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Anti-cancer activity of *Bacillus amyloliquefaciens* AK-0 through cyclin D1 proteasomal degradation *via* GSK3β-dependent phosphorylation of threonine-286

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Microorganisms have been regarded as important sources of novel bioactive natural products. In this study, we evaluated the anti-cancer activity and the potential mechanism of *Bacillus amyloliquefaciens* AK-0 newly isolated from the rhizosphere soil of Korean ginseng. The ethyl acetate fraction from the culture medium of *B. amyloliquefaciens* AK-0 (EA-AK0) inhibited markedly the proliferation of human colorectal cancer cells such as HCT116, SW480, LoVo and HT-29. EA-AK0 effectively decreased cyclin D1 protein level in human colorectal cancer cells, while cyclin D1 mRNA level was not changed by EA-AK0 treatment. Inhibition of proteasomal degradation by MG132 blocked EA-AK0-mediated cyclin D1 downregulation and the half-life of cyclin D1 was decreased in the cells treated with MRB. In addition, EA-AK0 increased threonine-286 (T286) phosphorylation of cyclin D1, and a point mutation of T286 to alanine attenuated cyclin D1 degradation by EA-AK0. Inhibition of GSK3β by LiCl suppressed cyclin D1 phosphorylation and downregulation by EA-AKO. From these results, EA-AK0 may suppress the proliferation of human colorectal cancer cells by inducing cyclin D1 proteasomal degradation through GSK3β-dependent T286 phosphorylation. These results indicate that EA-AK0 could be used for treating colorectal cancer and serve as a potential candidate for anticancer drug development. In addition, these findings will be helpful for expanding the knowledge on the molecular anti-cancer mechanisms of EA-AK0.

1. Introduction

Colorectal cancer (CRC) as one of the most common gastrointestinal tumors has a high incidence and mortality rate worldwide. Although diagnostic procedures and therapeutic strategies for CRC have been advanced, 50% of patients with CRC finally die because of this cancer (Corvinus et al. 2005). In addition, the use of current anti-cancer drugs has been limited in their efficacy and safety (Sukamporn et al. 2016). Thus, the study for searching the novel natural compounds as complementary and alternative medicines for managing cancer (Nobili et al., 2009).

Natural resources such as plants and microorganisms have been regarded as an important sources for bioactive compounds and various drugs have been developed from natural products (Amador et al. 2003). Since the discovery of penicillin as one of the microorganism-producing bioactive agents, microorganisms have been used as a valuable source of the bioactive agents (Phonnok et al. 2010). The bioactive compounds from microorganisms have been used as antibiotics (penicillin, erythromycin, streptomycin, amphotericin and polyketides), immunosuppressants (cyclosporine A, FK506 and rapamycin), cholesterol lowering agents (lovastatin and mevastatin) and anticancer agents (e.g., daunorubicin, doxorubicin, bleomycin and pentostatin) (Phonnok et al. 2010). In case of anti-cancer agents, 33% of the natural product-related anticancer drugs are from microorganisms and approved by the US Food and Drug Administration (FDA) (Giddings and Newman 2013).

Bacillus species such as *B. subtilis* and *B. amyloliquefaciens* as plant-growth promoting bacteria produce a variety of bioactive products with antifungal, antibacterial, immunosuppressive and antitumor activity (Koumoutsi et al. 2004; Thimon et al. 1992; Wu et al. 2014). From *B. amyloliquefaciens*, SQR9 has been reported to induce dendritic cell maturation and enhance the immune response against inactivated avian influenza virus (Huang et al. 2016). B-1895 showed DNA protection and antioxidant properties (Prazdnova et al. 2015). In addition, BACY1 induced cell death in human oral squamous carcinoma cells (Kuo et al. 2015) and fiply 3A induced apoptosis in human alveolar adenocarcinoma, renal carcinoma and colon adenocarcinoma cells (Hajare et al. 2013).

In this study, we evaluated the anti-cancer activity of *B. amyloliquefaciens* AK-0 newly isolated from the rhizosphere soil of Korean ginseng (Kim et al. 2017) and elucidated its potential molecular mechanism by which *B. amyloliquefaciens* AK-0 exerts anticancer activity. Here, we firstly report that *B. amyloliquefaciens* AK-0 reduced the proliferation of human colorectal cancer cells through cyclin D1 proteasomal degradation via GSK3β-dependent threonine-286 phosphorylation.

