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Restoration of cyclin D2 has an inhibitory potential on the proliferation of LNCaP cells

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ABSTRACT

Despite well known oncogenic function of G1–S cell-cycle progression, *cyclin D2* (*CCND2*) is often silenced epigenetically in prostate cancers. Here we show that CCND2 has an inhibitory potential on the proliferation of androgen receptor (AR)-dependent prostate cancer LNCaP cells. Forced expression of CCND2 suppressed the proliferative ability and induced cell death in LNCaP cells in a cdk-independent manner. Knocking down CCND2 restored the proliferation of LNCaP subclones with relatively high CCND2 expression and low proliferative profiles. Immunoprecipitation using deletion mutants of CCND2 indicated that a central domain of CCND2 is required for binding to AR. A deletion mutant lacking the central domain failed to hinder LNCaP cells. Collectively, our results indicated that CCND2 inhibits cell proliferation of AR-dependent prostate cancer through the interaction with AR. Our study suggests that restoration of CCND2 expression potentially prevents the carcinogenesis of prostate cancer, which is mostly AR-dependent in the initial settings.

Introduction

Prostate cancer is the most commonly diagnosed non-cutaneous malignancy among men in industrialized countries and the second leading cause of cancer related death in the United States [1,2]. Gene methylation and subsequent silencing of tumor suppressor genes are considered to play an important role in the molecular mechanisms for carcinogenesis of prostate cancer [3]. It is reported that certain epigenetic modulators including histone deacetylases (HDAC) and DNA methyltransferases (DNMT) are aberrantly activated in the early step of prostate carcinogenesis [4]. The absence of tumor suppressor genes including *GSTP1* [5], *PTEN* [6], *CDKN2* [7] and *E-cadherin* [8] which are frequently down-regulated in prostate cancer and adjacent precursor lesions, can be attributed at least partially to hypermethylation of the CpG island sequences encompassing the regulatory region resulting in prevention of the transcription of the genes, as well as allelic loss of the genes.

Cyclin D2 (CCND2) gene, a member of type-D cyclin family located at 12p, consists of five exons, holds CpG islands around exon 1, and is one of the common genes which are silenced by epigenetic modulation in prostate cancer [9,10]. Padar and colleagues examined 101 prostate cancer and 32 nonmalignant prostate tissue samples by methylation-specific PCR and reported that the frequency of methylation was significantly higher (32%) in prostate cancers than in nonmalignant prostate tissues [9]. Indeed, expression of CCND2 is progressively suppressed according to development and progression of prostate cancer opposite to that of CCND1 [11].

Well known function of D-type cyclins includes binding to and activating cyclin-dependent kinase (cdk) 4/6 [12]. Activated CCND2-cdk4/6 complex phosphorylates *retinoblastoma* (Rb) protein resulting in releasing and up-regulating transcription factor E2F, which targets following cell-cycle accelerators including cdk2, cdc25, cyclin A and cyclin E. This series of action is essential for cell-cycle progression from G1- to S-phase, and therefore, CCND2 is a potentially oncogenic protein.

There is an unexplained gap that *CCND2* is an oncogene but is frequently silenced in prostate cancer. Prostatic tissue developing adenocarcinoma is frequently in hypermethylation status and DNA methylation seems to be accumulated as it develops adenocarcinoma [13,14]. Therefore, CCND2 gene silencing can be just a result of hypermethylated tendency and has no significant role driving prostate carcinogenesis. On the other hand, it can be oncogenic and drive prostatic epithelial cells for carcinogenesis, if CCND2 has any anti-oncogenic function. Here we address this issue and demonstrate that CCND2 has a novel inhibitory function on

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androgen-dependent proliferation through biding to the androgen receptor.

Materials and methods

Antibody and reagents. Anti-HA antibody was purchased from Covance (Berkeley, CA), and anti-CCND2 (M-20, sc-593), anti-androgen receptor (C-19, sc-815), anti-PSA (C-19, sc-7638) and anti-β-tubulin (D-10, sc-5274) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin (ab6276) was purchased from Abcam (Cambridge, MA). Anti-phospho-pRb (Ser 780, #9307) antibody was obtained from Cell Signaling Technology (Beverly, MA). R1881 (methyltrienolone) was purchased from DuPont Merck Pharmaceutical (Boston, MA), 5-Aza-2′-deoxycytidine (5-aza-DC) and trichostatin A (TCA), from Sigma, and BD Matrigel, from BD Bioscience (Bedford, MA).

