Nuclear co-expression of p21 and p27 induced effective cell-cycle arrest in T24 cells treated with BCG

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Received: 27 October 2017 / Accepted: 8 November 2018 / Published online: 2 January 2019
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Abstract A proposed mechanism underlying the effect of bacillus Calmette–Guérin (BCG) treatment for bladder cancer cells is as follows: BCG-induced crosslinking of cell-surface receptors results in the activation of signaling cascades, including cell-cycle regulators. However, the clinical significance of cell-cycle regulators such as p21 and p27 is controversial. Here we investigated the relationship between BCG exposure and p21 and p27. We used confocal laser microscopy to examine the expression levels of pKi67, p21 and p27 in T24 cells (derived from human urothelial carcinoma) exposed six times to BCG. We performed dual immunofluorescence staining methods for p21 and p27 and observed the localization of nuclear and cytoplasmic expressions. We investigated the priority of p27 over p21 regarding nuclear expression by using p27 Stealth RNAiTM (p27-

siRNA). With 2-h BCG exposure, the nuclear-expression level of p21 and p27 was highest, while pKi67 was lowest. The percentage of double nuclear-expression of p21 and p27 in BCG cells was significantly higher than that in control cells during the 1st to 6th exposure ($P < 0.05$), and the expression of pKi67 showed the opposite of this pattern. Approximately 10% of the nuclear p21 was independent of p27, whereas the cytoplasmic p21 was dependent on p27. Our results suggested that the nuclear co-expression of p21 and p27 caused effective cell-cycle arrest, and thus the evaluation of the nuclear co-expression of p21 and p27 might help determine the effectiveness of BCG treatment.

Keywords BCG · Cell-cycle arrest · T24 cells · Nuclear co-expression · p27-siRNA

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Introduction

Intravesical bacillus Calmette–Guérin (BCG; a live attenuated strain of *Mycobacterium bovis*) is the standard adjuvant therapy after transurethral resection of high-grade non-muscle-invasive bladder cancer (Witjes 2006; Hall et al. 2007; Sato et al. 2011; Babjuk et al. 2015). A direct effect of BCG on the biology of urothelial carcinoma has been suggested; a BCG-induced crosslinking of cell surface receptors results in the activation of signaling cascades, which in turn

transactivate the expression of multiple genes. The identification of the BCG response markers that are clinically significant, disease-relevant, reproducible and early accessible in clinical settings is of key importance in the early treatment of non-muscle-invasive bladder cancer (Kiselyov et al. 2015). It was reported that the Cip/Kip family members p21 (Harper et al. 1993) and p27 (Toyoshima and Hunter. 1994) negatively regulate cyclin-dependent kinase (CDK) activity in cells, and many clinical reports have indicated that the CDK inhibitors p21^{Waf1/Cip1} (p21) and p27^{Kip1} (p27) are among the genes whose expression is increased in responses to BCG therapy (Zlotta et al. 1999; Cormio et al. 2009; Kim et al. 2010; Van Rhijn et al. 2012).

However, the prognostic significance of p21 and p27 is controversial. Several groups have reported findings about relationships between cell-cycle regulators other than p27 and responses to BCG exposure: p21 (Zhang et al. 2007; See et al. 2010), telomerase activity (Saitoh et al. 2002), integrin (Chen et al. 2005) and toll-like receptor 7 (Yu et al. 2015) but those studies used BCG conditions (a range of BCG concentrations and exposure durations, e.g., 6 h to 6 days) that are quite different from the conditions used in clinical treatment (40–80 mg/mL for 2 h/week, up to 6–8 times). The effects of p21 and p27 on cells exposed to BCG *in vitro* are not known, and to the best of our knowledge the significance of the co-expression of p21 and p27 in cells exposed to BCG has not been investigated. Here we investigated the optimum experimental conditions for investigating the relationship between BCG exposure and p21 and p27 and evaluated the expressions of p21 and p27, focusing on their effects on the cell-cycle arrest response to BCG treatment.

Materials and methods

Cell lines

All experiments were carried out with T24 cells derived from human urothelial carcinoma (courtesy of the Division of Urology, Kyushu University, Fukuoka, Japan). We cultured T24 cells in Dulbecco's modified Eagle's medium (DMEM) (05796, Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (S1820, BioWest, Nuaillé,

France) and 1% PSA (penicillin, streptomycin and amphotericin B) (complete media) in 35-mm culture dishes (35-0001, Becton–Dickinson, Lincoln Park, NJ, USA) and chamber slides (Falcon 354114, Becton–Dickinson). We used the same culture conditions (37 °C, 5% CO₂) for all of the experiments.