2. Investigations and results

2.1. EA-AK0 inhibits cell growth in human colorectal cancer cells

To investigate the effect of EA-AK0 on cell growth retardation in human colorectal cancer cells, HCT116, SW480, LoVo and HT-29 cells were treated with the indicated concentrations of EA-AK0 for 24 h, and cell proliferation was measured using MTT solution. As shown in Fig. 1A, the freeze-dried culture medium (AK0) without ethyl acetate fraction did not affect cell growth in HCT116 cells at 25-100 μg/ml, while the ethyl acetate fraction from the culture medium (EA-AK0) suppressed the proliferation of HCT116 cells by 27% at 50 μg/ml and 69% at 100 μg/ml, respectively. Thus, we selected EA-AK0 for the further study. In addition, EA-AK0 reduced the cell proliferation in SW480 cells by 33% at 50 μg/ml and 75% at 100 μg/ml, respectively. We also observed

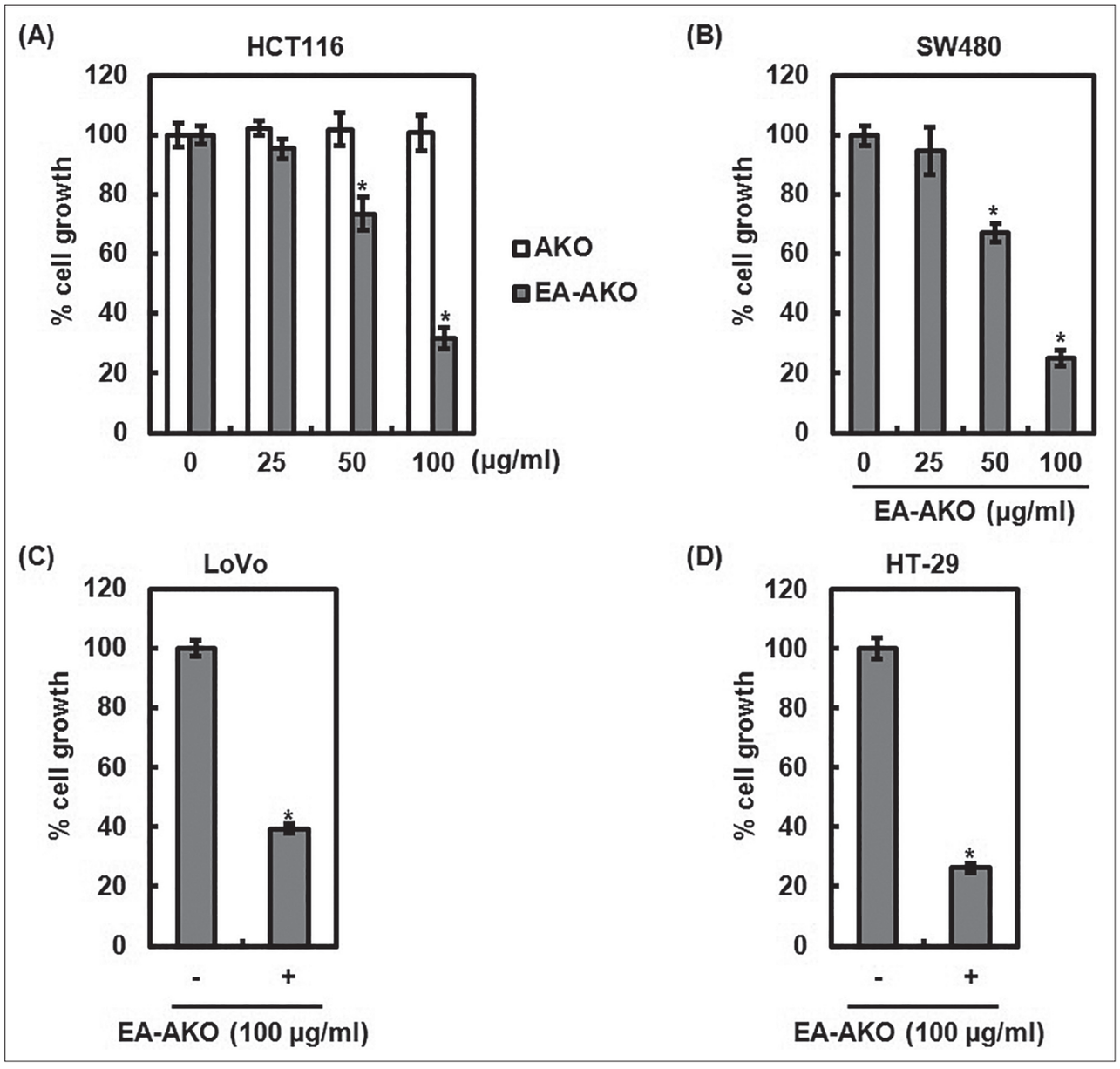

Fig. 1: Effect of EA-AK0 on the cell growth in human colorectal cancer cell lines such as HCT116, SW480, LoVo and HT-29 cells. (A-D) The cells were plated overnight and then treated with EA-AK0 for 24 h. Cell proliferation was measured using MTT assay as described in Materials and methods. *P < 0.05 compared to cell without EA-AK0 treatment.

EA-AK0-mediated inhibition of the cell proliferation in LoVo and HT-29 cells by 61% and 73% at 100 µg/ml, respectively.

