treated with laser ablation between 2004 and 2010 at the Tokyo University Hospital. Patients were followed up with cytology and colposcopy 3 months after laser ablation, and those who showed residual disease were excluded from this study. Patients with negative cytology and normal colposcopic findings 3 months after treatment were included in this retrospective study. A total of 144 patients (mean age, 36.9 years; range, 25–71 years) met these criteria and were included in this retrospective study. Patients were followed up for at least 5 years with cytological and colposcopic evaluations conducted at intervals of 3–4 months. No residual lesion was confirmed by satisfactory colposcopic findings with negative cytology on the ecto-endocervix together. The recurrence of CIN3 was regarded as the primary end-point. Referring to the previous publications,[9,10] date of recurrence was defined as the mid-point between the date of the examination when abnormal cytology or histology (such as moderate-severe dysplasia, atypical squamous cells that cannot exclude HSIL [ASC-H], or high-grade squamous intraepithelial lesion [HSIL]) was first detected with satisfactory colposcopy, and the most recent preceding examination in which the colposcopic evaluations and smear (ecto-endocervix) were normal.

In total, 83 patients (median age, 36 years) were further examined for the efficacy of laser ablation by HPV genotypes, identified by polymerase chain reaction (PCR)-based HPV DNA testing before and after ablation, comparing the post-treatment persistent infection and recurrence-free survival (RFS) rates. HPV genotyping was performed in each patient. Regarding the natural history of CIN in Japan, a recent prospective cohort study by Matsumoto *et al.*[14] showed that the cumulative progression rate for CIN3 within 5 years was 20.5% for eight types of high-risk HPV (16, 18, 31, 33, 35, 45, 52, and 58), which was significantly higher than the 6.0% observed for five other high-risk types (39, 51, 56, 59, and 68), demonstrating that differences in progression exists even in the 13 HPV types defined by IARC as high-risk. In our study, therefore, we classified the study population according to this report and focused on the eight 'higher-risk' types. Informed consent was obtained in all cases. The median follow-up period was 17 months, with a minimum of at least 6 months. Recurrence was defined as emergence of CIN3 in complete responders.

PCR-based HPV DNA testing

DNA was extracted from cervical smear samples by using the QIAGEN DNeasy Blood & Tissue Kits. PCR-based HPV DNA testing was performed using the PGMY-CHUV assay. Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer sets and HLA-dQ primer sets. Reverse blotting hybridization was subsequently performed as described previously.[15]

Laser ablation

Outpatient carbon dioxide laser procedures were carried out under colposcopic guidance, taking about 10 min, without anesthesia or premedication. Water in the tissue absorbs the laser energy, which destroys tissue by vaporization. To be effective, the lesion is typically ablated to a depth of 5 mm on the ectocervix and 8–9 mm around the endocervix. After ablation, the epithelium regenerates in 2–3 weeks. All cases were performed by gynecologic oncologists using CO2 laser, MEDILASER-30S (Model mel-30S, Mochida) with a power density of 8–12W in continuous mode.

Statistics

Date of recurrence was defined as the mid-point between the date of the examination when abnormal cytology was first detected and the most recent preceding examination in which the smear was normal. The log–rank test was used to assess differences in cumulative risk between study groups; tests of significance were carried out at the 5% two-sided level.

Results

We initially identified 144 patients with CIN3 who received laser ablation at Tokyo University Hospital between 2004 and 2010, and showed both negative cytology and normal colposcopic findings 3 months after treatment. Ten patients were excluded because of incomplete data. A total of 134 cases of CIN3 (median age, 37 years; range, 27–71 years, excluding CIS) were monitored every 3–4 months during the follow-up period (6–95 months; median, 38 months). Seven (5.2%) were censored at 1 year, 19 (14.2%) at 2 years, and 105 (78.4%) at 5 years after treatment. The recurrence of CIN3 was regarded as the primary end-point. All the patients were evaluated with satisfactory colposcopy and histological examination of transitional zone.

First, we investigated the efficacy of laser ablation against all CIN cases. During the follow-up period, recurrence was identified in 57 (42.5%) of the 134 patients, and the overall cumulative CIN3 recurrence rate in the first 12 months after treatment was 22.6%

with atypical squamous cells of undetermined significance (ASC-US) detected by screening cytology test; we recommend that HC2 should also be used widely for the surveillance of patients after treatment for CIN.

In summary, laser ablation is a useful conservative treatment for CIN3, especially for young women of reproductive age. It will continue to be necessary for gynecologists to tailor treatment strategies for young patients according to their health needs, balancing minimal adverse obstetric outcomes with minimal attenuation of efficacy. In addition, it is well established that the rate of developing high-grade disease, including invasive cervical cancer, remains elevated in these post-treatment patients, even after a very long time subsequent to treatment. Indeed, a study in 2006 warns that the rate of invasive disease remained at 56 per 100 000 woman-years, 2.8 times greater than expected, for at least 20 years after treatment.[19] Because of this substantial long-term risk, close monitoring remains critical, and combined cytological evaluation together with HPV testing should increase the safety of the surveillance. For more rigorous and detailed follow-up algorithms, including surveillance intervals, obstetric outcomes, and cost-effectiveness, larger and longer-term studies are warranted in the future.

