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$p21^{Cip/WAF1}$ activation is an important factor for the ERK pathway dependent anti-proliferation of colorectal cancer cells

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Abbreviations: BrdU, bromodeoxyuridine; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; MSSM, minimally serum starved medium; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PD98059, 2-(2'-amino-3'-methoxyphenyl) oxanaphthalen-4-one; SDS, sodium dodecyl sulfate

Abstract

$p21^{Cip/WAF1}$, an important regulator of cell proliferation, is induced by both p53- and extracellular signal regulated kinase (ERK) pathways. The induction of $p21^{Cip/WAF1}$ occurs by prolonged activation of the ERKs caused by extracellular stimuli, such as zinc. However, not all the cells appeared to respond to ERK pathway dependent $p21^{Cip/WAF1}$ induction. Here we investigated the cause of such difference using colorectal cancer cells. $p21^{Cip/WAF1}$ induction and concomitant reduction of bromodeoxyuridine (BrdU) incorporation were observed by zinc treatment within HT-29 and DLD-1. However, HCT-116 cells with high endogenous $p21^{Cip/WAF1}$ levels did not show any additional increment of $p21^{Cip/WAF1}$ levels by zinc treatment and did maintain high BrdU incorporation level. The $p21^{Cip/WAF1}$ induction by zinc depended upon prolonged activation of extracellular signal regulated kinase (ERK) was not observed in HCT-116 cells. The percentage of BrdU positive cells was 50% higher in $p21^{Cip/WAF1}$ -/- HCT-116 cells compared to $p21^{Cip/WAF1}$ +/+ HCT-116 cells, and no cells induced $p21^{Cip/WAF1}$ incorporated BrdU in its nucleus, yet confirming the importance of $p21^{Cip/WAF1}$ induction in anti-proliferation. These results again support that $p21^{Cip/WAF1}$ induction is a determinant in the regulation of colonic proliferation by the ERK pathway.

Keywords: anti-proliferation; colorectal cancer; ERK; MAP kinase; $p21^{Cip/WAF1}$; Zinc

Introduction

$p21^{Cip/WAF1}$ is an important cell cycle regulator that is induced by activation of both p53 and the extracellular signal regulated kinase (ERK) pathways (reviewed in Kerkhoff and Rapp, 1998). p53-independent induction of $p21^{Cip/WAF1}$ is acquired by activation of the ERK pathway involving activation of the Raf-1-MEK-ERK cascade (Liu *et al.,* 1996; Sewing *et al.,* 1997; Woods *et al.,* 1997; Mahyar-Roemer and Roemer, 2001; Park *et al.,* 2002). The inducers that can activate $p21^{Cip/WAF1}$ *via* activation of the ERK pathway include zinc (Park *et al.,* 2002; 2003), sulindac (Yip-Schneider and Schmidt, 2003), troglytazone (TRO) (Kim *et al.,* 2002), calmodulin inhibitor W13 (Bosch *et al.,* 1998), and butyrate (Archer *et al.,* 1998; Derjuga *et al.,* 2001). The ERK pathway is involved in growth arrest (Liu *et al.,* 1996; Sewing *et al.,* 1997; Woods *et al.,* 1997; Mahyar-Roemer and Roemer, 2001; Park *et al.,* 2002; Boucher *et al.,* 2004) as well as its well known function of growth stimulation (Blenis, 1993; Chang *et al.,* 2003). Activation of $p21^{Cip/WAF1}$, represented by induction and nuclear localization, is an important marker for anti-proliferation and growth arrest that occurs by prolonged activation of the ERK pathway (Nakano *et al.,* 1997; Archer *et al.,* 1998; Kim *et al.,* 2002; Park *et al.,* 2002; 2003; Eum *et al.,* 2003). On the other hand, $p21^{Cip/WAF1}$ was not induced by the transient and weak ERK activation that is associated with proliferation (Sewing *et al.,* 1997; Woods *et al.,* 1997; Park *et al.,* 2002). The differential activation of ERK that is related to different growth behaviors relies on signaling intensity and the serum concentration (Park *et al.,* 2003). Induction of $p21^{Cip/WAF1}$ is an important indicator for anti-proliferation in colorectal cancer cells (Nakano *et al.,* 1997; Archer *et al.,* 1998; Kim *et al.,* 2002; Park *et al.,* 2002). Percentages of cells incorporating BrdU were significantly reduced by zinc or TRO inducing $p21^{Cip/WAF1}$ (Kim *et al.,* 2002; Park *et al.,* 2002).