Bacillus Calmette–Guérin (BCG)

The Tokyo 172 strain BCG (Immunobladder 40 mg/vial; Japan BCG Laboratory, Tokyo, Japan) was used.

Optimum duration of BCG exposure

To investigate the optimum duration of BCG exposure, we grew T24 cells (5×10^4 cells/mL at the start) in chamber slides for 4 days. We then exposed the cells to BCG (40 mg/ml) for 1, 2 and 6 h. Based on the results, we identified the optimum duration of BCG exposure with the expression level of p21, p27 and pKi67.

Fluorescence immunocytochemistry

After harvest, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde (Wako, Osaka, Japan) for 15 min at -4 °C.

pKi67 immunofluorescence staining

We used Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 (1:100; #M7240, Dako, Glostrup, Denmark) as an anti-pKi67 antibody and Polyclonal Rabbit Anti-Mouse IgG/FITC Rabbit F(ab')₂ (1:10; #F0313, Dako) as a labeled antibody of pKi67. After pKi67 staining, propidium iodide (PI) (1:250; #P4864, Sigma Aldrich) was used for counterstaining. For the evaluation of pKi67, we observed nuclear-positive staining.

p21/p27 dual immunofluorescence staining

After fixation, on day 1, we performed immunofluorescence staining for cells with p27^{Kip1} (SX53G8.5) Mouse mAb (1:1600; #3698S, Cell Signaling Technology, Beverly, MA, USA) as an anti-p27 antibody, overnight. On day 2, we conducted immunofluorescence staining for cells with p21^{Waf1/Cip1} (12D1) Rabbit mAb Alexa Fluor 555 Conjugate (1:50;

#8493S, Cell Signaling Technology) as an anti-p21 antibody, overnight. On day 3, we performed immunofluorescence staining for cells by using polyclonal rabbit anti-mouse IgG/FITC Rabbit F(ab')₂ (1:10; #F0313, Dako) as a labeled antibody of p27 antibody for 1 h. After p21/p27 dual staining, 4',6-diamidino-2-phenylindole (DAPI) (1:1000; #0 236 276 001, Roche, Mannheim, Germany) was used for counterstaining.

For the evaluation of the cellular localization of p21 and p27, we discriminated the nuclear expression (N) from the cytoplasmic expression (C), and we observed the following four types: p21N(–)C(+)/p27N(–)C(+), p21N(+)C(+)/p27N(–)C(+), p21N(–)C(+)/p27N(+)C(+) and p21N(+)C(+)/p27N(+)C(+).

After the immunofluorescence staining, we observed the cells by confocal laser microscopy (LSM) (LSM700, Carl Zeiss MicroImaging, Jena, Germany) and calculated the expression levels with the positive staining percentage of p21/p27 and pKi67 using a minimum of 200 cells for each sample.

Six-times BCG exposure

First step: T24 cells seeded at 5×10^4 cells/mL were cultured on chamber slides and culture dishes for 4 days. Second step: The cells on the chamber slides and culture dishes were then exposed to BCG (40 mg/ml) for 2 h. Third step: BCG was removed from the cells by washing the cells with PBS, and the cells were then cultured for 4 days in DMEM. The first, second and third steps were repeated for a total of six times BCG exposure.

p27-siRNA

We performed in vitro transfection on day 2 as follows. p27 Stealth RNAiTM (p27-siRNA) was synthesized by Invitrogen (Tokyo, Japan). Standard siRNAs have been reported to exhibit off-target effects (Kawasaki et al. 2005). However, this stealth RNAi siRNA was modified with proprietary chemicals, and was different from standard siRNAs. By using this stealth RNAi siRNA, we were able to get results that essentially eliminated our concerns regarding off-target effects (<https://www.thermofisher.com>). The target sequence of the p27-siRNA was: UUG CAGGUCGCUUCCUUAUCCUGC. Negative Control Medium GC Duplex (12935112, Invitrogen)

(Negative Control) was used as a control. Transfection was performed with Lipofectamine[®] 2000 transfection reagent (#1166-019, Invitrogen). Briefly, Lipofectamine 2000 (3.6 µl/well) and p27-siRNA (2.16 µl/well) or Negative Control (2.16 µl/well) were incubated with DMEM + 10%FBS [PSA(–)medium] (360 µl/well) at 25 °C for 15 min. The p27-siRNA or Negative Control lipid mixture was added to the cultures. p27-siRNA was removed by changing the medium 6 h after the transfection.

