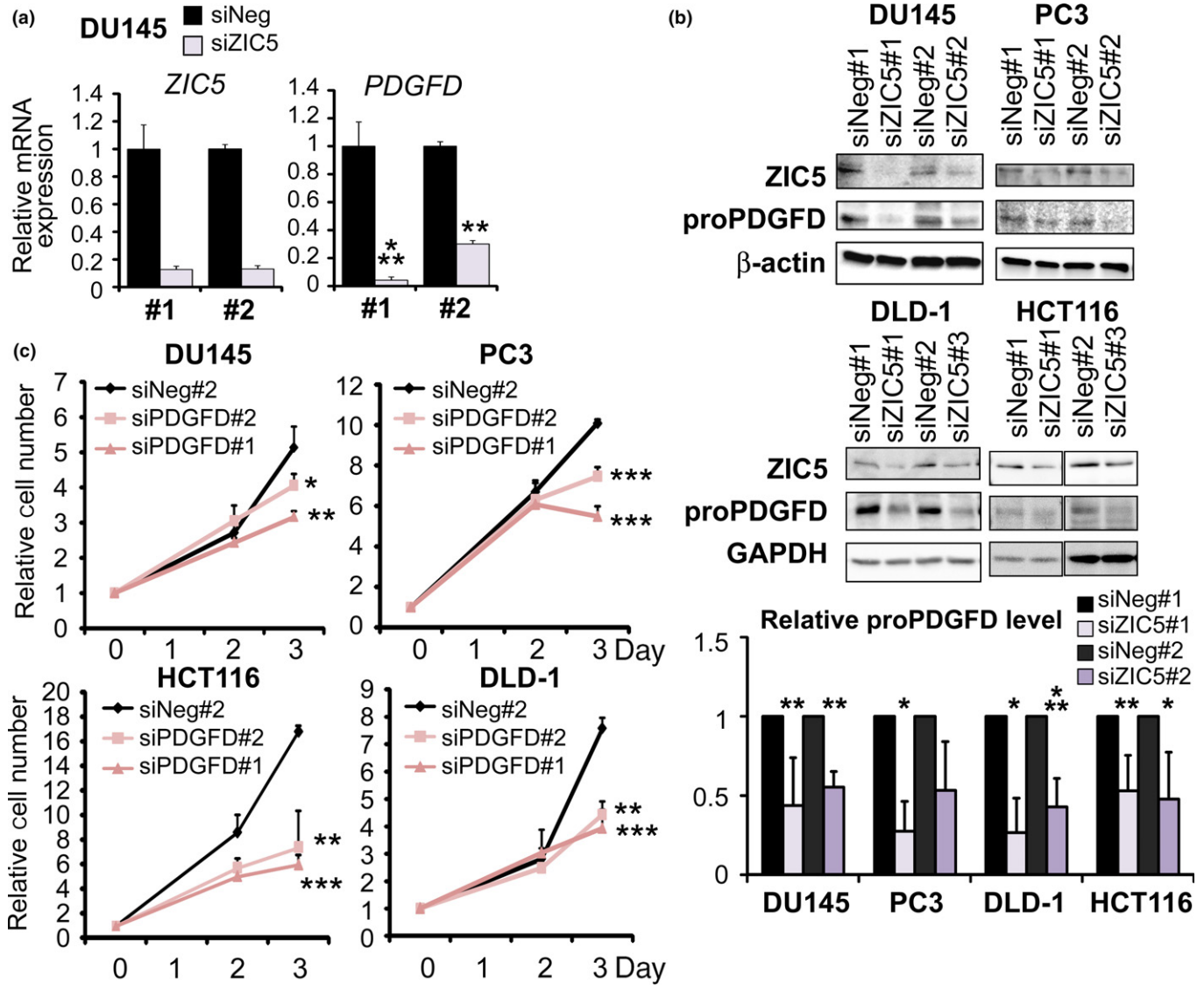

Fig. 3. Zinc finger protein of the cerebellum 5 (ZIC5) regulated platelet-derived growth factor D (PDGFD) expression in prostate cancer (PCa) and colorectal cancer (CRC) cells. (a) DU145 cells were transfected with negative control (siNeg) or ZIC5 (siZIC5) small interfering RNA (siRNA). *ZIC5* and *PDGFD* mRNA expression was determined in these cells by qPCR. Relative expression level was normalized to that of β-actin (*ACTB*) as an internal control. Statistical analysis was carried out using Tukey's multiple comparison test. (b) DU145, PC3, HCT116, and DLD-1 cells were transfected with siNeg or siZIC5 as indicated, after which the pro-PDGFD level was examined by Western blotting. GAPDH or β-actin level was determined as the internal control. Quantification of the pro-PDGFD levels normalized to GAPDH or β-actin from more than three independent experiments is shown in the bar graphs. (c) Cell proliferation was analyzed in DU145, PC3, HCT116, and DLD-1 cells transfected with siNeg or siRNA for *PDGFD* (siPDGFD). Target sequence of siRNA was different between #1 and #2. Statistical analysis was carried out using Dunnett's multiple comparison test (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