2.2. EA-AK0 decreases cyclin D1 protein level in human colorectal cancer cells

Because cell growth has been regarded to be related to cyclin D1 expression (Sukamporn et al. 2016), the alteration of cyclin D1 protein level by EA-AK0 treatment was investigated in HCT116, SW480, LoVo and HT-29 cells. As shown in Fig. 2A, cyclin D1 protein level of HCT116 and SW480 cells was significantly decreased at 50 and 100 µg/ml of EA-AK0, although low concentration (25 µg/ml) of EA-AK0 did not affect cyclin D1 protein level. In addition, the reduction of cyclin D1 protein level was observed in LoVo and HT-29 cells in presence of EA-AK0 (100 µg/ml) (Fig. 2B). Futhermore, cyclin D1 was downregulated at early time point in presence of EA-AK0 in HCT116 and SW480 cells, indicating that EA-AK0 reduced cyclin D1 protein level in a time-dependent manner (Fig. 2C).

2.3. EA-AK0 degrades cyclin D1 protein

To determine whether the decrease of cyclin D1 by EA-AK0 is attributed to transcriptional or post-translational regulation of the cyclin D1 gene, the mRNA level of cyclin D1 was investigated in HCT116, SW480, LoVo and HT-29 cells. As shown in Fig. 3A and 3B, the mRNA level of cyclin D1 was not changed by EA-AK0 treatment. This finding indicates that EA-AK0 may regulate cyclin D1 protein level through proteasomal degradation. To verify the proteasomal degradation of cyclin D1 by EA-AK0, HCT116 and SW480 cells were pretreated with MG132 (proteasome inhibitor) for 2 h, and then co-treated with 100 µg/ml of EA-AK0 for 6 h. As shown in Fig. 3C, EA-AK0-mediated degradation of cyclin D1 was restored in presence of MG132

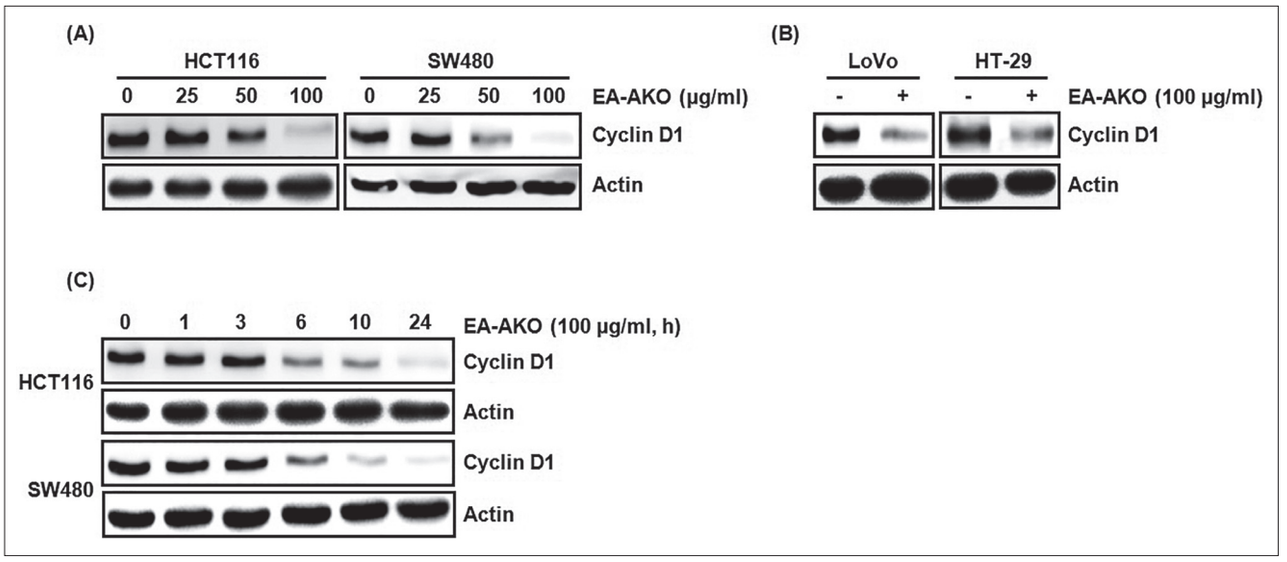

Fig. 2: Effect of EA-AK0 on cyclin D1 protein level in human colorectal cancer cell lines such as HCT116, SW480, LoVo and HT-29 cells. (A, B) HCT116, SW480, LoVo and HT-29 cells were plated overnight, and then treated with EA-AK0 at the indicated concentrations for 24 h. (C) HCT116 and SW480 cells were plated overnight, and then treated with EA-AK0 for the indicated times. Each cell lysate was subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. Actin was used as internal control.

