BS69 is involved in cellular senescence through the p53–p21Cip1 pathway

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The multidomain-containing cellular protein BS69 interacts with adenovirus E1A and several other viral and cellular factors, and acts as a transcription repressor. Here, we show that BS69 is involved in the p53–p21Cip1-mediated senescence pathway. Knockdown of BS69 by RNA interference in human primary fibroblasts results in elevated levels of p21Cip1 and the appearance of several senescent markers, including enhanced senescence-associated β -galactosidase activity and formation of senescence-associated heterochromatin foci. Importantly, knockdown of either p53 or p21Cip1, but not p16^{INK4a} or Rb, allows cells to bypass premature senescence that is induced by BS69 knockdown. Furthermore, we show that BS69 forms complexes with both p53 and p400, and that BS69 associates with the p21Cip1 promoter through p53. Together, our data indicate that BS69 is involved in cellular senescence mainly through the p53–p21Cip1 pathway.

Keywords: BS69; p53; p400; p21Cip1; senescence

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INTRODUCTION

BS69, a multidomain cellular protein containing PHD, Bromo, PWWP and MYND, was originally identified as an adenovirus E1A-binding protein that inhibits the transactivation function of E1A (Hateboer *et al*, 1995). The carboxy-terminal MYND domain of BS69 was shown to bind to the PXLXP motif present in E1A, the Epstein–Barr virus (EBV)-encoded EBNA2 and a Myc-related cellular protein MGA (Ansieau & Leutz, 2002). We recently showed that BS69 also interacts with latent membrane protein 1 (LMP1), another EBV-encoded protein, through its MYND domain and acts as a scaffold protein in the LMP1-mediated c-Jun amino-terminal kinase (JNK) pathway (Wan *et al*, 2006). Previous work by other groups also implicated BS69 in the regulation of gene

transcription. BS69 could function as a transcription repressor in association with various transcription factors such as c-Myb, B-Myb, Ets2 and MGA (Ladendorff *et al*, 2001; Masselink *et al*, 2001; Wei *et al*, 2003; Ekblad *et al*, 2005). BS69 was also shown to repress transcription by recruiting N-CoR (Masselink & Bernards, 2000). In addition, BS69 was found to associate with mitotic chromosomes and to interact with Brg1 (the catalytic subunit of the mammalian SWI/SNF complex), which suggests a role for BS69 in chromatin remodelling (Velasco *et al*, 2006).

Here, we provide evidence showing that knockdown of BS69 in human primary fibroblasts induces premature senescence. p53 and p21Cip1, but not p16^{INK4a} or Rb, act downstream of BS69 to mediate its effect. Furthermore, BS69 forms a complex with both p53 and p400, a nuclear protein implicated in cellular senescence (Chan *et al*, 2005). Collectively, our data indicate that BS69 is involved in cellular senescence mainly through the p53–p21Cip1 pathway.

RESULTS

Knockdown of BS69 induces cell-cycle arrest

To assess the functional role of BS69 in cell growth, we chose to knock down the expression of the endogenous BS69 gene by RNA interference (RNAi) in IMR90 cells, which are untransformed primary human diploid fibroblasts (Serrano *et al*, 1997). We generated two lentivirus-mediated short hairpin RNAs (shRNAs) that targeted distinct regions of human BS69 (Wan *et al*, 2006; Fig 1D; supplementary Fig S1 online). Another lentiviral vector expressing an shRNA that targeted the jellyfish green fluorescence protein (GFP) was used as a negative control. Knockdown of BS69 in IMR90 cells significantly inhibited cell proliferation (Fig 1A), increased the percentage of cells arrested in G1 phase (Fig 1B) and decreased bromodeoxyuridine (BrdU) incorporation (Fig 1C).

As BS69 can act as a transcription repressor (Ladendorff *et al*, 2001; Masselink *et al*, 2001; Wei *et al*, 2003; Ekblad *et al*, 2005), knockdown of BS69 was expected to change the expression levels of certain target genes, such as those involved in cell-cycle regulation, which could subsequently affect cell growth. Indeed, we detected induction of p21Cip1, but not p53 or p16^{INK4a}, in IMR90 cells infected with the lentivirus expressing BS69-shRNA (Fig 1D). Consistently, the levels of Bmi1, a member of the *polycomb* group transcription repressor, which negatively regulates the expression of p16^{INK4a} (Park *et al*, 2004), were not

