Published in final edited form as:
Oncogene. 2006 July 20; 25(31): 4370–4375.

Loss of the human polycomb group protein BMI1 promotes cancer-specific cell death

L Liu[1,2], **LG Andrews**[1], and **TO Tollefsbol**[1,2,3]

1*Department of Biology, University of Alabama at Birmingham, AL, USA*

2*Center for Aging, University of Alabama at Birmingham, AL, USA*

3*Comprehensive Cancer Center, University of Alabama at Birmingham, AL, USA*

Abstract

The polycomb group protein BMI1 has been shown to support normal stem cell proliferation via its putative stem cell factor function, but it is not known if BMI1 may also act as a cancer stem cell factor to promote cancer development. To determine the role of human BMI1 in cancer growth and survival, we performed a loss-of-function analysis of BMI1by RNA interference (RNAi) in both normal and malignant human cells. Our results indicate that BMI1 is crucial for the short-term survival of cancer cells but not of normal cells. We also demonstrated that loss of BMI1 was more effective in suppressing cancer cell growth than retinoid-treatment, and surviving cancer cells showed significantly reduced tumorigenicity. The cancer-specific growth retardation was mediated by an increased level of apoptosis and a delayed cell cycle progression due to the loss of BMI1. By comparison, BMI1 deficiency caused only a moderate inhibition of the cell cycle progression in normal lung cells. In both normal and cancer cells, the loss of BMI1 led to an upregulation of *INK4A-ARF*, but with no significant effect on the level of telomerase gene expression, suggesting that other BMI1-cooperative factors in addition to *INK4A-ARF* activation may be involved in the BMI1-dependent cancer-specific growth retardation. Thus, human BMI1 is critical for the short-term survival of cancer cells, and inhibition of BMI1 has minimal effect on the survival of normal cells. These findings provide a foundation for developing a cancer-specific therapy targeting *BMI1*.

Keywords

BMI1; cancer stem cells; telomerase; INK4A-ARF; PcG proteins

Introduction

The *BMI1* gene encodes a nuclear protein with an oncogenic potential via cooperation with MYC (Haupt et al., 1991; van Lohuizen et al., 1991). Elevated expression of human *BMI1* has been reported in multiple cancer samples and cancer cell lines (Bea et al., 2001; Vonlanthen et al., 2001; Dimri et al., 2002; Leung et al., 2004). Overexpression of BMI1 can also transform and immortalize normal fibroblasts and mammary epithelial cells via reactivation of the *human telomerase reverse transcriptase* (*hTERT*) gene in these cells (Jacobs et al., 1999a; Dimri et al., 2002; Leung et al., 2004). Such oncogenic effects of BMI1 are consistent with other studies showing an extended life span in both rodent and human fibroblasts overexpressing BMI1 (Cohen et al., 1996; Itahana et al., 2003; Takeda et al., 2004; Mori et al., 2005; Terai et al., 2005). More recently, BMI1 activity has been proposed to be an important marker for predicting

Correspondence: Dr L Liu, University of Alabama at Birmingham, Department of Biology, 175 Campbell Hall, 1300 University Boulevard, Birmingham, AL 35294-1170, USA. E-mail: liangliu@uab.edu.

the progression of myelodysplastic syndrome and the prognosis of patients (Mihara et al., 2006).

Analysis of *Bmi1*-null mice, however, suggests that *Bmi1* is important for normal development (van der Lugt et al., 1994; Molofsky et al., 2005). *Bmi1*-knockout mice are viable, but display reduced fitness and other developmental defects in hematological, neurological and skeletal systems. Such developmental abnormalities may be linked to a putative stem cell factor function of Bmi1 (Molofsky et al., 2003; Park et al., 2003; Shakhova et al., 2005), and therefore the loss of Bmi1 could lead to an exhaustion of tissue stem cells that compromises tissue growth. Furthermore, BMI11 is also implicated to sustain both self-renewal and proliferation of neural stem cells by repressing the function of Ink4a-Arf that can lead to growth arrest and premature senescence (Jacobs et al., 1999a; Bruggeman et al., 2005; Molofsky et al., 2005).