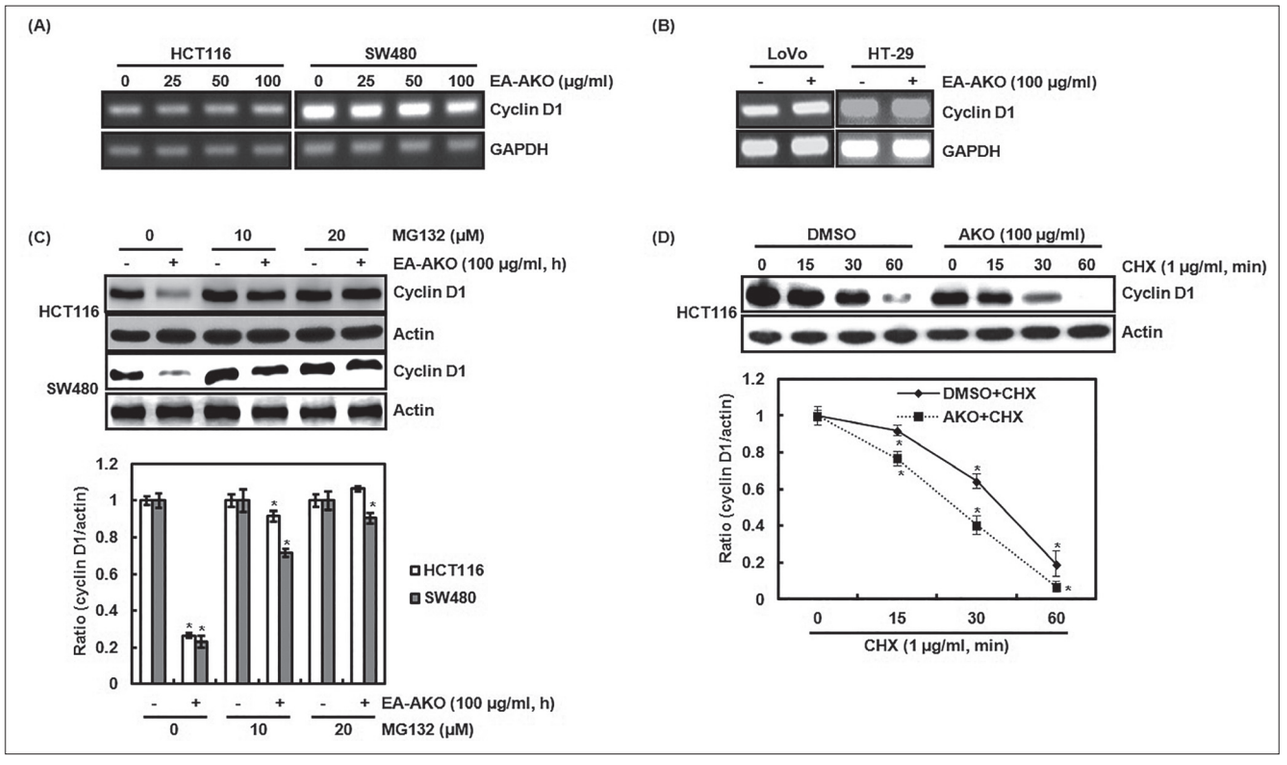

Fig. 3: Proteasomal degradation of cyclin D1 by EA-AK0. (A, B) HCT116, SW480, LoVo and HT-29 cells were plated overnight, and then treated with EA-AK0 at the indicated concentrations for 24 h. For RT-PCR analysis of cyclin D1 gene expression, total RNA was prepared. GAPDH was used as internal control. (C) HCT116 and SW480 cells were plated overnight. The cells were pretreated with MG132 for 2 h, and then co-treated with EA-AK0 for 6 h. (D) HCT116 cells were pretreated with DMSO or EA-AK0 for 3 h, and then co-treated with 10 μg/ml of cycloheximide (CHX) for the indicated times. Each cell lysate was subjected to SDS-PAGE and the Western blot was performed using antibody against cyclin D1. Actin was used as internal control. *P < 0.05 compared to cell without EA-AK0 treatment.

in both HCT116 and SW480 cells, which implies that EA-AK0 attenuates cyclin D1 protein level through proteasomal pathway. To confirm the effect of EA-AK0 on cyclin D1 proteasomal degradation, HCT116 cells were pretreated with 100 μg/ml of EA-AK0 for 3 h, and then co-treated with 10 μg/ml of CHX for the indicated times. As shown in Fig. 3D, EA-AK0 treatment decreased half-life of cyclin D1 protein in presence of CHX compared with DMSO-treated cells.