In previous studies, we identified that $p21^{Cip/WAF1}$ induction is a marker for ERK pathway dependent anti-proliferation due to extracellular zinc in HT-29 colorectal cancer cells (Oh *et al.,* 2002; Park *et al.,* 2002). However, we did not observe the zinc effect of anti-proliferation in other cell type, HCT-116. There-

fore, we investigated cell type specificity in regulation of colonic proliferation related with the ERK pathway dependent $p21^{Cip/WAF1}$ induction. The $p21^{Cip/WAF1}$ induction was identified as an essential marker for the ERK pathway dependent anti-proliferation in HT-29 and DLD-1 cells, and that was abolished in HCT-116 cells. We further confirmed importance of the role of $p21^{Cip/WAF1}$ in anti-proliferation by using $p21^{Cip/WAF1}$-/- HCT-116 cells. This study provides an evidence for an essentiality of $p21^{Cip/WAF1}$ induction in the regulation of proliferation of colorectal cancer cells by extracellular stimuli inducing prolonged ERK activation.

Materials and Methods

Cell culture

HT-29 (ATCC HTB-38) and DLD-1 (ATCC CCL-221) human colorectal cancer cells (Shirasawa *et al.,* 1993; Rowan *et al.,* 2000) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The HCT-16 $p21^{Cip/WAF1}$ +/+ human colon carcinoma cell line and the derivative line HCT-16 $p21^{Cip/WAF1}$-/-, in which both $p21^{Cip/WAF1}$ alleles are deleted by homologous recombination (Waldman *et al.,* 1995), were provided by Dr. Bert Vogelstein of John Hopkins Oncology Center. HT-29 and HCT-116 cells were maintained in McCoy's 5A medium supplemented with 10% (vol/vol) FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin in 5% CO_2 at 37°C. DLD-1 colorectal cancer cells were maintained in RPMI1640 supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin in 5% CO_2 at 37°C. Analyses were performed on cells at 70% confluence. To observe the effects of zinc, cells were serum-starved by growth for 16 to 20 h in a minimally serum starved medium (MSSM) containing 1% FBS, then either treated or not treated with 100 μM $ZnCl_2$. In required cases, PD98059 (Calbiochem, La Jolla, CA) was treated as 20 μM at 30 min prior to $ZnCl_2$ treatment. Lactacystin (Calbiochem) was applied to 2 μM at 9 h before harvest cells when required.

Western blot analysis

For preparation of proteins, attached cells were rinsed twice with ice-cold PBS, harvested, Then lysed directly in Laemmli SDS sample buffer (Laemmli, 1970). Samples were boiled and subjected to 8-10% sodium dodecyl sulfate-polyacrylamide gel electophoresis (SDS-PAGE) followed by Western blot analysis using anti-p-ERK (New England Biolabs, Beverly, MA), -ERK (Stratagene, La Jolla, CA), or $p21^{Cip/WAF1}$ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) primary antibodies followed by matching horse radish peroxidase conjugated secondary antibodies. Protein bands were visualized using enhanced chemiluminescence (Genepia, Seoul, Korea) according to the manufacturer's instructions.