the anti-cancer drugs docetaxel and oxaliplatin, which are therapeutic drugs used to treat PCa and CRC, respectively. Results showed that ZIC5 knockdown enhanced the death of DU145, PC3, HCT116, and DLD-1 cells induced by the anti-cancer drugs (Fig. 6a,b). PDGFD knockdown also enhanced cell death, with the exception of PC3 cells (Fig. 6a,b). We also confirmed the role of ZIC5 on cell survival by showing that its overexpression enhanced STAT3 phosphorylation and suppressed oxaliplatin-induced death of DLD-1 cells (Fig. 6c). These results suggest that ZIC5 and PDGFD abrogate drug susceptibility against anti-cancer drugs in PCa and CRC cells and, as such, are promising therapeutic targets for PCa and CRC.

ZIC5 but not PDGFD regulated heat shock protein 60 and p27^{kip1} in PC3 cells. ZIC5 but not PDGFD contributed to the survival of PC3 cells, which had no STAT3 expression (Figs 5,6b), suggesting that ZIC5 also regulates PDGFD- and STAT3-independent pathways to enhance cell survival. To determine novel ZIC5 downstream signaling molecules, we assessed PC3 cells with antibody arrays, and observed that the expression of p70S6K (T389) (RPS6KB1), p27 (T198) (CDKN1B), STAT3 (Y705) and HSP60 (HSPD1) significantly changed upon ZIC5 knockdown (Fig. 6d). p70S6K is regulated downstream of focal adhesion kinase[16] and promotes protein synthesis, cell growth, and cell proliferation;[17] thus, ZIC5 and PDGFD might regulate cell proliferation through the FAK/p70S6K pathway. Because STAT3 expression in PC3 cells was not observed by Western blotting (Fig. 5a) as a result of the complete genomic loss,[18] the STAT3 detected in this array might actually have been a non-specific signal. T198