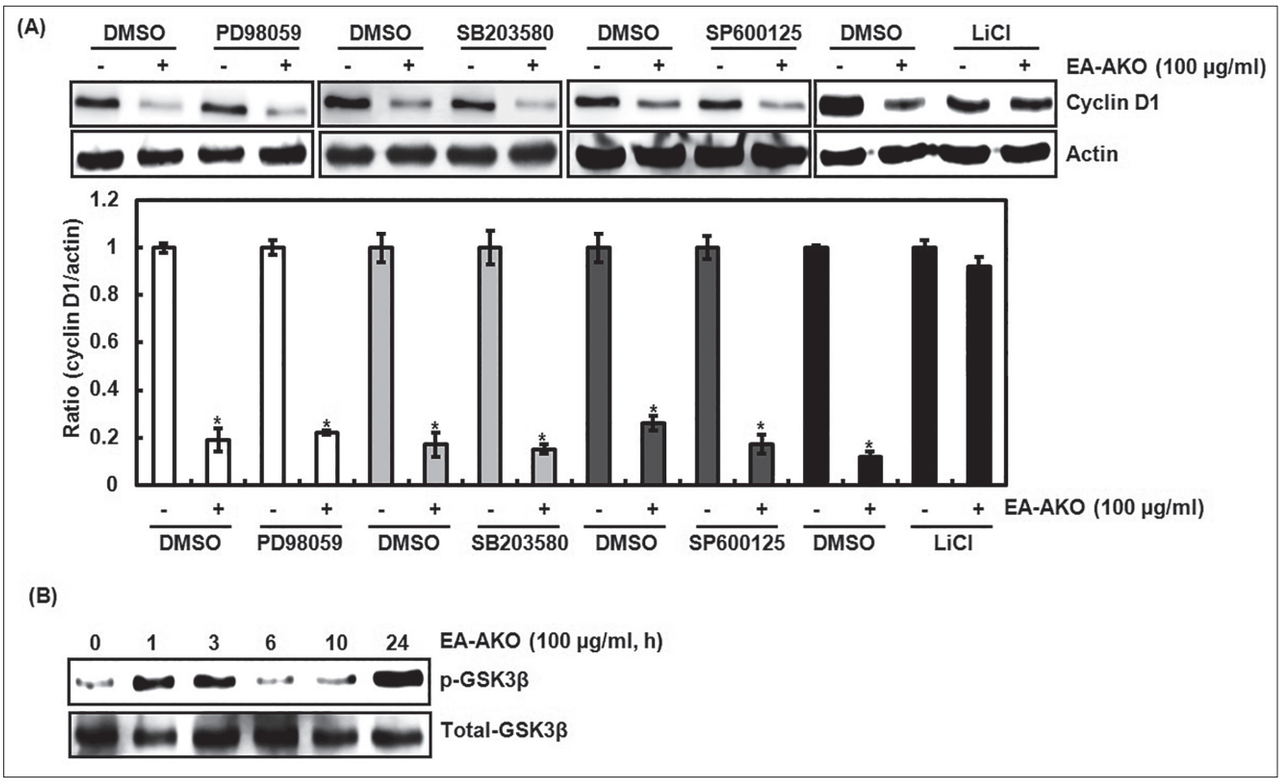

Fig. 4: GSK3β-dependent cyclin D1 degradation by EA-AK0. (A) HCT116 cells were pretreated with PD98059 (ERK1/2 inhibitor, 20 μM), SB203580 (p38 inhibitor, 20 μM), SP600125 (JNK inhibitor, 20 μM) or LiCl (GSK3β inhibitor, 20 mM) for 2 h and then co-treated with EA-AK0 for 6 h. (B) HCT116 cells were plated overnight, and then treated with EA-AK0 for the indicated times. Cell lysates were subjected to SDS-PAGE and the Western blot was performed using antibodies against cyclin D1, phospho-GSK3β and total GSK3β. Actin was used as internal control.

2.4. Degradation of cyclin D1 by EA-AK0 is dependent on GSK3β

To determine the kinases related to EA-AK0-mediated degradation of cyclin D1, HCT116 cells were pretreated with PD98059 (20 μM, ERK1/2 inhibitor), SB203580 (20 μM, p38 inhibitor), SP600125 (20 μM, JNK inhibitor) or LiCl (20 mM, GSK3β inhibitor) for 2 h, and then co-treated with 100 μg/ml of EA-AK0 for 6 h. As shown in Fig. 4A, the restoration of EA-AK0-mediated cyclin D1 degradation was only observed in presence of LiCl. However, other kinase inhibitors such as PD98059, SB203580 and SP600125 did not affect cyclin D1 degradation by EA-AK0. These data suggest that EA-AK0 induces cyclin D1 degradation at a GSK3β-dependent manner. Thus, we investigated whether EA-AK0 activates GSK3β. As shown in Fig. 4B, the phosphorylation of GSK3β as an active form was increased by EA-AK0.