Immunocytochemistry and BrdU incorporation

HT29, DLD-1 or $p21^{Cip/WAF1}$ +/+ $p21^{Cip/WAF1}$ -/- HCT-116 cells were plated onto cover slips at a density of 2×10^5 cells/coverslip within 6-well plates. Cells were grown overnight, serum starved for 16-20 h. Cells were treated or non-treated with 100 μM $ZnCl_2$ for 9 h before immunocytochemical analysis. Cells were then washed twice with PBS, fixed in a methanol/formaldehyde (99:1) mixture at -20°C for 15 min, permeabilized with PBS containing 0.2% Triton-X-100, then finally gently washed 5 times with PBS for 5 min. After treatment with a blocking solution (PBS containing 1% BSA, 0.1% gelatin, and 5% goat serum), the cells were further incubated with primary antibody (anti-$p21^{Cip/WAF1}$ antibody at a 1:100 dilution) for 2 h, then washed 5 times with PBS containing 1% BSA and 0.1% gelatin. Cells were then further incubated with goat anti-rabbit-Rhodamine Red TM-X-conjugated secondary antibody at a 1:100 dilution for 1 h, then washed 5 times with PBS. For a BrdU incorporation study, cells were grown in a medium containing 20 μM BrdU for 12 h before immunocytochemical analysis. The cells were then fixed in 3.7% formaldehyde for 10 min at room temperature and rinsed with PBS before being incubated for 10 min in 2 N HCl. Cells were then washed three times with PBS for 5 min. After blocking, the cells were incubated with anti-BrdU monoclonal antibody at a 1:20 dilution for 2 h and washed with PBS containing 1% BSA and 0.1% gelatin, followed by incubation with goat anti-mouse-CyTM2-conjugated secondary antibody at a 1:100 dilution for 1 h. The cells were then washed 5 times with PBS. Each analysis was performed at least three times. DAPI was then applied at a final concentration of 1 μg/ml in PBS for 10 min, then the cells were extensively washed with PBS and mounted for photography on a Radiance 2,000/MP multi-photon imaging system (Bio-Rad, UK).

Results and Discussion

$p21^{Cip/WAF1}$ activation was identified as an important marker for anti-proliferation and that related with strong and prolonged ERK activation (Sewing *et al.,* 1997; Woods *et al.,* 1997; Kim *et al.,* 2002; Park *et al.,* 2002; 2003). However, it is not clear whether $p21^{Cip/WAF1}$ activation is a required factor or just one of the indicators for anti-proliferation caused by extracellular stimuli that induce prolonged ERK activation.

In this study, we investigate the role of $p21^{Cip/WAF1}$ induction in the regulation of proliferation further