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Disclosure

The authors have no conflicts of interest related to this article.

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FULL PAPER

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Role of multifunctional transcription factor TFII-I and putative tumour suppressor DBC1 in cell cycle and DNA double strand damage repair

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Background: In multicellular organisms, precise control of cell cycle and the maintenance of genomic stability are crucial to prevent chromosomal alterations. The accurate function of the DNA damage pathway is maintained by DNA repair mechanisms including homologous recombination (HR). Herein, we show that both TFII-I and DBC1 mediate cellular mechanisms of cell-cycle regulation and DNA double strand damage repair.

Methods: Regulation of cell cycle by TFII-I and DBC1 was investigated using Trypan blue dye exclusion test, luciferase assay, and flow cytometry analysis. We also analysed the role of TFII-I and DBC1 in DNA double strand damage repair after irradiation by immunofluorescence study, clonogenicity assay, and HR assay.

Results: Flow cytometry analysis revealed a novel function that siRNA-mediated knockdown of endogenous DBC1 resulted in G2/M phase arrest. We also have shown that both endogenous TFII-I and DBC1 activate DNA repair mechanisms after irradiation because irradiation-induced foci formation of TFII-I-γH2AX was observed, and the depletion of endogenous TFII-I or DBC1 resulted in the inhibition of normal HR efficiency.

Conclusion: These results reveal novel mechanisms by which TFII-I and DBC1 can modulate cellular fate by affecting cell-cycle control as well as HR pathway.

TFII-I was originally identified as a transcription factor that could bind to two distinct promoter elements: the pyrimidine-rich initiator and the recognition site (E-box) for upstream stimulatory factor 1 (USF1). TFII-I stimulates transcription from the potent TATA- and initiator-containing adenovirus major late promoter synergistically with USF1 (Roy *et al*, 1997). TFII-I is a unique multifunctional factor that selectively regulates gene expression when activated by a variety of extracellular signals and can function both as a basal transcriptional factor and as an activator (Roy, 2012). The autosomal dominant genetic disorder

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Williams–Beuren syndrome is a multisystem disorder characterised by distinctive facial features, mental disability, diabetes mellitus, and supravalvular aortic stenosis. Humans that have a haploinsufficiency for the gene encoding TFII-I are characterised by a craniofacial phenotype along with cognitive deficits (Pober, 2010). The carboxyl terminus of BRCA1, referred to as the BRCT domain, possesses autonomic transcriptional activation functions, and the BRCT domain has been shown to be involved in DNA double strand damage repair and homologous recombination (HR) (Zhong *et al*, 1999). We previously reported that TFII-I stimulates the transactivation function of the BRCT domain and the BRCA1-mediated stimulation of SIRT1 promoter activity in the mammalian homologue of yeast Sir2 (silent information regulator 2) (Tanikawa *et al*, 2011).

DBC1 (deleted in breast cancer 1) is a nuclear protein that is thought to localise to the nucleus by virtue of its N-terminal nuclear localisation signal. The role of DBC1 as a transcriptional co-factor has been recently revealed. DBC1 directly inhibits the transcriptional activation function of the BRCT domain; thus, DBC1 may serve as a potent tumour promoter (Hiraike *et al*, 2010). In addition, DBC1 inhibits the BRCA1-mediated stimulation of SIRT1 promoter activity (Hiraike *et al*, 2010).

Whether TFII-I and DBC1 together play pivotal roles in tumour suppression or progression remains to be determined because our previous data showed anti-tumorigenic role of TFII-I (Tanikawa *et al*, 2011) and tumorigenic function of DBC1 (Hiraike *et al*, 2010). Especially, oestrogen-independent growth of MCF-7 breast cancer cells is provoked by DBC1, suggesting a tumorigenic function of DBC1 in cellular growth (Trauernicht *et al*, 2007). Contrary to this, DBC1 also prompts p53-dependent apoptosis by inhibiting SIRT1 (Kim *et al*, 2008; Zhao *et al*, 2008) and plays a role in DNA damage repair (Zannini *et al*, 2012). The analysis of DBC1 on cell-cycle progression and the HR function is lacking to date.

To better understand the physiological functions of TFII-I and DBC1, we studied the roles of TFII-I and DBC1 in cell-cycle regulation and DNA repair. Here, we analysed the effects of TFII-I and DBC1 on cell-cycle regulation. We further investigated the role of TFII-I and DBC1 in DNA repair given that both TFII-I and DBC1 were implicated in DNA repair (Hiraike *et al*, 2010; Tanikawa *et al*, 2011). Our findings establish a novel biological function of TFII-I and DBC1 as a modulator of cell cycle and HR.

MATERIALS AND METHODS

Cell culture. Human osteosarcoma U2OS (HTB-96) and human kidney 293T (CRL-11268) cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). SW480sn3 cell line harbouring a single integrated copy of a recombination substrate SCneo was kindly provided by Dr Thomas Helleday (Department of Medical Biochemistry and Biophysics, Karolinska Institute).