2.5. GSK3β-dependent threonine-286 (T286) contributes to cyclin D1 degradation by EA-AK0

Since T286 phosphorylation of cyclin D1 has been known to be involved in its proteasomal degradation, we tested whether EA-AK0 exerts T286 phosphorylation of cyclin D1 in HCT116 cells. As shown in Fig. 5A, T286 phosphorylation by EA-AK0 was increased at the earlier time point (1 h) compared to that of the decrease of cyclin D1 (6 h) (Fig. 2C). Next, we investigated the relation of the phosphorylation site (T286) to EA-AK0-mediated cyclin D1 degradation. For this study, HCT116 cells were transfected with HA-tagged wild type and T286A (point mutant of T286 to alanine) cyclin D1 plasmid for 24 h, and then treated with 100 μg/ml of EA-AK0. As shown in Fig. 5B, EA-AK0 reduced HA-cyclin D1 in the cells transfected with wild type-cyclin D1, while T286A transfection restored the reduction of HA-cyclin D1 by EA-AK0. These findings suggest that T286 phosphorylation of cyclin D1 by EA-AK0 results in the induction of cyclin D1 degradation. In addition, we examined the effect of GSK3β on T286 phosphorylation by EA-AK0. As a result, the inhibition of EA-AK0-mediated GSK3β activation by LiCl (20 mM) abolished T286 phosphorylation compared to the cells in absence of LiCl (Fig. 5C). These findings suggest that EA-AK0-mediated GSK3β activation contributes to T286 phosphorylation and subsequently induces cyclin D1 degradation. Because cyclin D1 degradation by GSK3β-dependent T286 phosphorylation is required to distribute cyclin D1 from the nucleus to the cytoplasm, we investigated the effect of the inhibition of nuclear export on cyclin D1 degradation by EA-AK0. HCT116 cells were pretreated with LMB as a nuclear export inhibitor and then co-treated with EA-AK0. As shown in Fig. 5D, the inhibition of cyclin D1 nuclear export by LMB attenuated EA-AK0-mediated cyclin D1 degradation.

3. Discussion

It has been reported that abnormal cell growth could be essential in cancer development and the control of cancer cell growth may be important for cancer prevention (Mori et al. 2001, 1999). Cyclin D1 as one of the oncogenic protein related to abnormal cell growth in cancer cells forms an active complex with cyclin-dependent kinases (CDK) 4 and 6, and phosphorylates retinoblastoma protein, which results in facilitating cell cycle progression from G1 to S phase via regulation of several transcriptional factors (Alao 2007). Overexpression of cyclin D1 was found in various cancer cells (Fu et al. 2004) and 68.3% of human colorectal cancer has been reported to be overexpressed (Bahnassy et al. 2004; Holland et al. 2001). Thus, cyclin D1 has been regarded as an important molecular target for the cancer chemoprevention and therapy in the human colorectal cancer. In this study, EA-AK0 (50 and 100 μg/ml) significantly reduced cyclin D1 protein levels and inhibited cell growth, while low-dose of EA-AK0 (25 μg/ml) did not affect