Cell culture, cell counting, colony-forming assay, apoptosis assay. Methods for cell culture are described elsewhere [15]. For cell count assay, cells were inoculated to 6- or 10-cm dishes at 5.0×10^5 cells/plate. After 24 h incubation for adhesion, the number of cells was counted for baseline (day 0) and on the days 2, 4, 6 and 8. Cells were collected by trypsinization and counted using hemocytometer. In colony-forming assays, cells (1×10^5) were seeded on 6-cm dishes and cultured in puromycin-containing (2 μg/ML) medium for 2 weeks. The cells were washed with phosphate-buffer saline (PBS), fixed with neutral-buffered formaldehyde (10%), stained with 0.1% of crystal violet-H_2O. Apoptosis assays were done as described previously [16].

Expression construct, transfection and retrovirus infection. Expression constructs including mammalian expression vectors and retrovirus vectors are described in Supplementary Information. Transfection of the plasmids was performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. To establish stable expression subclones from PC3 cells, cells were cultured in RPMI supplemented with 10% FBS containing 1 mg/ML G418 three days after transfection. Colonies were picked up and expression of target gene was confirmed by Western blotting. Retrovirus vectors were transfected along with VSVG (kind gifts of Dr. T. Era, Kobe, Japan) and gag/pol plasmids to obtain the virus using Lipofectamine 2000 reagents (Invitrogen) according to manufacturer's instruction. More than 70% of infection efficiency was confirmed by counting GFP-positive cells with fluorescence microscopy three days after retrovirus infection. Cells were cultured in puromycin-containing (2 μg/ML) medium for additional three days, and then subjected to cell count assay, colony-forming assay, reverse transcriptase PCR (RT-PCR) or SDS–PAGE.

In vivo xenograft model for tumorigenesis. PC3 cells were collected by trypsinization and 5×10^6 cells were suspended in 100 μL RPMI/BD Matrigel (1:1) and injected subcutaneously to the right flank of BALB/c AnNCrj nude mice. Tumor volumes were