Although BMI1 has been implicated as an oncogenic player, very little is known about the exact function of BMI1 in cancer growth and progression. We applied RNA interference (RNAi) to suppress BMI1 expression in both normal and cancerous human cells. BMI1-targeting dsRNA was transfected into human neuroblastoma (NB) cells to induce RNAi of BMI1. As shown in Figure 1a, RNAi-treated NB cells grew poorly with elevated apoptosis 3 days after treatment (Figure 1A-a, right). By comparison, the growth and survival of NB cells were not affected by the control treatment with a mixed pool of scrambled (scr) siRNAs (Figure 1A-a, left). In normal neurons, however, RNAi of BMI1 had no visible effects on their growth and survival (Figure 1A-b,). We applied the same studies on many other normal and cancerous human cells, and obtained similar results (Figure 1A-c, d, and Table 1). To confirm that the transfected dsRNA indeed caused a knockdown of BMI1 in RNAi-treated cells, both RT–PCR and Western blotting analysis were performed to confirm that there was an effective knock-down of BMI1 in all of the RNAi-treated cells (Figure 1B, C and data not shown). The target-specificity of RNAi was confirmed by RT–PCR analysis of several other genes, including *DNMTs* and *MYC*, which indicated no significant expression variations of these genes (data not shown). Collectively, these findings suggest that BMI1 is crucial for the short-term survival of cancer cells but not for normal cells despite that the loss of BMI caused a moderate growth inhibition of normal cells.

Another intriguing function of BMI1 during development is its role in maintaining the proliferation of tissue stem cells (Park et al., 2003; Shakhova et al., 2005). Cancer stem cells (CSCs) have been identified recently in several human cancers including acute myeloid leukemia, breast cancer, and brain tumors (Blair et al., 1997; Al-Hajj et al., 2003; Singh et al., 2004). These tumorigenic stem cells can both self-renew to form new tumorigenic cells and also differentiate to form abnormal cells with mixed phenotypes (Pardal et al., 2003). To determine whether loss of BMI1 will affect only the survival of cancer stem cells but not normal embryonic stem cells, we compared the effect of RNAi of BMI1 on NCCIT human embryonic carcinoma (EC) cells, which are malignant pluripotent stem cells, with that on normal embryonic stem (ES) cells. Both EC and ES cells grow in clumps known as foci (Figure 1A-c, d, × 200 magnification). RNA interference of BMI1 led to significant EC cell deaths and reduction in focus formation 3 days after treatment (Figure 1A-c, right); whereas no significant cell death or reduction in focus formation was observed in normal ES cells treated with or without RNAi (Figure 1A-d). Alkaline phosphatase (AP) activity is a biochemical marker for undifferentiated ES cells. Our AP-staining analysis also indicated no significant changes in the stemness of normal ES cells following RNAi of BMI1 during the experimental time-frame of this study, but we observed a reduced thickness of the colonies in the RNAi-treated ES cells compared with the scr-treated ES cells (Figure 1A-d). Since BMI1-deficiency affects the survival of EC cells but not normal ES cells, it is tempting to speculate that BMI1 may serve as a CSC factor to maintain the growth and survival of cancer.

Retinoids are commonly used drugs in clinical cancer therapy. We have previously reported that *all-trans* retinoic acid (ATRA) can induce cancer cell differentiation and death via telomerase inhibition (Liu et al., 2004). To determine that BMI1 can serve as a more effective molecular target for cancer therapy, we compared the efficacy of growth inhibition on EC cells treated by RNAi of BMI1 with that by ATRA. As shown in Figure 2a, growth kinetics of EC cells maintained in the presence of 2 μ_M ATRA were similar to those of normal control cells, whereas there was a significant decline in the number of surviving EC cells 3 days after RNAi treatment. Normal lung cells also displayed a moderate decrease in proliferation 3 days after RNAi treatment, but the decrease was much less profound than that seen in the EC cells (Figure 2b). Similar growth effects were observed in other cancer and normal cell cultures, respectively (data not shown), which is consistent with the findings presented in Table 1. These results clearly indicate that cancer therapy targeting BMI1 can be highly effective and specific in its inhibitory effects on cancer cell growth. To determine the tumorigenecity of cancer cells following RNAi treatment, we performed soft agar colony formation assays on RNAi-treated and control NB cells. Soft agar analysis of colony formation is one of the best *in vitro* determinations of cellular transformation (Kakunaga and Yamasaki, 1985; Casillas et al., 2003) because this assay tests for features essential to tumorigenesis such as growth independent of both cellular and extracellular matrix interactions and clonal growth. As shown in Figure 2c, the number of colonies formed by RNAi-treated NB cells was significantly less than those formed by either scr-treated NB cells or normal control NB cells during the first 5 days. By day 10, however, we observed a tendency of increased colony formation from the RNAi-treated NB cells, which may be due to the exhaustion of the dsRNA molecules and subsequent diminished RNAi effect. No colonies were formed by either RNAi-treated or scr-treated normal neurons (data not shown). These findings suggest that RNAi of BMI1 can effectively ablate the tumorigenicity of NB cells, but this effect requires long-term availability of dsRNA.