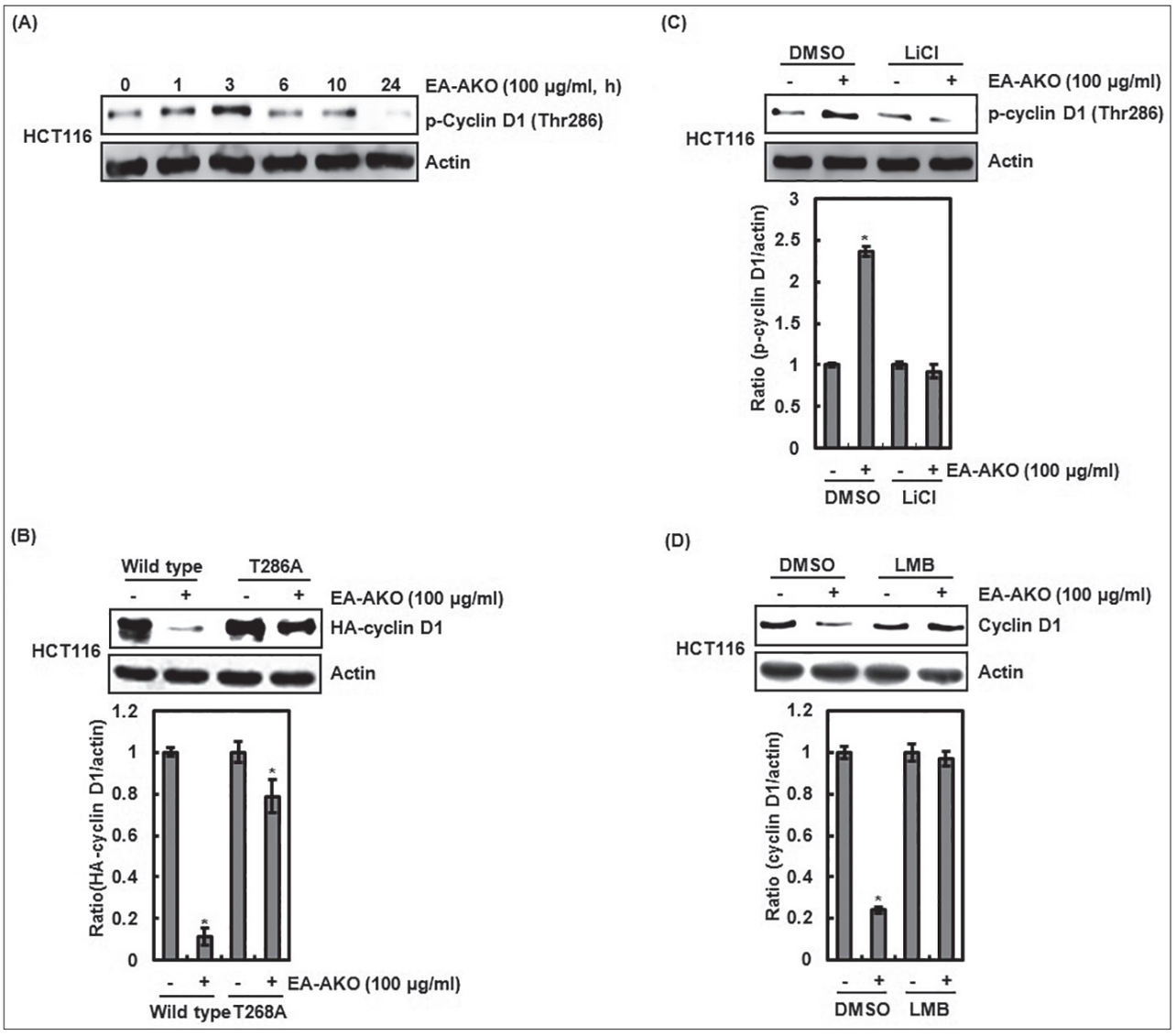

Fig. 5: Requirement of GSK3β-dependent T286 phosphorylation in EA-AK0-mediated cyclin D1 degradation. (A) HCT116 cells were plated overnight, and then treated with EA-AK0 for the indicated times. (B) HCT116 cells were transfected with wild type HA-tagged cyclin D1 or HA-tagged T286A cyclin D1 plasmid for 24 h and then treated with EA-AK0 for 6 h. (C) HCT116 cells were pretreated with LiCl (GSK3β inhibitor, 20 mM) for 2 h and then co-treated with EA-AK0 for 3 h. (D) HCT116 cells were pretreated with LMB (50 ng/ml) for 2 h and then co-treated with EA-AK0 for 6 h. Each lysate was subjected to SDS-PAGE and the Western blot was performed using antibodies against cyclin D1, phospho-cyclin D1 (T286) or HA-tag. Actin was used as internal control. *P < 0.05 compared to cell without EA-AK0 treatment.

the cyclin D1 level and the cell growth, which shows that the effect of EA-AK0 on cyclin D1 protein level is coincided with that on the cell growth.

There is growing evidence that many anti-cancer agents can block cyclin D1 expression through transcriptional and translational regulation (Sukamporn et al. 2016). Thus, the mechanistic study for the downregulation of cyclin D1 expression is required to understand how to better treat cancer and even to develop better anti-cancer drugs (Sukamporn et al. 2016). High level of cyclin D1 protein in human cancer can be regulated by its transcriptional pathway such as Wnt signaling (Shtutman et al. 1999). However, the decrease of cyclin D1 by EA-AK0 is thought to be independent on its transcriptional regulation because the mRNA level of cyclin D1 was not altered in presence of EA-AK0.

According some reports (Gillett et al. 1994; Russell et al. 1999), upregulated level of cyclin D1 solely results from a consequence of gene amplification and can be attributed to its deregulation at the post-translational level. Indeed, cyclin D1 degradation has been observed in cancer cells in the presence of many anti-cancer agents (Alao et al. 2004, 2006; Eo et al. 2015; Kim et al. 2015; Park et al. 2016). These studies suggest that cyclin D1 degradation may be the potential mechanism for anti-cancer activity. Our results showing that MG132 as a proteasome inhibitor restored EA-AK0-mediated degradation of cyclin D1 and co-treatment of EA-AK0 and CHX induced rapidly decreased cyclin D1 protein compared to the treatment of DMSO and CHX suggesting that downregulated cyclin D1 level by EA-AK0 may result from the degradation.

Cyclin D1 degradation has been reported to be followed by its modification such as RXXL motif, T286 and lysine residues (Alao 2007). Although many anti-cancer compound has been regarded to degrade cyclin D1 via T286 phosphorylation and RXXL motif-mediated ubiquitination, cyclin D1 degradation might not be strictly dependent on these modification (Sukamporn et al. 2016), Indeed, damnacanthal from the root of *Morinda citrifolia* L. affected polyubiquitination of cyclin D1 at the lysine sites (Sukamporn et al. 2016). However, our results showed that T286 phosphorylation is required for the induction of cyclin D1 degradation by EA-AK0. T286 phosphorylation regulates cyclin D1 protein level by regu-

lating ubiquitin-dependent proteasomal degradation (Diehl et al. 1997). For T286 phosphorylation and cyclin D1 degradation, there is additional evidence that mutation of T286 to alanine (T286A) greatly increased the stability of cyclin D1 (Alao 2007). In this study, we also demonstrated that T286A transfection abolished cyclin D1 degradation by EA-AK0.