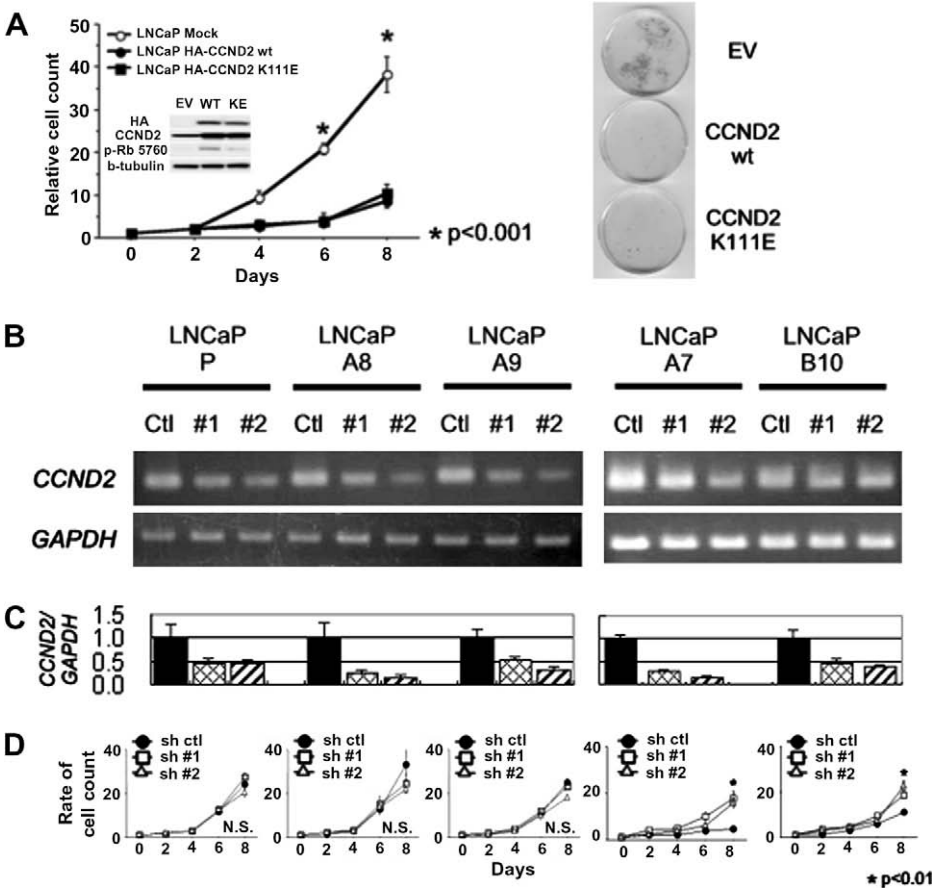

Fig. 1. Overexpression of CCND2 reduces the proliferation of LNCaP cells in culture (A). LNCaP cells were infected by retrovirus for empty vector (EV), HA-CCND2 wt (WT) and K111E mutant (KE) and protein expression was confirmed by Western blotting. For cell count assay, cells were inoculated to 6- or 10-cm dishes at 5.0×10^5 cells/plate and counted on the days 0, 2, 4, 6 and 8. Colony-forming ability of LNCaP cells expressing wild-type (CCND2 wt) or K111E mutant (CCND2 K111E) of CCND2 as well as control cells (EV) was also examined (right). Knocking down of CCND2 in LNCaP subclones with relatively higher expression of CCND2 potentiates their proliferation in culture (B, C and D). Clones A7 and B10 were selected as cells hyperexpressing *CCND2* (Supplementary Fig. 2), while parental LNCaP (LNCaP P) and subclones A8 and A9 served as controls. Results of semiquantitative (B) and quantitative (C) RT-PCR showed successful suppression of *CCND2* expressions by two short-hairpin RNAs. In cell count assays, the cell proliferation rates were significantly increased in clones A7 and B10 infected by shRNA expression retrovirus (D).

measured weekly with a caliper using the formula, $a \times b^2 \times 0.52$, where a is the largest diameter and b is the largest diameter perpendicular to a. All experiments involving laboratory animals were done in accordance with the Guideline for Animal Experiments of Kyoto University and approved by Animal Research Committee at Kyoto University Graduate School of Medicine.

Cell lysis, immunoprecipitation and immunoblotting. Cell lysis, immunoprecipitation and SDS–PAGE followed by immunoblotting were done as previously reported [15].

Semiquantitative and quantitative reverse transcription PCR. Semiquantitative and quantitative reverse transcription PCR were performed as described elsewhere [17]. The sequences of primers used for PCR analyses are described in Supplementary Information.

Statistical analysis. Data are presented as means ± SD of at least triplicate experiments. Statistical differences between two groups of data were analyzed using the Student's *t*-test. Statistical significance was applied to p values of <0.05.

Results and discussion

Forced expression of CCND2 inhibits the proliferation of LNCaP cells

In human prostate cancer LNCaP, PC3 and DU145 cells, expression of *CCND2* mRNA was restored by the treatment with epigenetic modulators deoxycytidine analog 5-aza-2′-deoxycitidine (5-aza-dC) or/and trichostatin A (Supplementary Fig. 1), indicating that CCND2 gene is epigenetically silenced in these cell lines consistent with previous reports [10].

LNCaP cells infected with retrovirus for wild-type and the KE mutant lacking the ability to activate cyclin-dependent kinases [18] of CCND2 showed lower proliferation and colony-forming abilities in comparison to their control counterparts (Fig. 1A), indicating that forced expression of CCND2 has an inhibitory effect on the growth of AR-dependent LNCaP cells in a cdk-independent manner.

Suppression of CCND2 expression restores the proliferation of LNCaP clones with relatively high CCND2 expression and lower proliferative profiles

Next, we examined whether endogenous expression of CCND2 has an inhibitory effect on prostate cancer cell proliferation similar to exogenously expressed *CCND2*. Since there was no available cell line with higher expression of endogenous *CCND2* (Supplementary Fig. 1), we isolated sublines of LNCaP and examined *CCND2* expression and proliferative ability according to the method previously reported by Iguchi et al. [19]. There was a certain degree of variety in the expression level of *CCND2* and proliferation rate among isolated subclones of LNCaP (Supplementary Fig. 2A). We selected two clones (A7 and B10) which expressed *CCND2* at relatively high levels (Supplementary Fig. 2B) and slow in their proliferation (Supplementary Fig. 2C). In addition to parental LNCaP cells, clones A8 an A9 were selected as controls with almost identical levels of *CCND2* expression and proliferation index to those of parental cells.