After transfection, PSA(–)medium was added and the cells were cultured for 24 h before BCG exposure. We determined the transfection efficiency by using BLOCK-iTTM Alexa Fluor[®] Red Fluorescent Control (#14750100, Thermo Fisher Scientific), and we calculated the ratio of transfection efficiency. The viability of the T24 cells after the transfection was determined by using toluidine blue.

For the evaluation of p27-siRNA, we observed the localization of expression; i.e., nuclear and/or cytoplasmic expression. We discriminated the expression patterns as follows. For nuclear expression: p21N(+)/p27N(+), p21N(+)/p27N(–), p21N(–)/p27N(+) and p21N(–)/p27N(–). For cytoplasmic expression: p21C(+)/p27C(+), p21C(+)/p27C(–), p21C(–)/p27C(+) and p21C(–)/p27C(–).

Statistical analysis

When appropriate, the data are expressed as the mean $\pm$ SD of three independent experiments. A minimum of 200 cells was counted for each sample. All statistical analyses, including a one-way analysis of variance (ANOVA) were performed using JMP (ver. 8.02) software. *P* values < 0.05 were considered significant.

Results

We investigated the relationship between BCG exposure and p21 and p27. We used confocal laser microscopy to examine the expression levels of pKi67, p21 and p27 in T24 cells exposed six times to BCG. To investigate the priority of p27 over p21, we performed the dual immunofluorescence staining methods and the p27 Stealth RNAiTM (p27-siRNA).

BCG exposure for 2 h caused effective cell-cycle arrest

We first performed experiments to determine the optimum duration of BCG exposure for T24 cells, by using the expression levels of pKi67, p21 and p27. The positive stainings of pKi67, p21 and p27 are shown in Fig. 1a. The cytoplasmic expressions of both p21 and p27 were higher than the corresponding nuclear expressions, and the cytoplasmic expression of p27 (p27C) was higher than that of p21 (p21C) (Fig. 1b). With the 1 h BCG exposure, the expression levels of pKi67, p21 (p21N) and p27 (p27N) were $94.0 \pm 0.7\%$, $27.0 \pm 1.9\%$ and $4.0 \pm 1.1\%$, respectively. With the 2 h BCG exposure, the expression levels of pKi67, p21 (p21N) and p27 (p27N) were $82.3 \pm 0.2\%$, $48.5 \pm 10.9\%$ and $25.5 \pm 8.8\%$,

respectively. With the 6 h BCG exposure, the expression levels of pKi67, p21 (p21N) and p27 (p27N) were $82.7 \pm 3.3\%$, $42.0 \pm 7.7\%$ and $2.0 \pm 0.1\%$, respectively.

Without BCG exposure (0 h), the expression levels of pKi67, p21 (p21N) and p27 (p27N) were $92 \pm 0.8\%$, $40.5 \pm 3.9\%$ and $10.0 \pm 2.6\%$, respectively. The expression levels of p21 (p21N) and p27 (p27N) with the 2-h BCG exposure were significantly higher than those in the other conditions. However, the expression level of pKi67 with the 2-h BCG exposure was significantly lower than the corresponding expression levels in the other conditions. We therefore decided that the optimum duration of BCG exposure was 2 h. We then incubated T24 cells with BCG for 2 h in the subsequent experiments.

Fig. 1 pKi67, p21 and p27 expression in T24 cells. **a** Immunostaining status observed by confocal laser microscope. pKi67 was localized in only the cells' nuclei, whereas p21 and p27 were observed in both nuclei and cytoplasm. **b** The pKi67, p21 and p27 expressions changed depending on the duration of BCG

exposure. With the 2-h BCG exposure, the nuclear-positive percentage of pKi67 was the lowest, and those of p21 and p27 were the highest. A min. of 200 cells was counted for each sample. Each result is the mean $\pm$ SD of three independent experiments. N nuclear expression, C cytoplasmic expression

The nuclear co-expression of p21 and p27 caused effective cell-cycle arrest

We first investigated the proliferation percentage of cells using pKi67, and we observed that the expression of pKi67 in BCG-treated T24 cells (hereafter, 'BCG cells') was approx. 20% lower than the expression in untreated cells (control cells) during the first to sixth exposures (Fig. 2a). This indicated that BCG exposure might induce cell-cycle arrest immediately. During the second to fourth BCG exposures, the nuclear-expression level of pKi67 was higher than that during the first exposure in both the control cells and BCG cells. This phenomenon seemed to be related to the biology of T24 cells rather than the BCG exposures.