Chemicals and antibodies. Rabbit polyclonal antibodies were anti-DBC1 (Hiraike *et al*, 2010; Koyama *et al*, 2010), anti-TFII-I, and anti-BRCA1 (Cell Signaling Technology, Inc., Temecula, CA, USA, catalogue nos. 4562 and 9010, respectively). Mouse monoclonal antibodies were anti-BRCA1 (Calbiochem, EMD Biosciences, Inc., La Jolla, CA, USA, catalogue no. OP93T), anti-SIRT1 (Abcam Ltd., Cambridge, UK, catalogue no. ab32441), and anti-Actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, catalogue no. sc-47778). Alexa Fluor 488-conjugated donkey anti-mouse IgG (A-21428) and Alexa Fluor 555-conjugated goat anti-rabbit IgG (A-21202) were purchased from Invitrogen (Carlsbad, CA, USA).

Western blot. The whole-cell extracts of U2OS cells transfected with indicated siRNAs were subjected for western blot assay (Hiraike *et al*, 2010; Koyama *et al*, 2010; Tanikawa *et al*, 2011). The proteins were separated by SDS–PAGE, transferred onto nitrocellulose membrane, and detected by western blotting using appropriate primary and secondary antibodies.

Luciferase assay. For luciferase assay, cells were transfected with indicated expression vectors and reporter plasmids. As an internal control to equalise transfection efficiency, phRL CMV-Renilla vector (Promega Co., Madison, WI, USA) was also transfected in all the experiments. Individual transfections, each consisting of triplicate wells, were repeated at least three times as described previously (Hiraike *et al*, 2010; Koyama *et al*, 2010; Tanikawa *et al*, 2011).

RNAi. The inhibition of TFII-I, DBC1, and SIRT1 was performed by transfection of Stealth siRNA duplex oligoribonucleotides. Control siRNA, TFII-I-specific siRNA (GTF2I-HSS142342, GTF2I-HSS142343, or GTF2I-HSS142344), DBC1-specific siRNA (KIAA1967-HSS126769, KIAA1967-HSS126771, or KIAA1967-HSS184064), and SIRT1-specific siRNA (SIRT1-HSS118729, SIRT1-HSS177403, or SIRT1-HSS177404) were synthesised by Invitrogen.

Fluorescence microscopy. U2OS cells were exposed to 8 gray (Gy) of gamma irradiation and fixed at the indicated time. After blocking, the cells were incubated sequentially with the appropriate primary and secondary antibodies. The slides were briefly counterstained with Hoechst 33342 and analysed under a confocal fluorescence microscope (Carl-Zeiss MicroImaging Inc., Oberkochen, Germany) using LSM7 series-ZEN200x software.

Cell-cycle synchronization and flow cytometry analysis. SW480sn3 cells were arrested twice at the G1/S boundary using a double incubation in the presence of 2.5 mM thymidine for 14–16 h, followed by a 9-h interval of growth without the drug. For mitotic arrest, SW480sn3 cells were first treated with 2.5 mM thymidine for 16 h and then treated with 50 $g\,ml^{-1}$ nocodazole for 8–10 h. The cells were released from the cell-cycle blocks and harvested at the indicted times. The fixed cells were stained with propidium iodide ($2\,\mu g\,ml^{-1}$) and analysed with a Becton-Dickinson FACScan (BD Biosciences, San Jose, CA, USA).

Clonogenicity assay. SW480sn3 cells were plated at a density of 2×10^3 cells per 60 mm dish and irradiated at the indicated dose of gamma irradiation with a ^{60}Co source. The cells were allowed to grow for 14 days, fixed, and stained with Giemsa.

DNA HR assay. The HR frequency was determined as previously described (Saberi *et al*, 2007; Yoshizawa-Sugata and Masai, 2009). Briefly, the assay was conducted in SW480sn3 cells harbouring a single integrated copy of a recombination substrate SCneo. 1.6×10^6 cells were seeded in 60-mm dishes on the day before siRNA transfection. Cells were transfected with siRNAs by Lipofectamine RNAi MAX (Invitrogen). At 48 h after siRNA transfection, cells were further transfected with pCMV3nls-I-SceI expression vector. At another 48 h after DNA transfection (96 h after siRNA transfection), double-stranded DNA breaks (DSBs)-introduced cells were either replated in selection media containing 1 mg G418 per ml (2×10^5 cells per dish) or non-selection media (500 cells per dish, for control of colony-forming efficiency). After two weeks for selection, colonies were fixed and stained with Giemsa dye. G418-resistant colonies of diameter over 250 μm (approximately over 100 cells) were counted. The recombination frequency was calculated as previously described (Yoshizawa-Sugata and Masai, 2009).