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Fig 1 | Knockdown of BS69 inhibits cell proliferation in IMR90 cells. Duplicate IMR90 cells were infected with the shRNA-expressing lentiviruses and selected with blasticidin for 6 days. (A) WST-1 assays were carried out at the indicated time points with the absorbance at day 6 set as 1. The experiment was repeated three times and the data shown represent mean $\pm$ s.d. (B) Cells were trypsinized and fixed, followed by the addition of propidium iodide. The samples were then subjected to FACS analysis. (C) BrdU was added to cells. The incorporated BrdU was detected using a fluorescein-conjugated BrdU antibody and visualized by fluorescence microscopy. The percentage of cells that incorporated BrdU was calculated from five randomly chosen fields ($\times 10$ magnifications). The experiment was repeated three times and the results are shown as mean $\pm$ s.d. (D) Cells were collected and 100 μ g of whole cell extracts were subjected to western blotting analysis. (E) Total RNA was extracted from cells. An equal amount of total RNA was subjected to both semiquantitative and quantitative RT-PCR analysis. *Student's *t*-test was carried out and $P < 0.05$ was considered statistically significant. BrdU, bromodeoxyuridine; FACS, fluorescent activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; RT-PCR, reverse transcription-PCR; shRNA, short hairpin RNA.

significantly altered by BS69-shRNA. To show whether *p21Cip1* messenger RNA levels were affected by BS69-shRNA, we collected total RNA from adenovirus-infected IMR90 cells and carried out both semiquantitative and real-time reverse transcription-PCR (RT-PCR). The *p21Cip1* mRNA levels in BS69-shRNA-infected cells were elevated by about fourfold compared with those in GFP-shRNA-infected cells (Fig 1E). Our data indicated that the induction of p21Cip1 protein expression by BS69-shRNA was mainly due to enhanced transcription of *p21Cip1* gene.

Knockdown of BS69 induces premature senescence

As a crucial cell-cycle inhibitor, p21Cip1 has been shown to be induced in senescent cells (Stein *et al*, 1999). Overexpression of *p21Cip1* can cause premature senescence (McConnell *et al*, 1998), whereas loss of *p21Cip1* expression can extend the lifespan of human fibroblasts (Brown *et al*, 1997). The induction of p21Cip1 by BS69-shRNA prompted us to test whether the knockdown of BS69 induces premature senescence. When we

infected IMR90 cells with the lentivirus expressing GFP-shRNA and then subjected the cells to 4',6-diamidino-2-phenylindole (DAPI) staining, a relatively uniform staining pattern was seen in the cell nuclei (Fig 2A, panel 1). By contrast, about 50% of the nuclei in cells infected with the lentivirus expressing BS69-shRNA showed a punctate DAPI staining pattern, resembling senescence-associated heterochromatin foci (SAHF; Narita *et al*, 2003; Fig 2A, panel 3). Consistently, these punctate DAPI staining regions colocalized with HP1 γ (Fig 2A, panel 4), an adaptor molecule that is required for heterochromatin assembly (Bannister *et al*, 2001; Lachner *et al*, 2001) and acts as one of the molecular markers for SAHF (Narita *et al*, 2006).

As senescent cells show high senescence-associated β -galactosidase (SA- β -Gal) activity, which is also widely used as a senescence marker (Dimri *et al*, 1995), we tested whether BS69-shRNA can induce SA- β -Gal activity. RasV12, an oncogenic mutant of Ras, was used as a positive control (Serrano *et al*, 1997). In a manner similar to the RasV12-infected cells, IMR90 cells infected with the lentivirus expressing BS69-shRNA showed

Fig 2 | Knockdown of BS69 induces premature senescence in IMR90 cells. (A,B) IMR90 cells were infected with either shRNA-expressing lentiviruses or a RasV12-expressing retrovirus and selected for 10 days with appropriate antibiotics. (A) Cells were fixed and subjected to immunostaining using antibodies against HP1 γ (red); DAPI (blue) was added to cells to stain nuclei. (B) Cells were fixed and subjected to X-gal staining. For both (A) and (B), the percentage of cells with SAHF or high SA- β -Gal activity was calculated from five randomly chosen fields ($\times 20$ magnification). The experiment was repeated three times and the results are shown as mean $\pm$ s.d. * $P < 0.05$. (C) Chromatin fragments were prepared from cells infected with different lentiviruses for various time points as indicated. Chromatin fractions from 5×10^5 cells were subjected to western blotting analysis. DAPI, 4',6-diamidino-2-phenylindole; GFP, green fluorescent protein; SA- β -Gal, senescence-associated β -galactosidase; SAHF, senescence-associated heterochromatin foci; shRNA, short hairpin RNA.

elevated SA- β -gal activity (Fig 2B). By contrast, cells infected with the lentivirus expressing GFP-shRNA showed no such effect. Furthermore, after BS69 knockdown, the total amount of the chromatin-bound HP1 γ increased substantially (Fig 2C), which is also a feature of senescence (Narita *et al*, 2006). In addition to IMR90 cells, we also repeated the above experiments in WI-38 cells, another strain of normal human fibroblasts, and similar results were obtained (supplementary Fig S1 online).