As summarized in Table 1, RNAi of BMI1 caused a moderate reduction in the proliferation of normal cells. This may be linked to the *INK4A-ARF* locus, which plays a major role in the regulation of cell proliferation via its p16 and p14 products (Jacobs et al., 1999a;Itahana et al., 2003;Bruggeman et al., 2005;Molofsky et al., 2005;Mihara et al., 2006). As BMI1 acts as a negative regulator of the *INK4A-ARF* locus, overexpression of BMI1 can repress p16 expression, and thus lead to an immortalization of murine fibroblasts and postpone cellular senescence in normal human cells (Jacobs et al., 1999a;Dimri et al., 2002;Takeda et al., 2004;Mori et al., 2005;Terai et al., 2005). To understand the cellular and molecular mechanisms underlying the growth retardation of cancer cells by RNAi of BMI1, we employed BrdU incorporation assays to detect the cell cycle positions of both BMI1 RNAi-treated and scrambled control-treated EC cells. As shown in Figure 3a, EC cells displayed a significant increase in the percentage of apoptotic cells (from 1.1 to 10.8%) together with a significant decrease in the percentage of actively dividing S-phase cells (from 94.7 to 50.4%) following RNAi treatment (Figure 3a, upper panel). By contrast, similar studies on normal lung cells showed that loss of BMI1 did not cause any significant apoptosis, but instead lead to a decrease in the percentage of S-phase normal lung cells (from 93.6 to 79.1%) (Figure 3a, lower panel). The decrease in the percentage of S-phase lung cells was less profound than that seen in EC cells, and it may well explain the moderate growth inhibition of normal cells in the absence of BMI1 as shown in Table 1. By means of real-time PCR analysis, we also showed that there was an elevated expression of INK4A-ARF after the knockdown of BMI1 in RNAi-treated normal and cancer cells except neurons (Figure 3b), which may explain the delayed progression into S-phase as described above in both EC cells and normal lung cells. hTERT expression in cancer cells, however, was not downregulated during the experimental time-frame of this study (Figure 3b), suggesting that cancer-specific cell death does not require the inhibition of telomerase activity. This is consistent with previous findings suggesting that BMI1 may well act upstream of telomerase activity during cellular transformation (Dimri et al., 2002).

Since upregulation of INK4A-ARF is observed in both RNAi-treated normal and cancer cells, the INK4A-ARF-mediated cell cycle delay does not explain the BMI1-dependent apoptosis of cancer cells. The severe growth retardation of cancer cells, therefore, should be mainly attributed to the increased apoptosis of cancer cells. As the loss of BMI1 does not acutely downregulate hTERT expression in human cancer cells, the cancer-specific acute cell death must involve pathways other than telomerase inhibition. One candidate pathway may be related to the synergistic relationship between MYC and BMI1 during tumor development as exemplified by mouse lymphomagenesis (Haupt et al., 1991; van Lohuizen et al., 1991; Jacobs et al., 1999b). It is possible that BMI1 may serve as a universal partner for MYC during cellular transformation. The loss of BMI1 will thus compromise the oncogenic activity of MYC in the maintenance of malignant growth (Adhikary and Eilers, 2005).

In summary, our studies have clearly shown that loss of BMI1 has an acute detrimental effect on the survival of human cancer cells that may involve its potential function as a candidate CSC factor. RNAi of BMI is highly effective in both retarding cancer growth and ablating the tumorigenicity of cancer cells, and has minimal effect on the survival of normal cells. Additional studies will be directed toward assessing the *in vivo* efficacy of RNAi of BMI1 that will facilitate the development of a new therapeutic protocol for treating cancer patients. Future studies to confirm BMI1 as a *bona fide* CSC factor are warranted and will greatly enhance our understanding of the very basic developmental aspects of cancer growth and progression. Identifying other BMI1-cooperative factors besides the oncogene MYC will also help elucidate the functional pathways of BMI1 in cancer development.

Acknowledgements

We would like to thank Amada Cunningham, Jessica Green, Joel Berletch, Serene Lai, and Drs Mitchell Pate and Richard Jope for help with some of the cell culture work. This work was funded in part by funding from the American Institute for Cancer Research (LL, Grant # 01A015) and an Evelyn F McKnight Brain Institute Grant (TOT).