RESULTS

Depletion of DBC1 retards G2-M progression. To study the cellular functions of TFII-I and DBC1, we tested the effect of siRNA-mediated knockdown of endogenous TFII-I and DBC1 on cellular growth using Trypan blue dye exclusion assay. Initially, we examined HeLa and MCF-7 cells but detected little effect as expected from the previous study (Ogura *et al*, 2006). We tested several cell lines and found that U2OS cells transfected with DBC1 siRNA enhanced the cell growth compared with control siRNA at 3 days after treatment, whereas TFII-I siRNA inhibited the cell growth (Figure 1A). Western blot analysis shows that the protein level of TFII-I (nos. 2, 3) and DBC1 (nos. 1, 3) significantly reduced after siRNA-mediated depletion of endogenous proteins (Figure 1B). Transient transfection assay was performed to examine the functions of TFII-I and DBC1 during cell cycle. 293T cells were transiently transfected with the indicated combinations of mammalian expression plasmids, and the transfected whole-cell lysates were assayed for luciferase activity. We examined p21 and GADD45 promoter regulation because the BRCT domain, which interacts with TFII-I and DBC1, is sufficient to activate the p21 promoter (Chai *et al*, 1999) and BRCA1 induces GADD45 expression through the activation of the GADD45 promoter (Jin *et al*, 2000). DBC1 and TFII-I potently activated p21 promoter activity in the presence of BRCA1 (Supplementary Figure S1). We also found that DBC1 repressed GADD45 promoter activity regardless of the presence of BRCA1, whereas TFII-I had no apparent effect (Figure 1C).

To evaluate the accelerated cell growth caused by siRNA, we examined the effects of siRNA-mediated knockdown of TFII-I and DBC1 on cell-cycle progression. It has been shown that TFII-I is important for G1–S phase transition because TFII-I promotes cell cycle by inducing cyclin D1 (Desgranges *et al*, 2005). To evaluate the effects on the progression of cell cycle by TFII-I and DBC1, we utilised siRNA-mediated knockdown of TFII-I and DBC1. We performed double thymidine block method to synchronise U2OS cells at the G1–S boundary after transfection of siRNA (Yoshizawa-Sugata and Masai, 2009) (Figure 1D, left panel). In cells depleted with endogenous TFII-I, as expected, increased G1–S boundary cell fraction was observed at 3–6 h after thymidine release. However, in cells depleted with endogenous DBC1, decreased fraction of postmitotic G1 peak was observed at 9 h after thymidine release, suggesting a prominent effect of DBC1. Then we tested the G2–M progression in cells depleted with TFII-I and DBC1. The cells treated with siRNA as indicated were synchronized by nocodazole block/release, and it has been revealed that siRNA-mediated knockdown of DBC1 resulted in an increased accumulation of G2–M fraction at 3–6 h after nocodazole release (Figure 1D, right panel). These data indicate that decreased expression of DBC1 in U2OS cells retards G2–M phase. Considering that GADD45 has been shown to play a role in the control of the G2–M checkpoint (Wang *et al*, 1999), these results indicate that DBC1 may have an inhibitory effect on the G2–M checkpoint.