Using quantitative RT-PCR, we also compared the endogenous mRNA levels of BS69 in young proliferating, RasV12-induced senescent and replication-induced senescent IMR90 cells. We found that the BS69 mRNA levels partly decreased in both senescent cells (supplementary Fig S3 online).

Key mediators in BS69-shRNA-induced senescence

At present, two connected yet distinct senescence-associated pathways have been identified: one involving p53/p21Cip1 and the second involving p16^{INK4a}/Rb (Beausejour *et al*, 2003; Chan *et al*, 2005; Herbig *et al*, 2006). To determine which pathway is used preferentially in BS69-shRNA-induced senescence, we individually knocked down p21Cip1, p53 and Rb in IMR90 cells in combination with BS69 knockdown. All shRNAs effectively knocked down their intended target genes (Fig 3A). It is important to note that, in response to BS69 knockdown, the

senescence-associated markers—such as elevated levels of the chromatin-bound HP1 γ , increased percentage of cells with SA- β -Gal activity and SAHF, and decreased BrdU incorporation—were all significantly inhibited when either p21Cip1 or p53 was knocked down simultaneously with BS69 (Fig 3A–D). By contrast, knockdown of Rb with BS69 had no such effects. In addition, we also noted that the BS69-shRNA-induced upregulation of p21Cip1 was completely eliminated when p53 was also knocked down simultaneously with BS69 (Fig 3A, compare lane 5 with 6), suggesting that p53 is an indispensable mediator in this process. To confirm the above results, we also repeated the experiments in WI-38 cells using two different BS69-shRNAs and a p16^{INK4a}-shRNA; similar results were obtained (supplementary Fig S1 online). Consistently, knockdown of p16^{INK4a}, like that of Rb, could not rescue BS69-shRNA-induced senescence.

To confirm further that a functional p53 is required to mediate the BS69-shRNA-induced molecular and cellular changes, we introduced BS69-shRNA into two immortalized human cell lines: U2OS, an osteosarcoma-derived cell line carrying the wild-type p53 alleles, and HeLa, a human cervical carcinoma-derived cell line in which the p53 function was compromised by the human papillomavirus-encoded E6 protein (Scheffner *et al*, 1991). When we knocked down BS69 in U2OS cells, we detected elevated levels of p21Cip1 expression (data not shown), an increased

Fig 3 | Knockdown of either *p53* or *p21Cip1*, but not *Rb*, inhibits BS69-shRNA-induced senescence. (A–D) IMR90 cells were infected with lentiviruses in different combinations as indicated and selected for 10 days with blasticidin. (A) 100 μ g of whole cell extracts or chromatin fragments from 5×10^5 cells were subjected to western blotting analysis. The experiments were repeated three times. The intensity of the bands was quantified by a densitometer with that from cells containing GFP-shRNA set as 1. The 'fold' increase in protein levels from test samples relative to the control was calculated and the results are shown as mean $\pm$ s.d. (B) Cells were fixed and subjected to X-gal staining. (C) Cells were fixed followed by the addition of DAPI. For both (B) and (C), the percentage of cells showing high SA- β -Gal activity and SAHF was calculated in the same way as described in Fig 2A,B, respectively. (D) BrdU incorporation was carried out and the data are presented the same way as described in Fig 1C. (E,F) U2OS and HeLa cells were separately infected with shRNA-expressing lentiviruses as indicated and selected with blasticidin for 6 days. (E) FACS analysis was carried out and the data are presented the same way as described in Fig 1B. (F) BrdU incorporation assays were carried out and the data are presented the same way as described in Fig 1C. * $P < 0.05$. BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; FACS, fluorescent activated cell sorting; GFP, green fluorescent protein; SA- β -Gal, senescence-associated β -galactosidase; SAHF, senescence-associated heterochromatin foci; shRNA, short hairpin RNA.