Using these cells, we examined whether knocking down of *CCND2* by shRNA expression affect the proliferation of LNCaP cells.

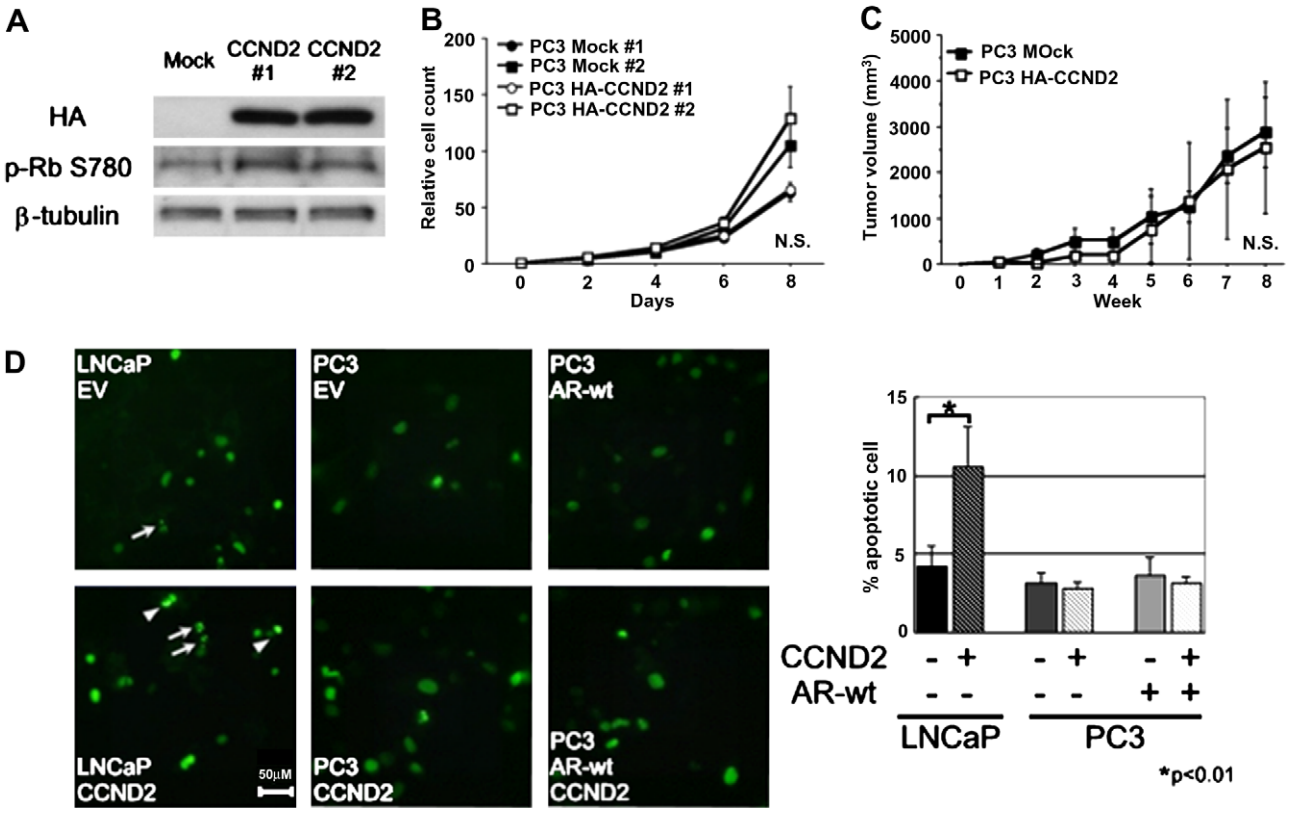

Fig. 2. Overexpression of CCND2 does not affect the proliferation and survival in AR-independent PC3 cells. Protein expression of HA-tagged CCND2 in PC3 Mock and HA-CCND2 cells was examined by SDS–PAGE followed by immunoblotting (A). *In vitro* proliferation was examined by cell count assays using two clones of PC3 Mock and two clones of PC3 HA-CCND2 (B). Xenograft formation was examined by subcutaneous injection of PC3 Mock and HA-CCND2 cells (C). PC3 Mock #2 and HA-CCND2 #2 cells were subcutaneously injected to the right flank of 11 and 10 mice, respectively. Influence of overexpression of CCND2 on apoptosis was examined by apoptosis assay (D). LNCaP cells were transfected with empty vector (EV) or pcDNA3.1(−) HA-CCND2 wt (CCND2). PC3 cells were transfected with empty vector (EV) or pcDNA3.1(−) HA-CCND2 (CCND2) alone, or along with equal amount of pCMV hAR-wt (total 7.2 μg). Nuclear fragmentation (arrows) and chromatin condensation (arrowheads) were scored 48 h later using fluorescent microscopy. The bar indicates 50 μm. Column chart represents the results of three independent experiments in which >100 fluorescent cells were scored.