References

Adhikary S, Eilers M. Nat Rev Mol Cell Biol 2005;6:635–645. [PubMed: 16064138]

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Proc Natl Acad Sci USA 2003;100:3983–3988. [PubMed: 12629218]

Bea S, Tort F, Pinyol M, Puig X, Hernandez L, Hernandez S, et al. Cancer Res 2001;61:2409–2412. [PubMed: 11289106]

Blair A, Hogge DE, Ailles LE, Lansdorp PM, Sutherland HJ. Blood 1997;89:3104–3112. [PubMed: 9129012]

Bruggeman SW, Valk-Lingbeek ME, van der Stoop PP, Jacobs JJ, Kieboom K, Tanger E, et al. Genes Dev 2005;19:1438–1443. [PubMed: 15964995]

Casillas MA, Brotherton SL, Andrews LG, Ruppert JM, Tollefsbol TO. Gene 2003;16:57–65. [PubMed: 14563552]

Cohen KJ, Hanna JS, Prescott JE, Dang CV. Mol Cell Biol 1996;16:5527–5535. [PubMed: 8816465]

Dimri GP, Martinez JL, Jacobs JJ, Keblusek P, Itahana K, Van Lohuizen M, et al. Cancer Res 2002;62:4736–4745. [PubMed: 12183433]

Haupt Y, Alexander WS, Barri G, Klinken SP, Adams JM. Cell 1991;65:753–763. [PubMed: 1904009]

Itahana K, Zou Y, Itahana Y, Martinez JL, Beausejour C, Jacobs JJ, et al. Mol Cell Biol 2003;23:389–401. [PubMed: 12482990]

Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. Nature 1999a;397:164–168. [PubMed: 9923679]

Jacobs JJ, Scheijen B, Voncken JW, Kieboom K, Berns A, van Lohuizen M. Genes Dev 1999b;13:2678–2690. [PubMed: 10541554]

Kakunaga, T.; Yamasaki, H. Transformation Assay of Established Cell Lines: Mechanisms and Applications. IARC Scientific Publications, International Agency for Research on Cancer; Lyon: 1985.

Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, Saremaslani P, et al. Nature 2004;428:337–341. [PubMed: 15029199]

Liu L, Berletch JB, Green JG, Pate MS, Andrews LG, Tollefsbol TO. Mol Cancer Ther 2004;3:1003–1009. [PubMed: 15299083]

Mihara K, Chowdhury M, Nakaju N, Hidani S, Ihara A, Hyodo H, et al. Blood 2006;107:305–308. [PubMed: 16160010]

Molofsky AV, He S, Bydon M, Morrison SJ, Pardal R. Genes Dev 2005;19:1432–1437. [PubMed: 15964994]

Molofsky AV, Pardal R, Iwashita T, Park IK, Clarke MF, Morrison SJ. Nature 2003;425:962–967. [PubMed: 14574365]

Mori T, Kiyono T, Imabayashi H, Takeda Y, Tsuchiya K, Miyoshi S, et al. Mol Cell Biol 2005;25:5183–5195. [PubMed: 15923633]

Pardal R, Clarke MF, Morrison SJ. Nat Rev Cancer 2003;3:895–902. [PubMed: 14737120]

Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL, et al. Nature 2003;423:302–305. [PubMed: 12714971]

Shakhova O, Leung C, Marino S. J Mol Med 2005;83:596–600. [PubMed: 15976916]

Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, et al. Nature 2004;432:396–401. [PubMed: 15549107]

Takeda Y, Mori T, Imabayashi H, Kiyono T, Gojo S, Miyoshi S, et al. J Gene Med 2004;6:833–845. [PubMed: 15293342]

Terai M, Uyama T, Sugiki T, Li XK, Umezawa A, Kiyono T. Mol Biol Cell 2005;16:1491–1499. [PubMed: 15647378]

van der Lugt NM, Domen J, Linders K, van Roon M, Robanus-Maandag E, te Riele H, et al. Genes Dev 1994;8:757–769. [PubMed: 7926765]

van Lohuizen M, Verbeek S, Scheijen B, Wientjens E, van der Gulden H, Berns A. Cell 1991;65:737–752. [PubMed: 1904008]

Vonlanthen S, Heighway J, Altermatt HJ, Gugger M, Kappeler A, Borner MM, et al. Br J Cancer 2001;84:1372–1376. [PubMed: 11355949]