percentage of cells arrested in G1 and decreased BrdU incorporation (Fig 3E,F); however, no such effects were observed in *p53*-defective HeLa cells. As BS69-shRNA seemed to upregulate *p21Cip1* through the activation of *p53*, it suggested that BS69

normally represses the function of *p53*. We showed, by chromatin immunoprecipitation (ChIP) assays, that knockdown of BS69 did not affect *p53* binding to the *p21Cip1* promoter (supplementary Fig S2 online). To assess whether BS69 affects the transcriptional

activity of p53, we co-transfected the p53-deficient Saos-2 cells with a luciferase reporter gene under the control of a 2.4 kb human *p21Cip1* promoter with two p53-binding sites (el-Deiry *et al*, 1995), a p53 expression vector and increasing amount of a BS69 expression vector. We found that BS69 repressed p53 transcriptional activity in a dose-dependent manner (supplementary Fig S2 online). In addition to *p21Cip1*, we also checked the protein levels of several other well-established p53 target genes in IMR90 cells infected with lentiviruses encoding either GFP-shRNA or BS69-shRNA. Similar to *p21Cip1*, we found that the protein levels of murine double minute 2 (MDM2), Fas and Bax were elevated when BS69 was knocked down. By contrast, the levels of plasminogen activator inhibitor 1 and GADD45 (Growth arrest and DNA-damage-inducible protein 45) were not affected (supplementary Fig S2 online). This suggested that BS69 selectively affects a subset of p53 target genes.

BS69 associates with p53 at the *p21Cip1* promoter

As BS69-shRNA upregulates *p21Cip1* expression through p53, we asked next whether BS69 interacts with p53 to modulate *p21Cip1* gene transcription in cells. First, we transfected 293 cells with a vector encoding Flag-tagged BS69. Co-immunoprecipitation experiments showed that BS69 indeed forms a complex with the endogenous p53 in 293 cells (Fig 4A, top two panels). Furthermore, in non-transfected 293 cells p53 could also be co-precipitated when we immunoprecipitated the endogenous BS69 (Fig 4A, bottom panel). As p53 directly regulates *p21Cip1* gene expression by binding to two consensus sites in the *p21Cip1* promoter (Fig 4B, sites 1 and 2; el-Deiry *et al*, 1995), we tested next whether p53 is necessary for BS69 to associate with the *p21Cip1* promoter. By using ChIP assays, we showed the coexistence of BS69 and p53 on two p53-binding sites, but not an adjacent control site (site no. 3), in the *p21Cip1* promoter in p53-positive IMR90 cells (Fig 4C, left panel; Chan *et al*, 2005). By contrast, no signals were detected at two p53-binding sites of the *p21Cip1* promoter in p53-defective HeLa cells (Fig 4C, middle panel). To confirm further that BS69 forms a complex with p53, we carried out re-ChIP assays by first immunoprecipitating BS69 from cell lysates with the BS69 antibody followed by re-immunoprecipitation of p53 with the p53 antibody. As a result, specific PCR-amplified signals were only detected at two p53-binding sites but not the control site (Fig 4C, right panel).

The protein p400 was recently shown to be involved in the p53–*p21Cip1* senescence pathway (Chan *et al*, 2005). The functional similarity between BS69-shRNA and p400-shRNA in inducing senescence through the p53–*p21Cip1* pathway prompted us to test whether BS69 and p400 coexist in the same complex. 293 cells were transfected with a vector encoding Flag–BS69. When the endogenous p400 was immunoprecipitated, we found that Flag–BS69 was also co-precipitated; by contrast, Flag–BS69 was not co-precipitated when a control antibody was used (Fig 4D).

DISCUSSION

BS69 has been shown to interact with several viral and cellular proteins, including E1A, EBNA2, LMP1, MGA and Myb (Hateboer *et al*, 1995; Ladendorff *et al*, 2001; Masselink *et al*, 2001; Ansieau & Leutz, 2002; Wan *et al*, 2006). In one case, BS69 (or an alternatively spliced isoform of BS69) acts to bridge EBV-encoded

Figure 4 | BS69 associates with the *p21Cip1* promoter and forms a complex with both p53 and p400. (A) 293 cells were either transfected with plasmids, as indicated, or left untransfected. Whole cell extracts were subjected to immunoprecipitation (IP) using the indicated antibodies. The co-precipitated p53 was detected by western blotting. (B) A schematic of the human *p21Cip1* promoter showing the positions of the p53 binding sites (numbers 1 and 2) and a control site (number 3). (C) IMR90 and HeLa cells were subjected to ChIP and re-ChIP analysis using antibodies and primers as indicated. (D) 293 cells were transfected with Flag–BS69. WCE were subjected to IP using either a control antibody or anti-p400. Co-precipitated Flag–BS69 was detected by immunoblotting (IB). IgG is the control antibody. (E) A schematic of the BS69/p400 complex involved in the p53–*p21Cip1* senescence pathway. ChIP, chromatin immunoprecipitation; WCE, whole-cell extracts.