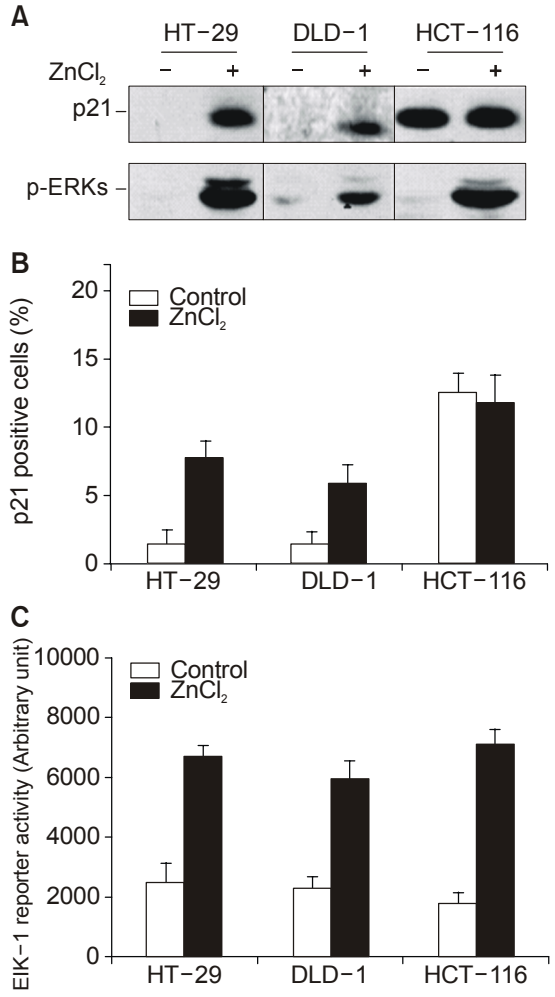

Figure 1. The effect of extracelluar zinc treatment on $p21^{Cip/WAF1}$ induction and ERK pathway activation in different colorectal cancer cells. HCT-116, HT-29 and DLD-1 cells were starved for 18 h by growth in MSSM followed by treatment or not treatment with 100 μM $ZnCl_2$ for 9 h. (A) Cells were harvested and subjected to Western blot analysis using anti-$p21^{Cip/WAF1}$ and -phospho-ERK antibodies as described in Materials and Methods. (B) $p21^{Cip/WAF1}$ positive cells were counted by the results of immunocytochemistry using anti-$p21^{Cip/WAF1}$ antibody followed by incubation with Rhodamine-Red-conjugated goat anti-rabbit IgG. Cell nuclei were stained with DAPI. $p21^{Cip/WAF1}$ positive cells were estimated by dividing $p21^{Cip/WAF1}$ positive cells with total DAPI stained cells. Error bars indicate the standard deviations of three independent experiments. (C) HCT-116, HT-29 and DLD-1 cells were grown as described in Figure 1A, and were co-transfected with 0.1 μg of luciferase reporter plasmid pFR-Luc and 25 ng of trans-activator pFA2-Elk-1. For normalization, the cells were co-transfected with 0.1 μg of plasmid containing the gene for β-galactosidase under the control of a CMV promoter. Cells were incubated in MSSM for additional 16 h. The medium was replaced with fresh medium with or without 100 μM $ZnCl_2$ and extracts were made 12 h after the zinc treatment. Luciferase activity was measured as described previously (Park *et al.,* 2002).

by investigating cell type specificity. We measured $p21^{Cip/WAF1}$ level in several colorectal cancer cells at 9 h after treatment of 100 μM $ZnCl_2$ in minimally serum starved medium (MSSM) to detect $p21^{Cip/WAF1}$ induction that occurs due to prolonged ERK activation (Park *et al.,* 2002; 2003). The $p21^{Cip/WAF1}$ induction observed both in HT-29 and DLD-1 cells, but that was not observed in HCT-116 cells (Figure 1A). The differential regulation of $p21^{Cip/WAF1}$ induction was also similarly observed by quantitative immunocytochemical analysis (Figure 1B). These results indicated $p21^{Cip/WAF1}$ induction by extracellular zinc varies dependent upon types of colorectal cancer cells. Differently from $p21^{Cip/WAF1}$ induction, ERK and Elk-1 reporter activities responding to ERK activity were increased by 100 μM $ZnCl_2$ treatment in all three different types of colorectal cancer cells grown in MSSM (Figure 1A and 1C). These results indicate that $p21^{Cip/WAF1}$ induction characteristic by extracellular zinc may be abolished in HCT-116 cells although ERK activation is induced. The $p21^{Cip/WAF1}$ induction in HT-29 cells was abolished by ERK inhibition by chemicals and genetic methods (Park *et al.,* 2002 and Oh *et al.,* 2002), indicating that the $p21^{Cip/WAF1}$ induction in HT-29 cells may be caused by prolonged ERK activation (Park *et al.,* 2002; 2003). Therefore, we measured effect of the MEK inhibitor PD98059 to know whether $p21^{Cip/WAF1}$ level can be regulated by PD98059 within HCT-116 cells.

The $p21^{Cip/WAF1}$ induction by 100 μM $ZnCl_2$ in HT-29 cells was abolished by pre-treatment of PD98059 in HT-29 cells (Oh *et al.,* 2002) and DLD-1 cells (Figure 2A). On the other hand, $p21^{Cip/WAF1}$ induction was not abolished in HCT-116 cells although ERK activities were significantly reduced by PD98059 pre-treatment (Figure 2B). These results indicated that the ERK pathway-dependent $p21^{Cip/WAF1}$ induction was abolished in HCT-116 cells. $p21^{Cip/WAF1}$ protein level in HCT-116 cells is abnormally high even without the zinc-dependent ERK activation (Figure 1A) by an unknown reason. It is known that $p21^{Cip/WAF1}$ is primarily regulated by transcription, and $p21^{Cip/WAF1}$ transcription is regulated by many different factors including p53, C/EBPα and β, EIA, and c-Myc (El-Deiry *et al.,* 1993; Timchenko *et al.,* 1996; Datto *et al.,* 1997; Claassen and Hann, 2000). $p21^{Cip/WAF1}$ expression is also tightly regulated at the level of protein degradation (Rousseau *et al.,* 1999; Sheaff *et al.,* 2000; Touitou *et al.,* 2001). We observed increase of $p21^{Cip/WAF1}$ protein level by treatment of the proteasome inhibitor, lactacystin (Xiao *et al.,* 2004), and the level of increase is similar to the level acquired by 100 μM $ZnCl_2$ in MSSM (Figure 3). In addition, level of $p21^{Cip/WAF1}$ was synergistically increased by co-treatment of lactacystin and zinc (Figure 3). These results indicate that the $p21^{Cip/WAF1}$ protein may be