GSK3β has been reported to be closely associated with cyclin D1 degradation (Diehl et al. 1998). It has been reported that GSK3β phosphorylates T286 phosphorylation and subsequently redistributes cyclin D1 from the nucleus to the cytoplasm, which results in cyclin D1 degradation (Diehl et al., 1998). There is growing evidence that T286A blocks cyclin D1 degradation through maintaining a nuclear localization of cyclin D1 (Alt et al. 2000). These findings indicate that GSK3β-dependent T286 phosphorylation can induce the nuclear export of cyclin D1 and subsequent degradation of cyclin D1 with in the cytoplasm (Amador et al. 2003; Diehl et al. 1998). In this study, EA-AK0 was investigated to induce GSK3β-dependent phosphorylation of T286 and cyclin D1 degradation. In addition, we observed that the inhibition of nuclear export of cyclin D1 by LMB restored cyclin D1 degradation by EA-AK0.

Although the ethyl acetate fraction was analyzed with GC/MS, we did not determine the chemical components produced by *B. amyloliquefaciens* AK-0 for anti-cancer activity. Thus, we intend to continue our research for the isolation of the potential anti-cancer compounds produced by *B. amyloliquefaciens* AK-0. In addition, it will be necessary to perform *in vivo* studies to confirm the anti-cancer activity of *B. amyloliquefaciens* AK-0 in an animal model.

In this study, we demonstrated that EA-AK0 may induce cyclin D1 proteasomal degradation through GSK3β-dependent Thr-286 phosphorylation, and EA-AK0-mediated cyclin D1 degradation may contribute to the inhibition of the proliferation in human colorectal cancer cells. The importance of this study is that *B. amyloliquefaciens* AK-0 with anti-cancer activity in human colorectal cancer is newly isolated, and the potential mechanism for anti-cancer activity of B. amyloliquefaciens AK-0 is elucidated. These findings can provide detailed information for a preclinical study to determine the use of *B. amyloliquefaciens* AK-0 as a therapeutic and chemopreventive agent for the treatment of human colorectal cancer.

4. Experimental

4.1. Cell line and reagents

Human colorectal cancer cell lines such as HCT116, SW480, LoVo and HT-29 were purchased from Korean Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM/F-12 (Lonza, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2. PD98059 (ERK1/2 inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor) and LiCl (GSK3β inhibitor), MG132, cycloheximide (CHX), leptomycin B (LMB, nuclear export inhibitor) and 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). Antibodies against cyclin D1, phospho-cyclin D1 (Thr286), HA-tag, phospho-GSK3β, total-GSK3β and β-actin were purchased from Cell Signaling (Bervely, MA, USA). All chemicals were purchased from Fisher Scientific (Hampton, NH, USA), unless otherwise specified.

4.2. Plasmids and transient transfection

HA-tagged cyclin D1 plasmids such as wildtype and point mutation of threonine-286 to alanine (T268A) were provided from Addgene (Cambridge, MA, USA). Each vector was transfected to HCT116 cells for 24 h using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction.

4.3. Sample preparation and treatment

B. amyloliquefaciens AK-0 isolated from the rhizosphere soil of Korean ginseng (Kim et al. 2017) was cultured in brain-heart infusion (BHI) broth at 28 °C for 7 days under shaking conditions at 180 rpm. After day 7, the culture medium was fractionated with ethyl acetate (v/v=1:1). The ethyl acetate fraction was separated from the mixture, evaporated by a vacuum evaporator, and prepared aseptically and kept in a refrigerator (-80 °C). The ethyl acetate fraction from the culture medium of *B. amyloliquefaci*ens AK-0 (EA-AK0) was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration did not exceed 0.1% (v/v).

4.4. Cell proliferation assay

Cell proliferation assay was performed using MTT solution. Briefly, cells were plated onto 96-well culture plates and treated with the varying concentrations of EA-AK0 for 24 h. Then, the cells were incubated with 50 μl of MTT solution (1 mg/ml) for an additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 nm.

4.5. Western blot analysis

After each treatment, cells were washed with 1×phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 4 °C. Then, cell lysates were centrifuged at 15,000 rpm for 10 min at 4 °C. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The equal proteins were separated on SDS-PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1h at room temperature and then incubated with specific primary antibodies in 5% non-fat dry milk at 4 °C overnight. After three washes with TBS-T, the membranes were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences, Piscataway, NJ, USA) and visualized on a Polaroid film.

4.6. Reverse transcription-polymerase chain reaction (RT-PCR)

After EA-AKO treatment, total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 μg) was reverse-transcribed using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was carried out using PCR Master Mix Kit (Promega, Madison, WI, USA) with human primers for cyclin D1 and GAPDH as followed : cyclin D1: forward 5'-aactacctggaccgcttcct-3' and reverse 5'-ccacttgagcttgttcacca-3', GAPDH: forward 5'-acccagaagactgtggatgg-3' and reverse 5'-ttctagacggcaggtcaggt-3'.

4.7. Statistical analysis

All the data are shown as mean±SEM (standard error of mean). Statistical analysis was performed with the Student unpaired t-test. Results were considered statistically significant at $^*P < 0.05$.

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Conflicts of interest: Non declared

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