Two distinct sequences of shRNA for *CCND2* were introduced with retrovirus vector and they showed successful suppression of *CCND2* expression (Fig. 1B and C). In this condition, proliferation rates of LNCaP A7 and B10 were increased compared to those infected with control vector (Fig. 1D). This indicates that, at least in these two subclones isolated from LNCaP cells, endogenous *CCND2* played an inhibitory role and was responsible for their lower proliferative abilities at least partially. On the other hand, knocking down *CCND2* did not affect control cells including parental cells and A8 and A9 clones (Fig. 1D), suggesting that further suppression of *CCND2* no longer affects prostate cancer cell proliferation in which CCND2 is already epigenetically silenced.

Forced expression of CCND2 little affects the proliferation of AR-independent prostate cancer PC3 cells

Next we examined whether forced expression of *CCND2* affects another prostate cancer cell line, PC3. We established two monoclones engineered to express *CCND2* (PC3 HA-CCND2, Fig. 2A) that showed no differences in the growth *in vitro* (Fig. 2B) and *in vivo* (Fig. 2C) compared to the control cells.

Furthermore, we examined whether CCND2 affects cell death of these prostate cancer cell lines by apoptosis assay using plasmid encoding histone-EGFP fusion protein [16]. In PC3 cells, proportion of dead cells was not significantly different irrespective of the presence or absence of AR or CCND2 (Fig. 2D), whereas significantly more nuclei of the LNCaP cells transfected with CCND2 displayed the hallmarks of apoptotic cell death (10.5 ± 2.6 vs $4.2 \pm 1.3\%$, $p < 0.01$).

Collectively, CCND2 does not appear to have significant inhibitory effect on AR-independent PC3 cells irrespective of the presence of AR suggesting that the inhibitory effect of CCND2 is limited to AR-dependent prostate cancer cells. Therefore, silencing CCND2 gene may contribute to prostate cancer progression at its very early stage since most prostate cancer is initially androgen-dependent. It is supported by observations that prostate cancer cells contain changes in DNA methylation at the time of diagnosis [3].

CCND2 interacts with AR but it scarcely affects the expression of AR-responsive gene products

Since there has been a report that cyclins D1 and D3 bind to AR and inhibit its transcriptional activity [20], we examined the interaction between CCND2 and AR. HEK293 cells were transfected with expression vector for HA-tagged CCND1 or CCND2 along with expression vector coding human wild-type AR, and then cell lysates were immunoprecipitated using antibody to HA or AR. HA-CCND2 and AR were co-immunoprecipitated with AR and HA-CCND2, respectively, to a similar extent to CCND1-transfected cells (Fig. 3A). Significantly less AR was co-immunoprecipitated with a deletion mutant of CCND2 (CCND2 ΔXMN in Fig. 3B), correspondent to previously reported CCND1 mutant lacking a central domain that is required and sufficient for the interaction with AR [21], indicating that the central domain of CCND2 is responsible for the interaction with AR as previously reported for CCND1 (Fig. 3C).

Although there have been some reports that D1- and D3-cyclins modulate AR transactivity through direct interaction [20] or downstream target molecules including E2F1 [22] or cdk6 [23], there have been few reports on CCND2 with regard to modulation of AR. Unexpectedly, reporter activity of endogenous AR in LNCaP cells and exogenous wild-type AR in PC3 cells was not significantly altered by transient transfection of wild-type, KE and ΔXMN mutants of CCND2 (Fig. 3D). Additionally, expression levels of PSA mRNA and protein were not significantly different among LNCaP cells expressing these CCND2 mutants (Fig. 3E). Moreover, there were no significant differences in the mRNA expression of AR-responsive genes including PSA, FKBP5, Nkx3.1, TMPRSS2, and RHOU between LNCaP cells overexpressing CCND1 and CCND2 (data not shown).