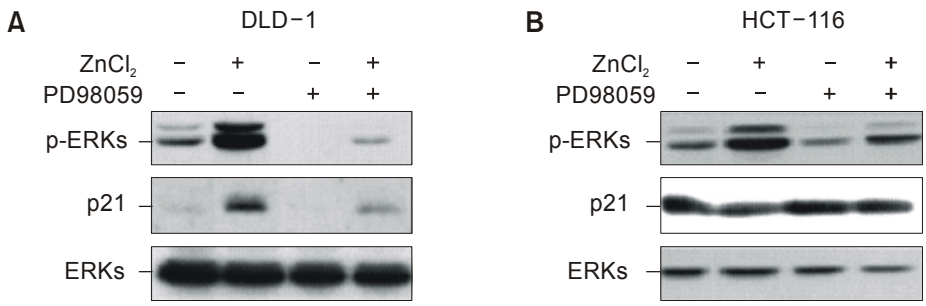

Figure 2. Effects of PD98059 on zinc-dependent induction of $p21^{Cip/WAF1}$ in different colorectal cancer cells. DLD1 and HCT-116 cells were grown and treated with 100 μM $ZnCl_2$ as described in Figure 1A. When required, 10 μM of PD98059 was also treated for 30 min before zinc treatment. The cells were harvested at 9 h after zinc treatment for assay. ERK activities and ERK and $p21^{Cip/WAF1}$ protein levels were measured by Western blot analysis using anti-phospho-ERK, -ERK and -p21 antibodies, respectively.

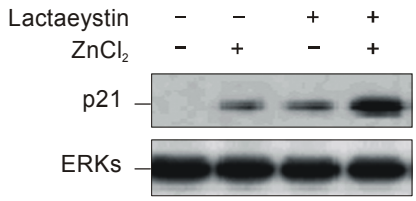

Figure 3. Effects of lactacystin, proteasome-dependent degradation inhibitor, on $p21^{Cip/WAF1}$ protein stability within HT-29 cells. HT-29 cells were grown in MSSM for 16 h. The cells were harvested 9 h after treatment with 100 μM $ZnCl_2$ with or without co-treatment of 2 μM of lactacystein. The $p21^{Cip/WAF1}$ and ERK protein levels were detected by Western blot analysis as described in Materials and Methods.

regulated at least partly by protein stability level involving proteasome. Maintenance of low $p21^{Cip/WAF1}$ protein levels in HT-29 and DLD-1 cells indicate that the $p21^{Cip/WAF1}$ level is probably tightly regulated in those cells, and the regulatory mechanism may be released in HCT-116 cells.

Cellular proliferation was significantly reduced in cells induced $p21^{Cip/WAF1}$ by zinc treatment, and $p21^{Cip/WAF1}$ induction was abolished by MEK inhibitors (Oh *et al.*, 2002; Park *et al.*, 2002; 2003). However, essentiality of $p21^{Cip/WAF1}$ in anti-proliferation by prolonged ERK activation caused by extracellular stimuli is not fully understood. In addition, most of the above studies related with anti-proliferation were performed by overexpression of $p21^{Cip/WAF1}$ or by induction of $p21^{Cip/WAF1}$ by extracellular zinc stimulus.