We then used the dual staining method to determine the expressions of p21 and p27 in the cell nuclei (N) and cytoplasm (C). We did not observe nuclear expression without cytoplasmic expression of both p21 and p27 (data not shown). We divided the expression patterns into the following four patterns. Double-negative: p21N(–)C(+)/p27N(–)C(+). Single-p21: p21N(+)C(+)/p27N(–)C(+). Single-p27: p21N(–)C(+)/p27N(+)C(+). Double-positive: p21N(+)C(+)/p27N(+)C(+).

The percentage of the double-negative patterns in the BCG cells was lower than that in the control cells during the first to sixth exposures (Fig. 2b). The percentage of the single-p21 pattern in both the BCG cells and control cells was very low, and there was no difference in the single-p21 expression between these two cell groups (Fig. 2c). The percentage of the single-p27 pattern in the BCG cells was lower than that in the control cells, and the double-negative pattern was similar (Fig. 2b, d). The percentage of the double-positive pattern in BCG cells was significantly higher than that in the control cells during the first to sixth exposures, whereas this double-positive expression of pKi67 was the reverse; i.e., this pattern was higher in the control cells than in the BCG cells (Fig. 2a, e).

These results revealed two interesting phenomena. (1) The nuclear co-expression of p21 and p27 could be important for effective cell-cycle arrest. (2) Few of the cell nuclei expressed p21 without p27. We thus suggest that regarding nuclear expression, the expression of p27 might be a prerequisite to that of p21 (Fig. 2c).

Some of the nuclear expression of p21 was irrespective of p27 expression

To investigate the priority of p27 and p21 in nuclear expression, we used p27-siRNA (siRNA). The effectiveness percentage of siRNA injection and the viability of T24 cells after injection were $95.8 \pm 2.6\%$ and $90.1 \pm 2.6\%$, respectively (data not shown). The nuclear expression of p27 would thus be approx. 5% in the conditions using siRNA (Fig. 3a, c). The nuclear double-positive percentages were as follows: siRNA(–)/BCG(–), $18.2 \pm 3.0\%$; siRNA(–)/BCG(+), $32.2 \pm 8.0\%$; siRNA(+)/BCG(–), $4.2 \pm 2.5\%$; and siRNA(+)/BCG(+), $6.7 \pm 5.2\%$ (Fig. 3a). The nuclear single-p21 percentages were siRNA(–)/BCG(–), $10.8 \pm 3.7\%$; siRNA(–)/BCG(+), $13.2 \pm 2.5\%$; siRNA(+)/BCG(–), $10.3 \pm 4.3\%$; and siRNA(+)/BCG(+), $14.7 \pm 11.7\%$ (Fig. 3b).

The nuclear single-p27 percentages were as follows: siRNA(–)/BCG(–), $15.3 \pm 1.3\%$; siRNA(–)/BCG(+), $22.0 \pm 8.3\%$; siRNA(+)/BCG(–), $5.3 \pm 0.8\%$; and siRNA(+)/BCG(+), $5.2 \pm 5.1\%$ (Fig. 3c). The nuclear double-negative percentages were: siRNA(–)/BCG(–), $55.7 \pm 5.1\%$; siRNA(–)/BCG(+), $32.7 \pm 3.5\%$; siRNA(+)/BCG(–), $80.2 \pm 7.0\%$; and siRNA(+)/BCG(+), $73.5 \pm 8.4\%$ (Fig. 3d).

These results revealed the following three interesting points. (1) BCG exposure induced the nuclear expression of not only single-p27 expression but also the co-expression of p21 and p27 (Fig. 3a, c). (2) p27-siRNA decreased the nuclear expression of not only single p27 but also the co-expression of p21 and p27 (Fig. 3a, c). (3) In nuclear expression, approx. 10% of the p21 expression would be independent of p27 expression (Fig. 3b).