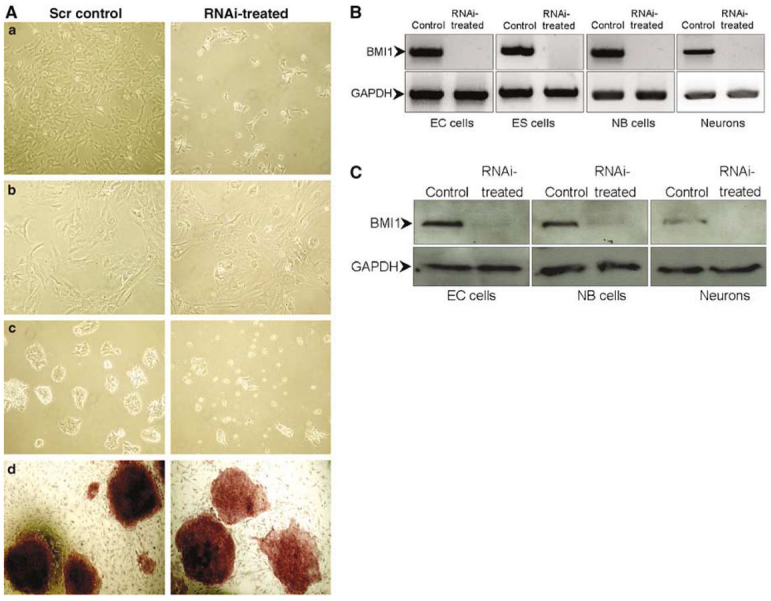

Figure 1.

Morphological changes and gene expression analyses of BMI1 and GAPDH in RNA interference (RNAi)-treated and scrambled (scr)-treated control cells. (A) Representative morphological changes of SH-SY5Y neuroblastoma (NB) cells (a), HCN-2 cortical neurons (b), NCCIT embryonic carcinoma cells (c), and H1 ES cells (d) 3 days after RNAi treatment (magnification: × 200). All cells were grown following providers' guidelines. For all experiments, the cells were seeded at 5×10^4 cells/ml in fresh medium. To generate dsRNA targeting BMI1 mRNA, a DNA fragment was PCR amplified from human leukemic cell cDNA using primer sequences specific for the *BMI1* gene (see below) but with an addition of T7 primer sequences at the 5′ end of both forward and reverse primers. The amplified DNA fragment was used to generate dsRNA targeting BMI1 mRNA using the MEGAscript RNAi kit (Ambion) following the manufacturer's instructions. Purified dsRNA was transfected into cultured cells (8 μg dsRNA/10^6 cells) using the X-tremeGENE siRNA Transfection Reagent (Roche Applied Science). For RNAi specificity control, an equal amount of mixed pool of scrambled (scr) siRNA molecules (commercially available from Ambion) was used for transfection in place of the BMI1 dsRNA. Detection of alkaline phosphatase activity was performed using a semi-quantitative Alkaline Phosphatase Histochemical Staining system (Sigma Diagnostics) according to the manufacturer's instructions. (**B**) Representative gene expression analysis of BMI1 and GAPDH in RNAi-treated and scr-treated control EC cells, normal ES cells, NB cells, and normal neurons. Extraction of total cellular RNA and cDNA synthesis were performed as described previously (Liu et al., 2004). cDNA was amplified with primers specific for either BMI1 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR conditions were: 94°C for 4 min, 28–30 cycles of 94°C for 45 s, 56°C for 30 s, 72°C for 40 s, and a final extension step at 72°C for 5 min. The primer sequences for BMI1 are 5′-GTCCAAGTTCACAAGACCAGACC-3′ and 5′-ACAGTCATTGCTGCTGGGCATCG-3′. GAPDH primers were the same as used previously (Liu et al., 2004). (**C**) Representative Western blotting analysis of BMI1 and GAPDH in RNAi-treated and scr-treated control EC cells, NB cells, and normal neurons. Western blotting was performed using primary antibody against GAPDH (Santa Cruz Biotechnology) and monoclonal antibody against BMI1 (Abcam). Bound antibody was detected with the Immun-Star™ HRP chemiluminescent system following the manufacturer's instructions (Bio-Rad).