We used $p21^{Cip/WAF1}$ -/- HCT-116 cells in mea-

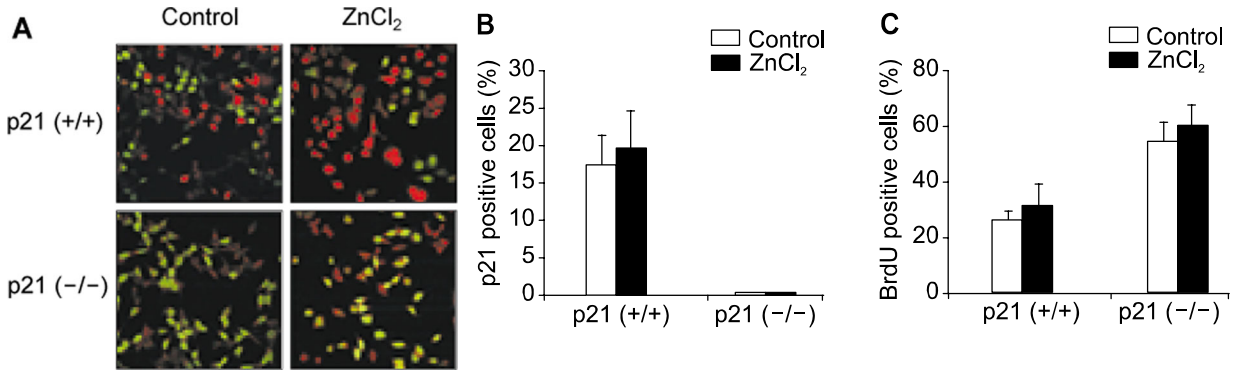

Figure 4. Effects of extracellular zinc treatment on the induction of $p21^{Cip/WAF1}$ and BrdU incorporation in $p21^{Cip/WAF1}$+/+ and $p21^{Cip/WAF1}$-/- HCT-116 cells. $p21^{Cip/WAF1}$+/+ and $p21^{Cip/WAF1}$-/- HCT-116 cells were cultured for 48 h by growth in MSSM. The cells were labeled with 20 μM BrdU for the last 5 h before imunocytochemical assay (A) BrdU-incorporated and $p21^{Cip/WAF1}$ positive cells were visualized after the immunostaining of $p21^{Cip/WAF1}$ +/+ and $p21^{Cip/WAF1}$ -/- HCT-116 cells culture in MSSM for 48 h against BrdU and $p21^{Cip/WAF1}$. (B) $p21^{Cip/WAF1}$ positive cells were estimated by counting the number of $p21^{Cip/WAF1}$ positive cells out of the total number of DAPI stained cells. Analyses were repeated at least three times. (C) BrdU-incorporated cells were counted after the immunostaining of $p21^{Cip/WAF1}$ +/+ and $p21^{Cip/WAF1}$ -/- HCT-116 cells culture in MSSM for 48 h against BrdU and $p21^{Cip/WAF1}$. Analyses were repeated at least three times.

surement of an effect of zinc to clarify the role of $p21^{Cip/WAF1}$ in the proliferation of colorectal cancer cells. Because $p21^{Cip/WAF1}$ induction ability was lost in the HCT-116 cells, it is an ideal cell type to compare with HT-29 and DLD-1 cells and to confirm whether $p21^{Cip/WAF1}$ induction is an essential factor in the regulation of the proliferation by extracellula stimuli inducting prolonged ERK activation. None of the $p21^{Cip/WAF1}$ -/- HCT-116 cells accumulated $p21^{Cip/WAF1}$ in nuclear although 17% of $p21^{Cip/WAF1}$ +/+ HCT-116 cells actively expressed $p21^{Cip/WAF1}$ by 100 μM $ZnCl_2$ treatment (Figure 4A and 4B). Percentage of cells induced $p21^{Cip/WAF1}$ was not significantly increased by extracellular zinc treatment for both $p21^{Cip/WAF1}$ +/+ and $p21^{Cip/WAF1}$ -/- HCT-116 cells (Figure 4B).

In a resting state, 28% of $p21^{Cip/WAF1}$ +/+ HCT-116 cells actively incorporated BrdU in its nuclei, and percentage of cells incorporated BrdU was increased to 55% in case of $p21^{Cip/WAF1}$ -/- HCT-116. These results confirm importance of the $p21^{Cip/WAF1}$ induction in the regulation of the proliferation of colorectal cancer cells. Interestingly, BrdU incorporation level was not reduced by 100 μM $ZnCl_2$ treatment for both $p21^{Cip/WAF1}$ +/+ and $p21^{Cip/WAF1}$ +/+ HCT-116 cells grown in MSSM. Therefore, HCT116 cells, which abolished the ERK pathway dependent $p21^{Cip/WAF1}$ induction, have lost the regulatory function of proliferation. On the other hand, HT-29 cells which retain the ERK pathway dependent $p21^{Cip/WAF1}$ induction function retained the regulatory function of proliferation (Park *et al.,* 2002).

Overall, $p21^{Cip/WAF1}$ induction is an essential factor for the ERK pathway dependent anti-proliferation of colorectal cancer cells. The cell type specificity in the ERK pathway dependent growth inhibition of colorectal cancer cells may be attributed by induction of $p21^{Cip/WAF1}$ by prolonged activation of the ERK pathway.

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