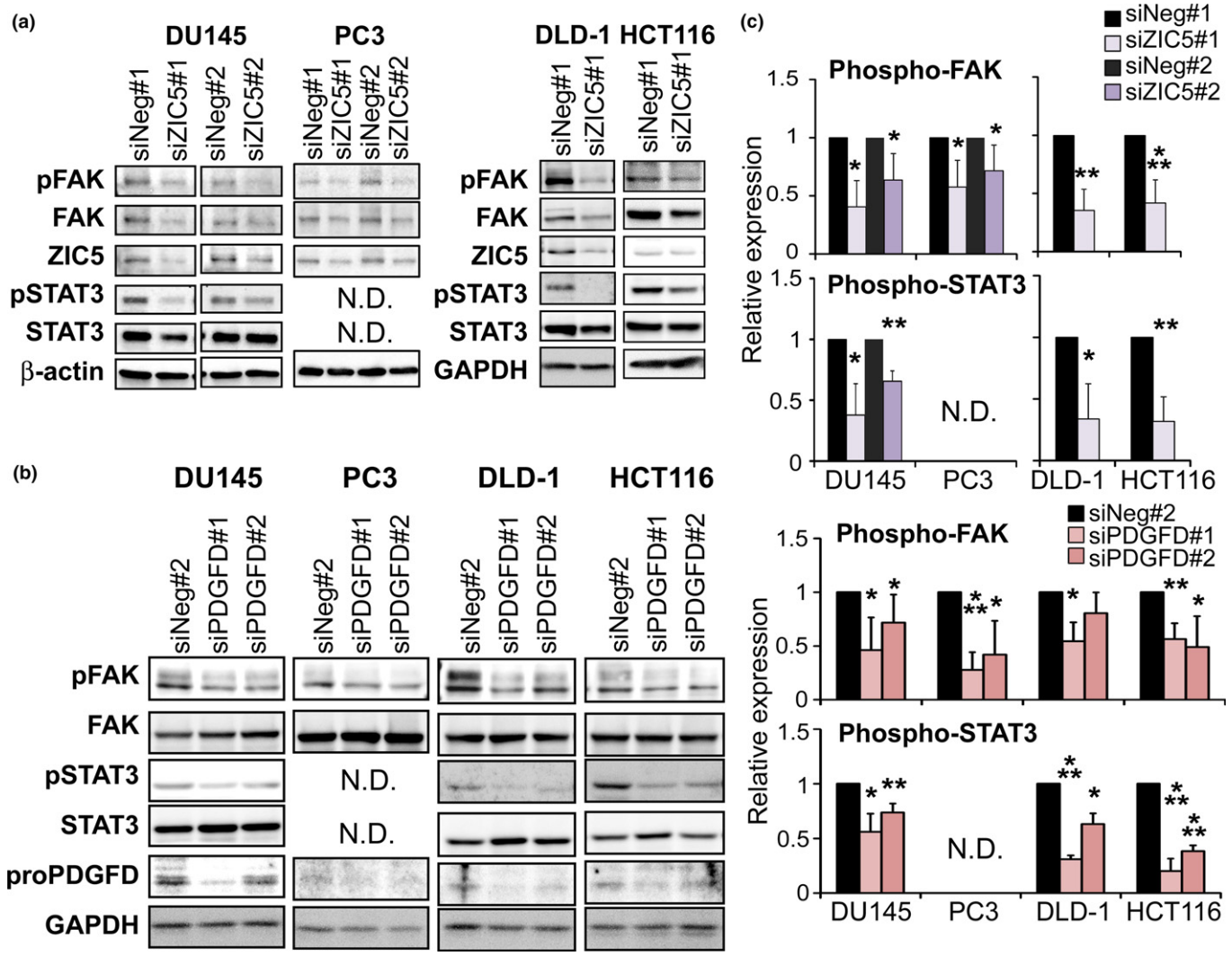

Fig. 4. Zinc finger protein of the cerebellum 5 (ZIC5) and platelet-derived growth factor D (PDGFD) positively regulated the phosphorylation of FAK and STAT3. (a) Levels of phosphorylated FAK (Tyr576/Tyr577) and STAT3 (Tyr705) as well as levels of total FAK and STAT3, ZIC5, and β-actin (ACTB) or GAPDH were examined in DU145, PC3, DLD-1, and HCT116 cells transfected with negative control (siNeg) or ZIC5 (siZIC5) small interfering RNA (siRNA). N.D., not detected. (b) Levels of phosphorylated FAK (Tyr576/Tyr577) and STAT3 (Tyr705) as well as total levels of FAK and STAT3, pro-PDGFD and GAPDH were examined in DU145, PC3, DLD-1, and HCT116 cells transfected with siNeg or siPDGFD. N.D., not detected. (c) Quantification of phosphorylated FAK or STAT3 levels normalized to GAPDH or β-actin in (a) and (b) from more than three independent experiments is shown in the bar graphs (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$).