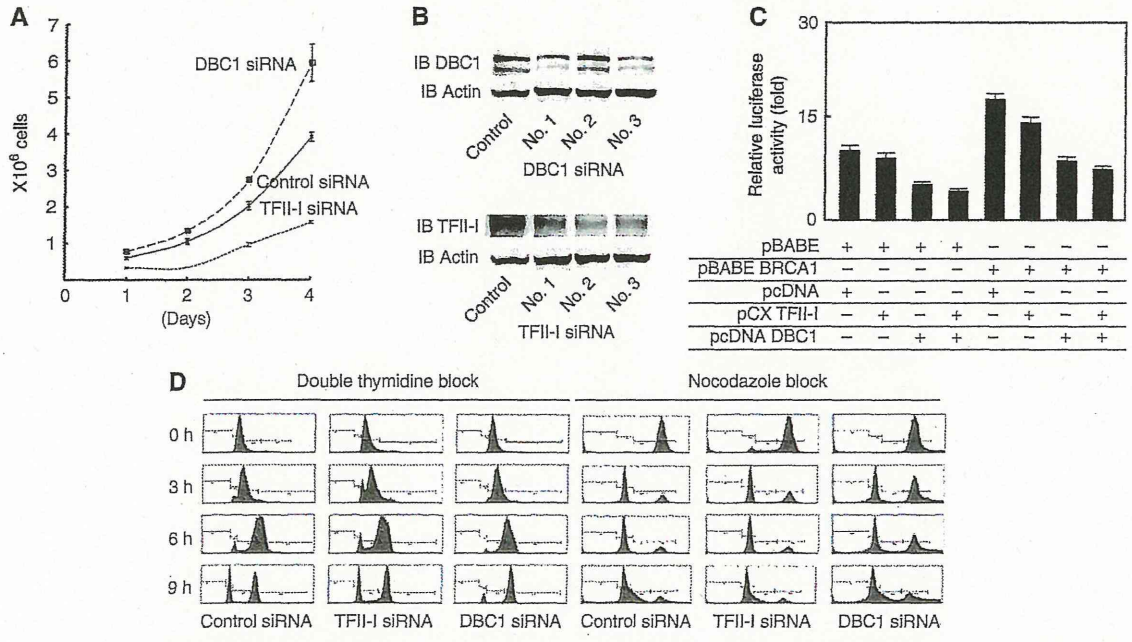

Figure 1. The cell-cycle regulation by TFII-I and DBC1. (**A**) Trypan blue dye exclusion test was performed to examine the effect on cellular growth in U2OS cells. In this assay, siRNA-mediated knockdown of endogenous DBC1 or TFII-I was performed and 5×10^5 U2OS cells were allowed to grow for a subsequent 3 days. The knockdown of DBC1 resulted in an increase in cell numbers compared with control siRNA 3 days after treatment, whereas the depletion of TFII-I resulted in a decrease in cell numbers compared with control. (**B**) Efficiency of siRNA-mediated knockdown of TFII-I and DBC1 is demonstrated by western blot. (**C**) Transient transfection assays were performed to examine the functions of TFII-I and DBC1 in BRCA1-mediated cell-cycle regulation. 293T cells were transfected with the indicated combinations of mammalian expression plasmids, and the transfected whole-cell lysates were assayed for luciferase activity. DBC1 repressed GADD45 promoter activity regardless of the presence of BRCA1. The error bars represent the standard deviations. (**D**) Flow cytometry analysis was performed using U2OS cells synchronized using a double-thymidine block. The cells were subsequently released into S phase. In cells depleted with endogenous TFII-I, increased G1–S boundary cell fraction was observed at 3–6 h after thymidine release. In cells depleted with endogenous DBC1, decreased fraction of postmitotic G1 peak was observed at 9 h after thymidine release. The cells treated with siRNA as indicated were also synchronized by a nocodazole block/release. The siRNA-mediated knockdown of DBC1 resulted in increased accumulation of the G2/M fraction 3–6 h after nocodazole release.

TFII-I and DBC1 play a role in the repair of DSBs induced by irradiation. Our previous data indicated that DSBs caused by irradiation led to the colocalization of BRCA1 and TFII-I in nuclei of HeLa cells, possibly at sites of the DNA damage because BRCA1 accumulates at the DSBs and forms nuclear foci with γH2AX (Tanikawa *et al*, 2011). Therefore, we hypothesised that TFII-I may play an important role in DSB repair. We examined the subcellular distribution of TFII-I and DBC1 in U2OS cells using immunofluorescence analysis. U2OS cells were exposed to 8 Gy of gamma irradiation, and the subcellular localisation of TFII-I, DBC1, and γH2AX was determined by confocal microscopy. It has been suggested that SIRT1 aids DNA damage repair and maintains genome integrity by binding to DSBs (Jeong *et al*, 2007; Wang *et al*, 2008). Additionally, Rad51 and NBS1, critical components of the homologous HR process, are recruited to chromatin concomitantly with SIRT1 in response to exposure to DSB-inducing ionising irradiation (Oberdoerffer *et al*, 2008). We confirmed that SIRT1 and γH2AX formed nuclear foci after induction of DSBs (Figure 2A). We also showed that TFII-I and γH2AX formed nuclear foci after irradiation, but the colocalization of DBC1 and γH2AX after irradiation remained unclear because it was difficult to distinguish individual foci (Figure 2A). The effect of the siRNA-mediated depletion of endogenous TFII-I and DBC1 was further investigated. Although the depletion of endogenous DBC1 showed no prominent effects on the nuclear focus formation of γH2AX-BRCA1 and γH2AX-SIRT1, the depletion of TFII-I completely inhibited the formation of these nuclear foci (Figure 2B).

We further investigated the effect of the siRNA-mediated depletion of endogenous TFII-I and DBC1, and siRNAs tested did not induce significant apoptosis compared with control siRNA (data not shown). U2OS cells were treated with siRNA as indicated and we examined the effects of the siRNA-mediated depletion of TFII-I or DBC1 using a clonogenicity assay. As shown in Figure 3A, the depletion of endogenous TFII-I or DBC1 abrogated colony formation efficiency similar to SIRT1 after irradiation. These results strongly suggested that TFII-I plays a central role in stimulating DSB repair, and the role of DBC1 in DSB repair remained relatively elusive.

TFII-I and DBC1 enhance HR. We next postulated that the aberrant DNA damage response was caused by the inactivation of HR repair; therefore, HR was analysed after the depletion of endogenous TFII-I or DBC1. For this purpose, SW480sn3 cell line specifically designed to investigate HR efficiency was used because SW480sn3 cell line harbour a single integrated copy of a recombination substrate SCneo. Irradiation causes DSBs, and DSBs that are formed during S and G2 phases are predominantly repaired through HR mechanisms (Branzei and Foiani, 2008). The DNA repair functions of BRCA1 mainly involve HR (Narod and Foulkes, 2004); therefore, we investigated HR activity after the inhibition of TFII-I and DBC1. Colony formation efficiency without selection media remained unchanged by siRNA-mediated knockdown of endogenous TFII-I, DBC1, and SIRT1 (Supplementary Figure S2). Surprisingly, after normalisation of colony formation efficiency, both TFII-I and DBC1 were shown to play a crucial role in HR similar to SIRT1 (Figure 4). Thus, our data demonstrate that both TFII-I and DBC1 play a critical role in regulating the DNA damage response, suggesting a role for TFII-I and DBC1 in the HR pathway.