It is unclear why the consequent effects of binding on the AR transactivity are different between cyclins D1 and D2 despite similar molecular basis of physical interaction through the central domains which harbor significant identity and similarity. There can be some differences in the modulation of ligand-dependent conformational changes, modification of chromatin binding activity, or

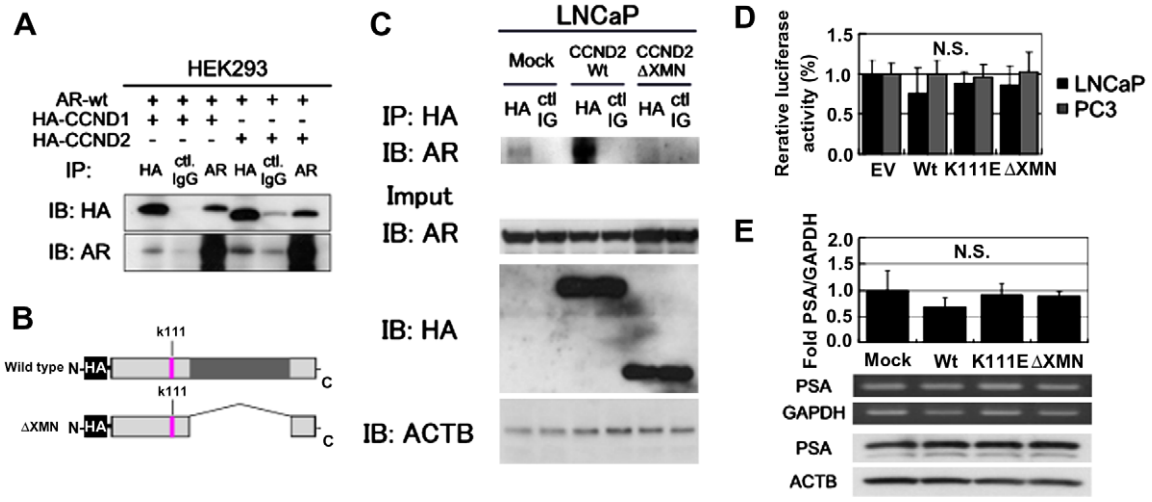

Fig. 3. Overexpressed CCND2 binds to the AR but does not affect the AR transcriptional activity significantly. HEK293 cells were transfected with pCMV AR-wt (AR-wt) along with pcDNA3.1(−) HA-CCND1 or HA-CCND2 (A). Cell lysates containing 1 mg of total protein were immunoprecipitated using control IgG or antibody to HA or AR. Schematic models representing wild-type, HA-tagged CCND2 (HA-CCND2) and HA-CCND2 ΔXMN utilized for binding analysis by immunoprecipitation (B). Shaded part represents the central domain (XMN, aa 136–252) of CCND2. Co-immunoprecipitation of endogenous AR of LNCaP cells was significantly reduced with CCND2 ΔXMN mutant (C). Effect of CCND2 mutants on AR transactivity was examined by dual-luciferase reporter assay (D). LNCaP and PC3 cells were transfected with 500 ng of empty vector (EV) or pcDNA3.1(−) HA-CCND2 wt, K111E or ΔXMN. Expression of *PSA* was examined by quantitative RT-PCR and Western blotting (E). LNCaP cells were infected with retrovirus for control (Mock) or CCND2 wt, K111E or ΔXMN, and then RNA and protein were extracted. In column chart, results are shown as the mean and S.D. values of *PSA* mRNA relative to *GAPDH*.

recruitment of other co-factors interacting with AR, which can cause distinct influences on the transcriptional function of AR. This discrepancy can be possibly associated with inconsistent observations on changes in expression between *CCND1* and *CCND2* through prostate carcinogenesis; expression of *CCND1* is reported to increase according to prostate carcinogenesis and progression opposite to that of *CCND2* [11].