phosphorylation of $p27^{kip1}$ regulates its stability[19] and $p27^{kip1}$ negatively regulates PCa aggressiveness[20] and drug resistance.[21] HSP60 expression is significantly increased in PCa,[22] and HSP60 promotes the survival of cancer cells.[23] So, we focused on $p27^{kip1}$ and HSP60, and confirmed the antibody array results by Western blot analysis. When ZIC5 expression was suppressed, the level of HSP60 was reduced, whereas $p27^{kip1}$ expression was increased (Fig. 6e). Because PDGFD knockdown did not affect HSP60 and $p27^{kip1}$ levels, ZIC5 may regulate these factors in a PDGFD-independent way.

Discussion

Combined application of chemotherapeutic drugs is generally carried out in PCa and CRC patients who are unable to undergo surgery. For drug selection, drug sensitivity is critical for preventing survival and drug resistance of cancer cells. Although the development of molecular targeted drugs has become popular in many diseases, DNA replication-inhibiting drugs such as fluorouracil and platinum-containing drugs are commonly used for aggressive PCa and CRC in which intolerable side-effects are serious problems.[1,2,24,25] Thus, the identification of novel therapeutic targets to enhance drug sensitivity is required for the development of efficacious strategies. In this study, we found that ZIC5 positively regulated the proliferation, migration (Fig. 2), and survival (Fig. 5) of PCa and CRC cells, showing that ZIC5 is a promising novel molecular target for these diseases. We also found that ZIC5 induced PDGFD expression (Fig. 3), and inhibition of the ZIC5/PDGFD axis sensitized PCa and CRC cells to docetaxel and oxaliplatin, respectively (Fig. 6). PDGFD is the most recently identified member of the PDGF family, and has been found to promote aggressiveness in many types of cancer.[26,27] PDGFD and platelet derived growth factor subunit B (PDGFB) bind and activate platelet derived growth factor receptor beta (PDGFRβ),