DISCUSSION

TFII-I is considered to be involved in the regulation of the expression of genes as a signal-induced multifunctional transcription

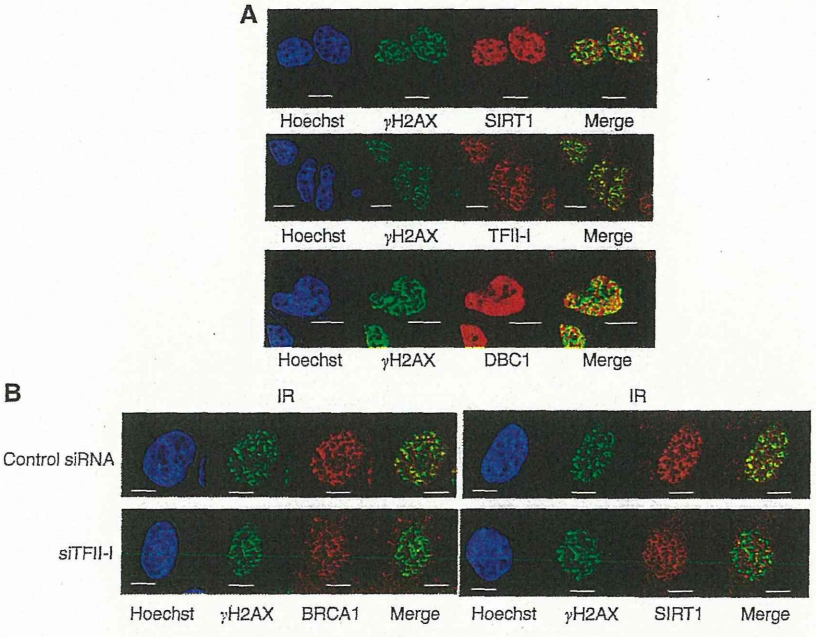

Figure 2. Nuclear foci formation of TFII-I with γH2AX after gamma irradiation. (A) U2OS cells were exposed to 8 Gy of gamma irradiation and fixed within 15 min. The cells were prepared for confocal microscopy as described in the Materials and Methods and then were incubated with primary antibodies followed by secondary antibodies. Representative immunofluorescence images are shown. The bars indicate a length of 10 μm. (B) The siRNA-mediated depletion of endogenous TFII-I inhibited γH2AX-BRCA1 and γH2AX-SIRT1 nuclear focus formation. γH2AX nuclear focus formation was analysed after the inactivation of endogenous TFII-I. U2OS cells were fixed 15 min after irradiation. The cells were prepared for confocal microscopy as described in the Materials and Methods and were exposed to the appropriate antibodies. Representative immunofluorescence images are shown. The bars indicate a length of 10 μm.

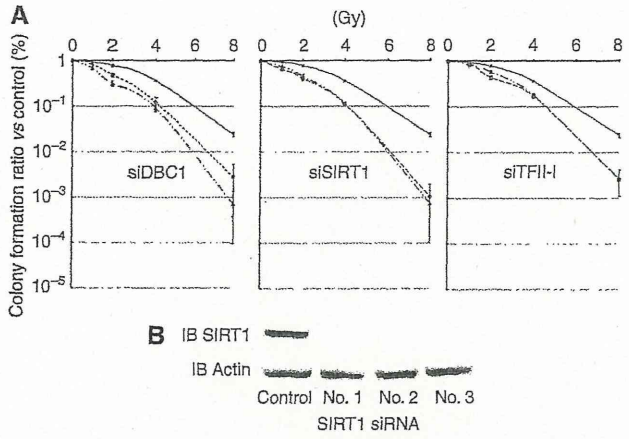

Figure 3. TFII-I and DBC1 play a role in the repair of DSBs induced by irradiation. (**A**) TFII-I and DBC1 siRNA inhibit colony formation efficiency after irradiation. U2OS cells were transfected with the indicated siRNAs and irradiated. The cells were allowed to grow for 14 days and stained with Giemsa. The knockdown of TFII-I or DBC1 resulted in a significant inhibition of colony formation efficiency. The solid line represents control siRNA and the broken lines represent three independent siRNA. The siRNA-mediated knockdown of SIRT1 serves as a positive control because SIRT1 has been shown to maintain genomic stability and is required for efficient DSB (Jeong *et al*, 2007; Wang *et al*, 2008). (**B**) Efficiency of siRNA-mediated knockdown of SIRT1 is demonstrated by western blot.