Effect of CCND2 mutants on the proliferation and survival of LNCaP cells

The proliferative ability and colony-forming ability of LNCaP cells infected by retrovirus for CCND2 ΔXMN were not significantly different from those of the control cells, whereas the cells infected by retrovirus for wild-type or the K111E mutant of CCND2 showed impaired proliferative ability (Fig. 4A and B). Similarly, rate of cells displaying the hallmarks of apoptotic cell death was not significantly increased by transfection with plasmid encoding CCND2 ΔXMN, whereas the rates of dead cells significantly increased by wild-type or K111E mutant of CCND2 (Fig. 4C). These results indicate that the inhibitory effects of CCND2 on the proliferation and survival of LNCaP cells are independent of its cdk-activating ability but dependent on interaction with AR. So, it is unlikely that forced, isolated overexpression of downstream oncogenes without upstream mitogenic signals causes apoptosis as proposed by Blagosklonny [24]. It is possible that some specific genes induced or suppressed by CCND2–AR complex may cause activation of upstream mitogenic signals and cell death in cooperation with activation of downstream proliferation signals.

Collectively, overexpression of CCND2 results in cell death through interaction with AR although precise molecular mechanisms should be further elucidated in future studies possibly by looking into comparative gene expression profiles between prostate cancer cells expressing CCND1 and CCND2. And it can provide us an important implication for future establishment of a novel strategy for the prevention of prostate cancer.

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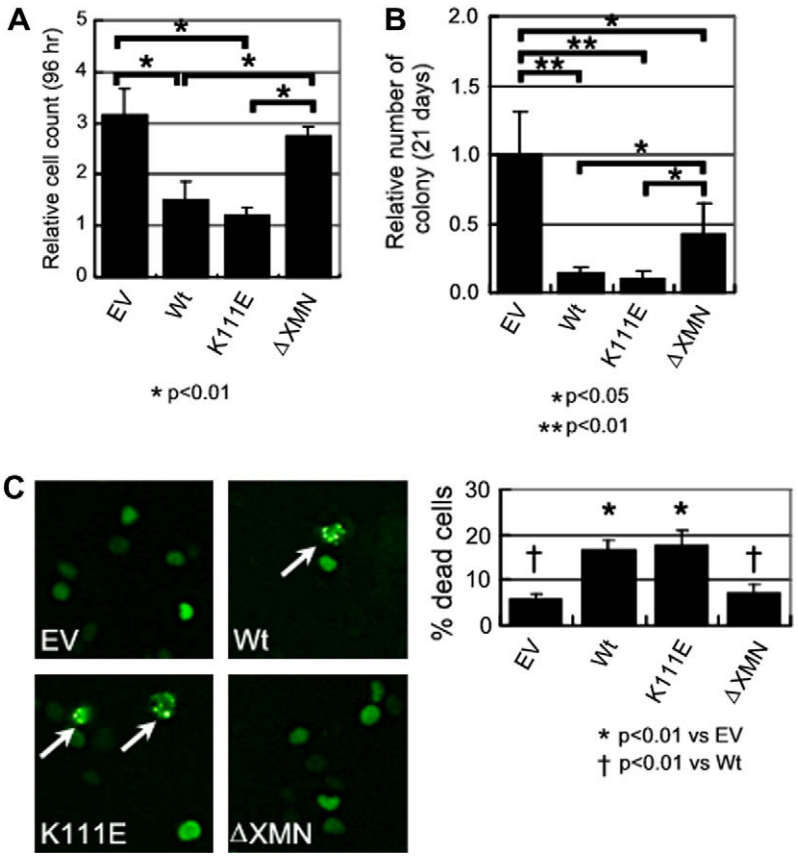

Fig. 4. Overexpressed CCND2 inhibits the cell proliferation and induces cell death in LNCaP cells independently of cdk-activating function but dependently on binding to AR. LNCaP cells were infected with retrovirus for control (EV) or HA-CCND2 wt, K111E or ΔXMN and cells were subjected to cell count assay (A). The column chart indicates relative cell count at 96 h (day 4) to baseline (day 0). LNCaP cells were transfected with empty vector (EV) or pcDNA3.1(−) HA-CCND2 wt, K111E or ΔXMN and subjected to colony-forming assay (B). Relative numbers of colonies to cells transfected with EV are indicated in the column chart. Apoptosis assay in LNCaP cells was conducted using empty vector (EV) or pcDNA3.1(−) HA-CCND2 wt, K111E or ΔXMN (C). Arrows indicate nuclear fragmentation. The column chart indicates the proportion of dead cells in fluorescent cells. $^{*}p < 0.01$ in comparison to EV. $^{\dagger}p < 0.01$ in comparison to HA-CCND2 wt.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.06.146.

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