The cytoplasmic expression of p21 is dependent on p27

To investigate the dependence and dominance of p27 to p21 in cytoplasmic expression, we identified the four patterns mentioned above: double-positive, single-p21, single-p27, and double-negative. The cytoplasmic double-positive percentages were as follows. siRNA(–)/BCG(–), $38.5 \pm 20.9\%$; siRNA(–)/BCG(+), $45.0 \pm 19.9\%$; siRNA(+)/BCG(–), $15.6 \pm 7.0\%$; and siRNA(+)/BCG(+),

25.8 ± 11.6% (Fig. 4a). The cytoplasmic single-p21 positive percentages were: siRNA(-)/BCG(-), 0.8 ± 0.3%; siRNA(-)/BCG(+), 0.2 ± 0.3%; siRNA(+)/BCG(-), 0.3 ± 0.3%; and siRNA(+)/BCG(+), 0.0 ± 0.0% (Fig. 4b). The cytoplasmic

single-p27 positive percentages were: siRNA(-)/BCG(-), 47.7 ± 11.7%; siRNA(-)/BCG(+), 51.3 ± 18.5%; siRNA(+)/BCG(-), 47.7 ± 7.0%; and siRNA(+)/BCG(+), 49.5 ± 5.0% (Fig. 4c). The cytoplasmic double-negative percentages were as

Fig. 2 The nuclear and cytoplasmic expressions depended on the number of BCG exposures. **a** shows the expression level of pKi67 and **b–e** show the percentages of each condition in each BCG exposure. **a** During the first to sixth BCG exposures, the nuclear-positive percentage of pKi67 of the BCG cells was approx. 20% lower than that of the control cells. **b** The nuclear double-negative percentages of p21 and p27 (which were exclusively localized to the cytoplasm of the BCG cells) were lower than those in the control cells. **c** The nuclear-positive percentage of single p21 in both the BCG cells and control cells was very low. **d** The nuclear-positive percentage of single p27 of the BCG cells was lower than that of the control cells. **e** The nuclear double-positive percentage of p21 and p27 of the BCG cells was approx. 20% higher than that of the control cells during the first to sixth BCG exposures. This pattern was the reverse of the pattern of the nuclear-positive percentage of pKi67 (**a**). A min. of 200 cells was counted for each sample. Each result is the mean $\pm$ SD of three independent experiments. N(+) nuclear expression, C(+) cytoplasmic expression. * $P < 0.05$

follows: siRNA(-)/BCG(-), $13.0 \pm 10.8\%$; siRNA(-)/BCG(+), $3.5 \pm 3.3\%$; siRNA(+)/BCG(-), $36.4 \pm 14.2\%$; and siRNA(+)/BCG(+), $24.7 \pm 6.5\%$ (Fig. 4d).

These results indicated that: (1) The cytoplasmic expression of p21 might be dependent on p27 (Fig. 4b). (2) The p27-siRNA-decreased co-expression resulted from the decrease of p21 (Fig. 4a, b, d). (3) The cytoplasmic expression of p27 might not be influenced by p27-siRNA (Fig. 4c).

Discussion

We investigated the relationship between BCG and cell-cycle arrest using pKi67, p21, p27 and p27-siRNA with T24 cells, which are derived from human urothelial carcinoma. pKi67 is well established in investigative and diagnostic pathology, since the expression of the Ki67 protein is tightly linked with cell proliferation (MacCallum and Hall 2000). p21 (Harper et al. 1993) and p27 (Toyoshima and Hunter, 1994) are reported as the Cip/Kip family members which negatively regulate CDK activity. Therefore, we used pKi67 as an operational marker of proliferation and p21 and p27 as markers of cell-cycle arrest.

As noted in the Introduction, since the previous relevant studies used BCG conditions that differ from those used in clinical treatment, it has been difficult to estimate the effects of BCG exposure on cells. We thus first identified the optimal duration of BCG exposure,

and found that 2-h exposure was the most effective for cell-cycle arrest. With 2 h of BCG exposure, the nuclear expressions of p21 and p27 were highest, and the nuclear expression of pKi67 was lowest (Fig. 1b). In clinical treatment, 2-h BCG exposure has been used to treat non-muscle-invasive urothelial carcinoma as recommended by the Immunobladder[®] vaccine drug information. Our present findings support the concept that 2-h BCG exposure is the best for clinical treatment.

On the other hand, the cytoplasmic expression of both p21 and p27 was higher than the corresponding nuclear expression, and the cytoplasmic expression of p27 was higher than that of p21 in all conditions (Fig. 1b).