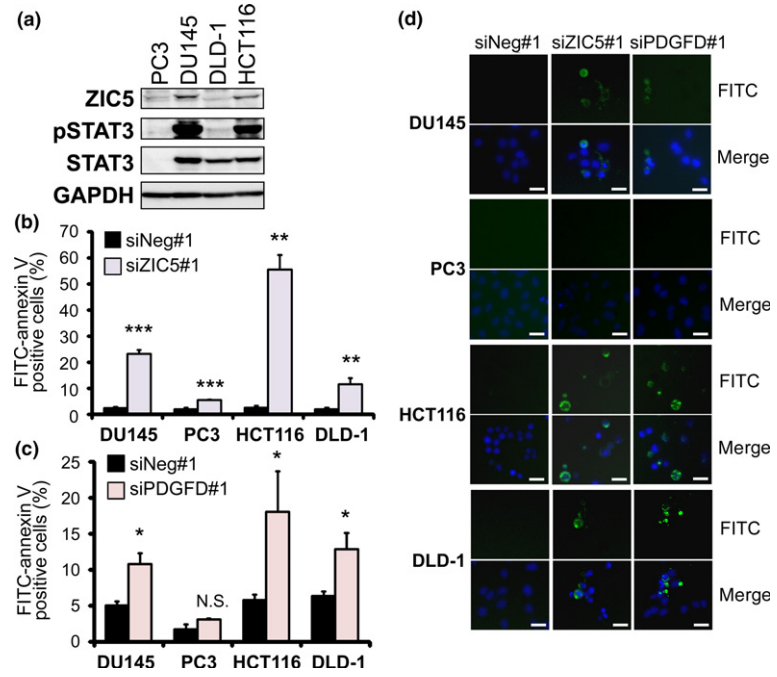

Fig. 5. Suppression of zinc finger protein of the cerebellum 5 (ZIC5) or platelet-derived growth factor D (PDGFD) promoted apoptosis in prostate cancer (PCa) and colorectal cancer (CRC) cells. (a) After culturing PC3, DU145, DLD-1, and HCT116 cells for 4 days, expression levels of ZIC5, phosphorylated STAT3, STAT3, and GAPDH were assessed. (b–d) DU145, PC3, HCT116, and DLD-1 cells were transfected with negative control (siNeg) of small interfering RNA (siRNA), siRNA for ZIC5 (siZIC5), or siRNA for PDGFD (siPDGFD). After 3 days, these cells were incubated with FITC-labeled annexin V and Hoechst33342. (b) Percentage of annexin V-positive cells transfected with siNeg or siZIC5 was determined. (c) Percentage of annexin V-positive cells transfected with siNeg or siPDGFD was determined. Statistical analysis was carried out using Student's *t*-test (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). N.S., not significant. (d) Representative images of FITC-annexin V staining (green) and Hoechst (blue). Scale bar, 30 μm.