LMP1 and TRAF6 in the LMP1-induced JNK pathway (Wan *et al*, 2006). However, in many other cases, BS69 functions to repress the transcriptional activity of its associated factors (Masselink & Bernards, 2000; Wei *et al*, 2003; Ekblad *et al*, 2005). How BS69 represses transcription is unclear. Presumably, it does so by recruiting molecules such as histone deacetylases, as BS69 does not contain domains with intrinsic repressive activity (Velasco *et al*, 2006). Other than the features above, the normal cellular function of BS69 remains unclear. Furthermore, we do not know the actual target genes controlled by BS69; therefore, the present work represents the first example, to our knowledge, in which several BS69 target genes, for example, *p21Cip1*, *MDM2* and *Fas*, are identified. We have shown that BS69 regulates these genes through p53; however, as not all known p53 target genes are affected by BS69, it suggests that BS69 only selectively associates with a fraction of p53-responsive promoters. By fine-tuning the

expression levels of *p21Cip1*, BS69 could function as a 'sensor' in the normal cellular growth programme. In response to the loss of BS69 function—either owing to downregulation of its expression levels by some yet to be identified extracellular signals or to sequestration by another protein—cells will enter the senescence state as an intrinsic cellular defense mechanism. The presence of other transforming genes might result in full-blown cell transformation (Land *et al*, 1983; Serrano *et al*, 1997).

Two connected yet distinct senescence pathways have been identified: one consisting of p53/p21Cip1 and the second p16^{INK4a}/Rb (Beausejour *et al*, 2003; Chan *et al*, 2005; Herbig *et al*, 2006). Although RasV12-induced senescence involves both pathways (Serrano *et al*, 1997), cellular senescence induced by the knockdown of either *BS69* or *p400* involves only p53/p21Cip1 (Fig 3; supplementary Fig S1 online; Chan *et al*, 2005). However, it remains unclear which upstream stimuli preferentially use the p53/p21Cip1 pathway to trigger senescence. This might explain why *BS69* mRNA levels were only partly decreased in both RasV12-induced and replication-induced senescent cells (supplementary Fig S3A online). Consistently, overexpression of *BS69* is not sufficient to bypass RasV12-induced and replication-induced senescence (supplementary Fig S3B,C online). As to the relationship between BS69 and p400, in many aspects, BS69 behaves like p400 as both proteins interact with adenovirus E1A protein (Hateboer *et al*, 1995; Fuchs *et al*, 2001) and knockdown of either *BS69* or *p400* in IMR90 cells induces senescence (Chan *et al*, 2005). Interestingly, we found that BS69 forms a complex with p400 in cells. We propose that a transcription repressive complex, consisting of p400, BS69 and possibly some other yet to be identified proteins, interacts with p53 to repress its transcriptional activity (Fig 4E). Loss of either protein relieves the repression on p53 and promotes cells to enter the senescence state through the p53/p21Cip1 pathway. In the future, it is important to identify other protein components in the p400–BS69 complex, to understand how these proteins interact with each other in the complex, and to find out exactly how these proteins interact with p53 and repress its transcriptional activity. Only by doing so can we obtain a clearer picture of the role of BS69 in cellular senescence and get more insights into the normal physiological function of BS69.

METHODS

Cell culture and virus infection. IMR90, WI-38, U2OS, Saos-2, BING, 293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Lentivirus-based shRNA was generated using the BLOCK-iT lentiviral RNAi expression system (Invitrogen, Carlsbad, CA, USA). Amphotropic retrovirus was produced in BING cells. The infected cells were selected using either 10 µg/ml of blasticidin for lentivirus or 100 µg/ml of hygromycin for retrovirus.

Cell proliferation and SA-β-gal assays. Pre-mix WST-1 cell proliferation assay kit (Takara, Otsu, Japan) was used to generate the cell growth curve. SA-β-gal activity was detected as described previously (Dimri *et al*, 1995; Serrano *et al*, 1997; Narita *et al*, 2003).

Chromatin immunoprecipitation assay. ChIP assays were carried out as described previously (Shang *et al*, 2000) with slight modifications. Quantitative ChIP was carried out using the Stratagene (La Jolla, CA, USA) Mx3000P QPCR system.

SYBR green-based quantitative RT–PCR. Total RNA was isolated from cells with TRIzol reagent (Invitrogen). The first strand complementary DNA was synthesized with the ImProm-II reverse transcription system (Promega, Madison, WI, USA). The 2 × SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) was used in 25 µl quantitative PCR reactions, according to the manufacturer's instructions.

Other methods. Detailed methods are described in the supplementary information online.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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