In general, bladder cancer patients are treated with BCG exposure once per week for 6–8 weeks after the transurethral resection of non-muscle-invasive bladder cancer. However, there are no reports from experimental studies in which cells were exposed to BCG many times. Here, to investigate the relationship between the expression of proteins and the number of BCG exposures, we exposed human urothelial carcinoma-derived (T24) cells to BCG six times and used double staining methods to observe the nuclear and cytoplasm protein expressions, with LSM. First, from the first to the sixth BCG exposure, we observed that the nuclear co-expression of p21 and p27 of BCG cells was higher than that of the control cells, and that the BCG cells' expression of pKi67 was lower than that of the control cells. In short, the nuclear co-expression of p21 and p27 was the opposite of the expression of pKi67 from the first to sixth exposures. Our results also demonstrated that cell-cycle arrest was induced in approx. 20% of the T24 cells by BCG exposure (Fig. 2a, e). These results suggest that BCG exposure might induce cell-cycle arrest immediately, and that the co-expression of p21 and p27 caused effective cell-cycle arrest.

Cormio et al. (2009) reported that changes involving at least two cell-cycle regulators among pRb, p53, p21 and p27 were necessary for the bladder cancer progression in 27 non-muscle-invasive bladder cancers. In experiments, Zhang et al. (2007) reported that BCG induces p21 expression. However, See et al. (2010) reported that p21 expression is sufficient but not necessary for BCG-induced cell-cycle arrest, and their results thus indicated that the existence of some factors other than p21 is related to the cell-cycle arrest.

Fig. 3 Influence of p27-siRNA on the nuclear expressions of p21 and p27. **a–d** show the percentages of the each experimental condition. **a** p27-siRNA significantly decreased the nuclear double-positive percentage. **b** The nuclear single-positive percentage of p21 was approx. 10% in all conditions. There were no significant differences between all sets. **c** p27-siRNA

significantly decreased the nuclear single-positive percentage of p27. **d** p27-siRNA significantly increased the double-negative percentage. A min. of 200 cells was counted for each sample. Each result is the mean $\pm$ SD of three independent experiments. ** $P < 0.001$; * $P < 0.05$

To the best of our knowledge however, no previous studies have evaluated expression of p27 or the co-expression of p21 and p27 in BCG experiments.

Stefano et al. (2011) reported that double siRNA had a greater effect on cardiomyocytes than the single siRNA of p21 and p27, and Orlando et al. (2015) reported that the expression of target genes was much higher in double p21/p27 null cells than in single knock-out or control cells, confirming the collaborative role of p27 and p21 in the regulation of transcription. These reports indicated that the co-expression of p21 and p27 was more effective than the single expression of either p21 or p27, which appears to be supported by our present results.

Clinical studies revealed contrasting findings regarding p27; a prognostic factor for urothelial carcinoma (Shariat et al. 2009; van Rhijn et al. 2003) but not a prognostic factor for bladder cancer (Park et al. 2013). These differences might be due to the quite different roles of p21 and p27 between the cell nucleus and cytoplasm. The cytoplasmic roles of p21 and p27, which have been investigated for over 20 years, are to mediate the assembly and nuclear import of Cdk4(6)-cyclin D complexes. The nuclear roles of p21 and p27 are to inhibit Cdk-cyclin complexes (Yoon et al. 2012). p27 and p21 have also been reported to be nuclear tumor suppressors and cytoplasmic oncogenes (Philipp-Staheli et al. 2002; Blagosklonny 2002). The cytoplasmic localization of

Fig. 4 Influences of p27-siRNA on cytoplasmic expressions. **a–d** showed the percentages of the each experimental condition. **a** p27-siRNA decreased the cytoplasmic double-positive percentage. However, there were no significant differences between all sets. **b** There was a very low cytoplasmic single-

positive percentage of p21 in all conditions. **c** In all conditions, there was no influence on the cytoplasmic single-positive percentage of p27. **d** p27-siRNA significantly increased the cytoplasmic double-negative percentage. The results are the mean $\pm$ SD of three independent experiments. * $P < 0.05$

p27 inactivates the tumor suppressor function of the protein (Kossatz and Malek 2007).