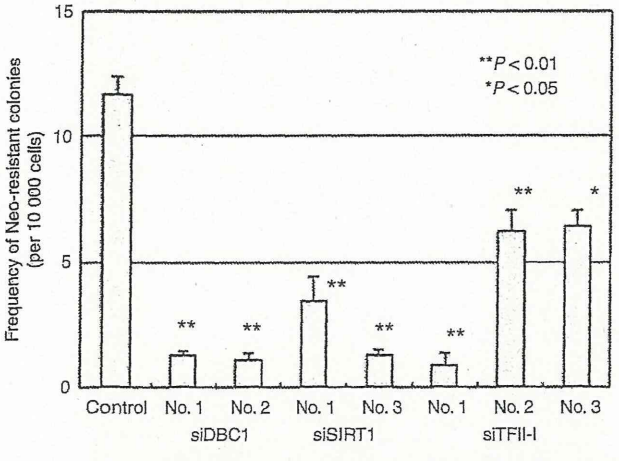

Figure 4. TFII-I and DBC1 enhance DSB repair following irradiation by stimulating homologous recombination. The efficiency of HR repair was analysed after the depletion of endogenous TFII-I or DBC1. The siRNA-mediated knockdown of TFII-I or DBC1 was performed prior the transfection of I-Sce-I expression vectors, and the neomycin-resistant colony formation of SW480sn3 cells was investigated. The effect of the depletion of endogenous SIRT1 was also investigated as a positive control. *$P < 0.05$; **$P < 0.01$.

factor (Hakre *et al*, 2006; Roy, 2012); however, the data regarding the functions of TFII-I in carcinogenesis are limited. The physiological role of DBC1 has been extensively investigated, and caspase-dependent cleavage of DBC1 may work as a positive feedback mechanism to promote apoptosis (Sundararajan *et al*, 2005). This would be an explanation how DBC1 functions as a tumour suppressor (Hamaguchi *et al*, 2002). Furthermore, DBC1 possesses both tumorigenic (Trauernicht *et al*, 2007; Hiraike *et al*, 2010; Koyama *et al*, 2010) and anti-tumorigenic properties (Kim *et al*, 2008; Zhao *et al*, 2008) similar to SIRT1, and we characterised DBC1 as a possible transcriptional factor because oestrogen receptor β (Koyama *et al*, 2010) and BRCA1 (Hiraike *et al*, 2010) are involved in the basal transcriptional machinery. Our previous studies suggested the possibility that TFII-I and DBC1 may have a role in modulating the physiological activity of the BRCT domain, and the importance of the BRCT domain in growth suppression is highlighted by the fact that the BRCT domain is found in a diverse group of proteins implicated in DNA repair and cell-cycle checkpoint control (Bork *et al*, 1997; Callebaut and Mornon, 1997), and we determined to pursue the functions of TFII-I and DBC1 in cell-cycle control and DNA repair processes.

Depletion of TFII-I or DBC1 could show various effects upon cellular growth. Although TFII-I knockdown accelerated the growth of MCF-7 cells, the flow cytometric analysis showed modest effects on cell-cycle progression in MCF-7 cells (Ogura *et al*, 2006), and depletion of endogenous DBC1 inhibited oestrogen-independent proliferation in MCF-7 cells (Trauernicht *et al*, 2007). TFII-I transcriptionally regulates the cyclin D1 status, and thus the cell cycle, by binding to the promoter region of cyclin D1 containing an initiator element (Eto, 2000; Desgranges *et al*, 2005). The cell-cycle regulation by TFII-I was also manifested by our data that siRNA-mediated depletion of endogenous TFII-I resulted in an accumulated G1–S boundary cell fraction after thymidine release (Figure 1D). We hypothesise that TFII-I primarily has a role in the regulation of the G1–S transition and our flow cytometric analyses were consistent with the previous observations (Desgranges *et al*, 2005). In addition, here we demonstrated a novel function that DBC1 modulates cell cycle similar to TFII-I and plays a role at G2-M transition because DBC1 repressed GADD45 promoter activity (Figure 1C) and the flow cytometric data showed G2/M transition delay in DBC1 knockdown cells (Figure 1D). Contrary to this novel function, deletion of DBC1 increase cell growth (Figure 1A). These data are apparently inconsistent with each other. Considering the BRCA1-mediated activation of p21 promoter of these proteins (Supplementary Figure S1), we might speculate that TFII-I and/or DBC1 could affect the control of multiple cell-cycle regulators, thus resulting in peculiar cell growth pattern. The crucial question that must be addressed in the future is whether the recruitment of TFII-I and DBC1 is a signal-regulated process and, if so, how to identify the signals involved.

When cells are exposed to ionising radiation, both BRCA1 and RAD51 localise to the damaged regions, and both initiate HR, resulting in the repair of DSBs. Our previous data suggested the possibility that both BRCA1 and TFII-I participate in the DNA damage repair pathway (Tanikawa *et al*, 2011), and this observation is consistent with the previous data that TFII-I influences the persistence of γH2AX foci and thus affects DSB repair (Desgranges and Roy, 2006). Although these data suggested a role for TFII-I in DNA repair, the precise mechanism underlying DSB repair remained to be solved. We clearly demonstrated a novel mechanism by which endogenous TFII-I promotes DSB repair after irradiation by participating in the HR process in this study. These results also suggest another possible mechanism underlying how TFII-I regulates DNA damage machinery because SIRT1 possesses DNA repair activity (Jeong *et al*, 2007; Wang *et al*, 2008) and TFII-I could serve as a transcription factor, thereby inducing genes such as SIRT1 (Tanikawa *et al*, 2011). Collectively, we hypothesise that TFII-I functions to affect DNA repair in addition to its many other roles. In the future, we intend to address the effects of loss-of- and gain-of-function of TFII-I on DNA damage response using human samples.