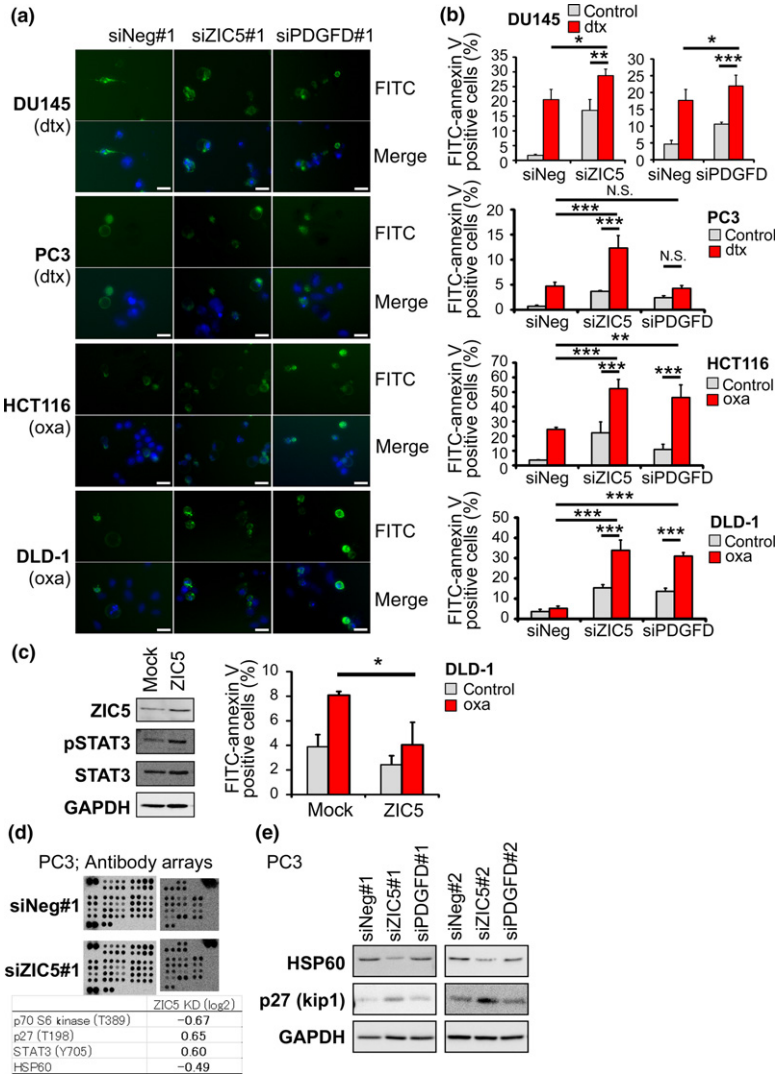

Fig. 6. Suppression of zinc finger protein of the cerebellum 5 (ZIC5) or platelet-derived growth factor D (PDGFD) sensitized prostate cancer (PCa) and colorectal cancer (CRC) cells to anti-cancer drugs. (a) Cells transfected with negative control (siNeg) of small interfering RNA (siRNA), siRNA for ZIC5 (siZIC5), or siRNA for PDGFD (siPDGFD) were treated with docetaxel (dtx, 5 nM) or oxaliplatin (oxa, 20 μM) for 24 h (HCT116) or 48 h (DU145, PC3, and DLD-1), and then stained with FITC-annexin V (green) and Hoechst (blue). Scale bar, 30 μm. (b) Percentage of annexin V-positive cells is shown. (c) DLD-1 cells transfected with ZIC5-expression vector or relevant empty vector (mock) were treated with oxaliplatin for 48 h, and then stained with FITC-annexin V. Percentage of annexin V-positive cells is shown (left). Levels of phosphorylated STAT3 (Tyr705) and total STAT3, ZIC5 and GAPDH were assessed by Western blotting (right). Statistical analysis was carried out using Tukey's multiple comparison test (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$). (d) PC3 cells were transfected with negative control (siNeg) or ZIC5 (siZIC5) small interfering RNA (siRNA). After 3 days, cells were harvested and analyzed using the Proteome Profiler (Human Phospho-Kinase Array). Level of each spot was quantified and the fold change (siZIC5/siNeg) was determined. The top four candidates are shown. (e) Expression levels of HSP60 and $p27^{kip1}$ were assessed in PC3 cells transfected with siNeg or siZIC5. GAPDH was used as the loading control.

but PDGFD promotes tumorigenesis more effectively than PDGFB in PCa, suggesting the existence of PDGFD-specific mechanisms of signal transduction.[28,29] Elucidation of these mechanisms may lead to the identification of novel and selective therapeutic targets. Although ZIC5 is a transcription factor, it is not clear whether it directly regulates the promoter activity of PDGFD, which could make the ZIC5/PDGFD axis a potential therapeutic target, as the signaling pathway of ZIC5 and PDGFD is quite different from replication-targeted drugs. Thus, inhibitors of the ZIC5/PDGFD axis in combination with existing drugs could enhance efficacy of anti-cancer drugs and reduce side-effects.

STAT3 is persistently activated in various cancers and is involved in tumor growth, survival, drug resistance, and stem cell properties.[7] We found that ZIC5 enhanced PDGFD expression, and ZIC5 and PDGFD contributed to STAT3 activation in DU145, HCT116, and DLD-1 cell lines (Fig. 4). In these cells, ZIC5 and PDGFD knockdown reduced STAT3 phosphorylation and induced significant cell death. ZIC5 knockdown slightly induced cell death in PC3, in which STAT3 expression was not observed. These observations suggest that ZIC5 and PDGFD promote the survival of PCa and CRC cells, mainly by maintaining STAT3 activation. However, ZIC5 may also promote cell survival in a STAT3-independent way, as ZIC5 knockdown enhanced the death of PC3 cells upon treatment with docetaxel (Fig. 6). In addition, because PDGFD knockdown did not enhance PC3 cell death by docetaxel in (Fig. 6), other ZIC5 targets except PDGFD might also have contributed to the survival of these cells. Antibody arrays showed that ZIC5 but not PDGFD regulated $p27^{kip1}$ and HSP60, which are involved in cell survival[20,21,23] (Fig. 6).

Taken together, our results show that inhibiting ZIC5 and PDGFD is a promising strategy for disrupting the intrinsic resistance of PCa and CRC cells to therapeutic reagents. Notably, ZIC5 suppression promoted cell death by anti-cancer drugs in all cell lines examined, whereas PDGFD suppression promoted cell death in STAT3-positive cell lines. Therefore, the role of ZIC5 as a survival factor appears to be consistent across cancer types.

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Disclosure Statement

Authors declare no conflicts of interest for this article.

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