In addition, the exclusion of p27 from the nucleus is frequently found in breast cancer tissues due to an increased phosphorylation by AKT (Besson et al. 2004; Kossatz and Malek 2007). Moreover, the expression of a mutant form of p27 that localizes exclusively to the cytoplasm (p27deltaNLS) down-regulated RhoA and increased the motility, survival, and Akt levels without an effect on cell-cycle distribution in MCF7 breast cancer cells (Wu et al. 2006).

Our present findings demonstrated that the positive percentage of p27C was higher than that of p21C (Fig. 1b), and there were very few cells with single p21N (Fig. 2c). We therefore propose that the expression of p27 might be a higher priority compared to that of p21. To evaluate the prerequisite of p27, we performed the experiment using p27-siRNA, and the

results revealed different expression patterns of p21 and p27 between the nuclear expression and cytoplasm expression. Standard siRNAs have been reported to exhibit off-target effects (Kawasaki et al. 2005). Therefore, in the present study, we used the stealth RNAi siRNA that was modified for essential elimination from off-target effects. The use of p27-siRNA decreased the nuclear co-expression, which was concurrent with a decrease in the single p27N expression (Fig. 3a, c). Interestingly, approx. 10% of the single p21N would express whatever exposure of p27-siRNA and BCG. Generally, the expression of p21 depends on p53, but T24 cells have a mutation in p53 as an in-frame deletion of tyrosine 126 (Cooper et al. 1994). Zhang et al. (2007) reported that p21 transactivation in response to BCG occurs via an immediate early pathway that does not require p53.

On the other hand, in the cytoplasm, we observed that the positive percentage of single cytoplasmic p21 expression (p21C) was very low in all conditions (Fig. 4b), whereas the percentage of single cytoplasmic p27 expression (p27C) was approx. 50% (Fig. 4c). In light of these results, we suggest that the cytoplasmic expression of p21 might depend on the cytoplasmic expression of p27. This dependence of p21C on p27C might be a reason for our observation that the p27C expression was higher than that of p21C in all conditions (Fig. 1b).

The nuclear localization of p21 and p27 inhibits cyclin-CDK complexes and controls the cell cycle. When p21 and p27 are phosphorylated by AKT/PKB, their cytoplasmic localization is induced (Yoon et al. 2012). In short, the phosphorylation of p21 at Thr145 (Zhou et al. 2001) and the phosphorylation of p27 at Thr157 (Liang et al. 2002), Ser10 (McAllister et al. 2003) and Thr198 (Larrea et al. 2009) induce their cytoplasmic localization, and this causes cell proliferation and migration (Besson et al. 2004; Larrea et al. 2009; Liang et al. 2002).

Interestingly, in the present study, the positive percentage of p27C was approx. 50% despite the use of p27-siRNA. This phenomenon was also reported by Stefano et al. (2011). Blagosklonny (2002) noted that mutations and deletions of p27 have not been detected even in cancers (which is very different from pRb, p53 and p16), suggesting that the mechanism of p27 expression is complex. Non-CDK-bound p27 was reported to play an active role in tumour formation (Björklund et al. 2010).

We conducted five investigations using T24 cells in the present study: (1) BCG exposure for 2 h was able to immediately induce cell-cycle arrest. (2) The cell-cycle arrest induced by BCG exposure might be related to the nuclear co-expression of p21 and p27. (3) Inhibition of p27 with the use of p27-siRNA decreased the nuclear expression of p27, whereas this inhibition did not influence the cytoplasmic expression of p27. (4) BCG exposure and p27-siRNA had no influence on the single nuclear expression of p21. (5) The cytoplasmic expression of p21 might depend on the cytoplasmic expression of p27.

We used only p27-siRNA, and further investigation using p21-siRNA, AKT/PKB, p53, pRb and CDKs are thus necessary to broaden our understanding of the roles of p21 and p27. The incidence of non-muscle-invasive bladder cancer patients who fail to respond to

BCG is approx. 30% (Herr et al. 2015). The elucidation of the mechanism of BCG treatment could contribute to the selection of the optimum treatment for high-grade non-muscle-invasive bladder cancer patients.

To our knowledge, the present study is the first to demonstrate the priority of p27 expression over p21 expression in response to BCG exposure, and that the co-expression of p27 and p21 effectively induced cell-cycle arrest. The evaluation of the nuclear co-expression of p21 and p27 might be an indicator decision of the effectiveness of BCG treatment.

Acknowledgements This study was supported by JSPS KAKENHI Grant Nos. #JP25460459 and #JP17K08744.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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