Meanwhile DBC1 may function as a G2–M checkpoint factor, two recent studies suggested a function of DSB repair properties of DBC1, and these studies established the importance of phosphorylation at Threonine 454 residue of DBC1 by ATM/ATR following genotoxic stress (Yuan *et al*, 2012; Zannini *et al*, 2012). Although we were unable to detect a colocalization between γH2AX and DBC1, which was distributed throughout the chromatin, it can be attributed to the fact that phosphorylated DBC1 did not show a colocalization with γH2AX (Zannini *et al*, 2012). Here we revealed that DBC1 could serve as a part of DNA repair machinery, and this novel function of DSB repair by DBC1 depends on its HR ability because DBC1 can generally compete with SIRT1. As a result of competition, DBC1 might have an ability to modulate DNA repair functions (Figure 4). This finding is translated as an anti-tumorigenic property of DBC1, and one hypothetical explanation of our findings about DBC1 could be a checkpoint factor enabling cells to enter mitosis after the repair of damaged DNA. This hypothesis should be further examined by *in vivo* works using animal models in the future. Williams–Beuren syndrome is estimated to occur in approximately 1 in 10 000 persons, and most patients have a shortened life expectancy, due to complications. With the improvement of life expectancy, it may be apparent that the patients of Williams–Beuren syndrome may have an increased tumour predisposition in the near future.

In conclusion, our data indicate that TFII-I and DBC1 govern anti-tumourigenic processes and play important roles in regulating BRCA1-related functions. Because the biological and functional relationship of the TFII-I–DBC1 axis remains unanswered, we must further test the expression of TFII-I and DBC1 in tumour tissues. An analysis of the expression levels of TFII-I, SIRT1, and DBC1 would be beneficial because they can affect different BRCA1-mediated regulatory pathways, and the inhibition of BRCA1 functions by TFII-I and DBC1 may be a key event in cancer predisposition.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Review Article

PDZ Domains and Viral Infection: Versatile Potentials of HPV-PDZ Interactions in relation to Malignancy

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Cervical cancer is caused by high-risk human papillomaviruses (HPVs), and a unique characteristic of these is a PDZ (PSD-95/Dlg/ZO-1-)binding motif in their E6 proteins. Through this motif HPV E6 interacts with a variety of PDZ domain-containing proteins and targets them mainly for degradation. These E6-PDZ interactions exhibit extraordinarily different functions in relation to HPV-induced malignancy, depending upon various cellular contexts; for example, Dlg and Scrib show different distribution patterns from what is seen in normal epithelium, both in localization and in amount, and their loss may be a late-stage marker in malignant progression. Recent studies show that interactions with specific forms of the proteins may have oncogenic potential. In addition, it is interesting that PDZ proteins make a contribution to the stabilization of E6 and viral episomal maintenance during the course of HPV life cycle. Various posttranslational modifications also greatly affect their functions. Phosphorylation of hDlg and hScrib by certain kinases regulates several important signaling cascades, and E6-PDZ interactions themselves are regulated through PKA-dependent phosphorylation. Thus these interactions naturally have great potential for both predictive and therapeutic applications, and, with development of screening tools for identifying novel targets of their interactions, comprehensive spatiotemporal analysis is currently underway.

1. Introduction

PDZ domain-containing proteins are ubiquitous protein interaction modules and possess multiple functions that are essential to maintain cellular homeostasis. PDZ domain recognition motifs are often encoded by pathogenic viruses, including high-risk HPVs, and through this motif they target various cellular proteins via protein-protein interactions; these HPV-PDZ interactions are in part associated with virally induced cancer progression. However, as this two-way interplay exhibits diverse characteristics and the activities are often combinatorial, depending upon various cellular contexts, extensive understanding of their roles in diverse aspects of cancer has a long way to go. In this paper we focus on the multiple functions that HPV E6-PDZ interactions potentially have in relation to HPV-induced malignancy, depending upon their localizations, stages along the time-axis of malignant progression, and various posttranslational modifications such as phosphorylation by various kinases and we also consider their potential for both predictive and therapeutic applications in HPV-induced malignancy.

2. HPVs as the Cause of Cervical Cancer

When exposed to infectious viruses, humans may eliminate infection by their innate immune system, or the infection may progress in stages from mild symptoms to serious, long-term impairment. Some human viruses have been found to cause cancer, accounting for 10–15% of human cancers worldwide [1], and specific viral oncogenes, such as Tax in human T-cell leukemia virus, are identified as responsible for causing tumors in the infected cells. Perhaps the best studied among them are the E6 and E7 oncoproteins in HPVs, the cause of cervical cancer.

2.1. Characteristics of Cervical Cancer. Cervical cancer is the third most common female cancer, with 530,000 new cases