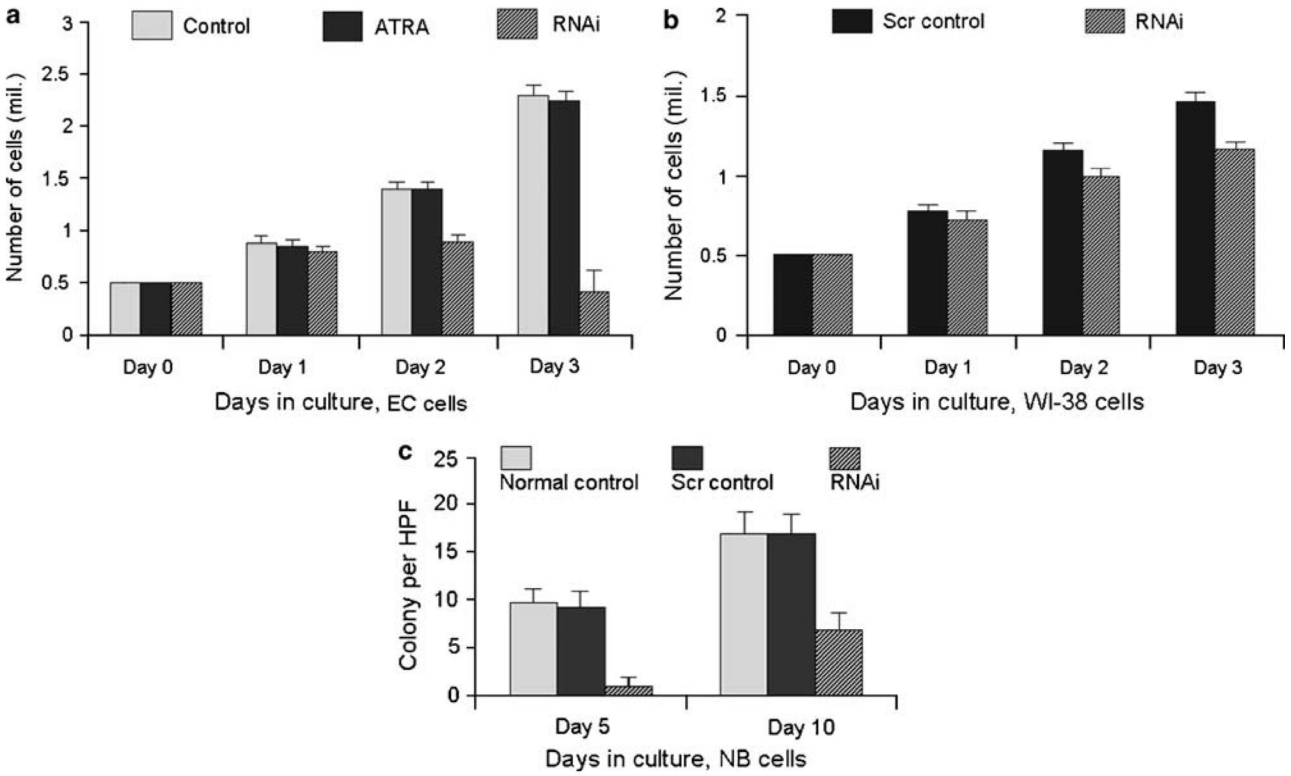

Figure 2.

Effects of RNA interference (RNAi) of BMI1 on cell proliferation and tumorigenicity. (a) Histogram of the number of live cells remaining in the culture of control, RNAi-treated, and ATRA-treated EC cells, respectively, during the first 3 days of treatment. The cells used for growth curve studies were grown for a total of three days, and cells were counted on days 0, 1, 2 and 3 following transfection using trypan blue (0.25%) for monitoring viability. (b) Histogram of the number of live cells remaining in the culture of control and RNAi-treated normal WI-38 cells, respectively; (c) Histogram of the number of colonies formed by control and RNAi-treated neuroblastoma (NB) cells on days 5 and 10, respectively. For soft agar colony formation assays, two sets of human NB cells were prepared for normal control cells, cells treated with scrambled siRNA, and cells treated with dsRNA, respectively. Cells (1×10^4) were suspended in 1.5 ml growth medium (10% FBS-DMEM) containing 0.4% Bacto agar. The cell suspension was overlaid onto a hard agar base composed of growth medium containing 0.7% Bacto agar in six-well plates. Plates were maintained in a humidified incubator at 37°C for 5 days and 10 days, respectively. Plates were subsequently stained with 0.005% Crystal Violet (Sigma) solution for 4 h at room temperature and colonies were counted under a microscope at × 200 magnification on the fifth day and 10th day, respectively. Vertical bars represent standard deviation (s.d.) from at least three sets of experiments. HPF: high-power field.

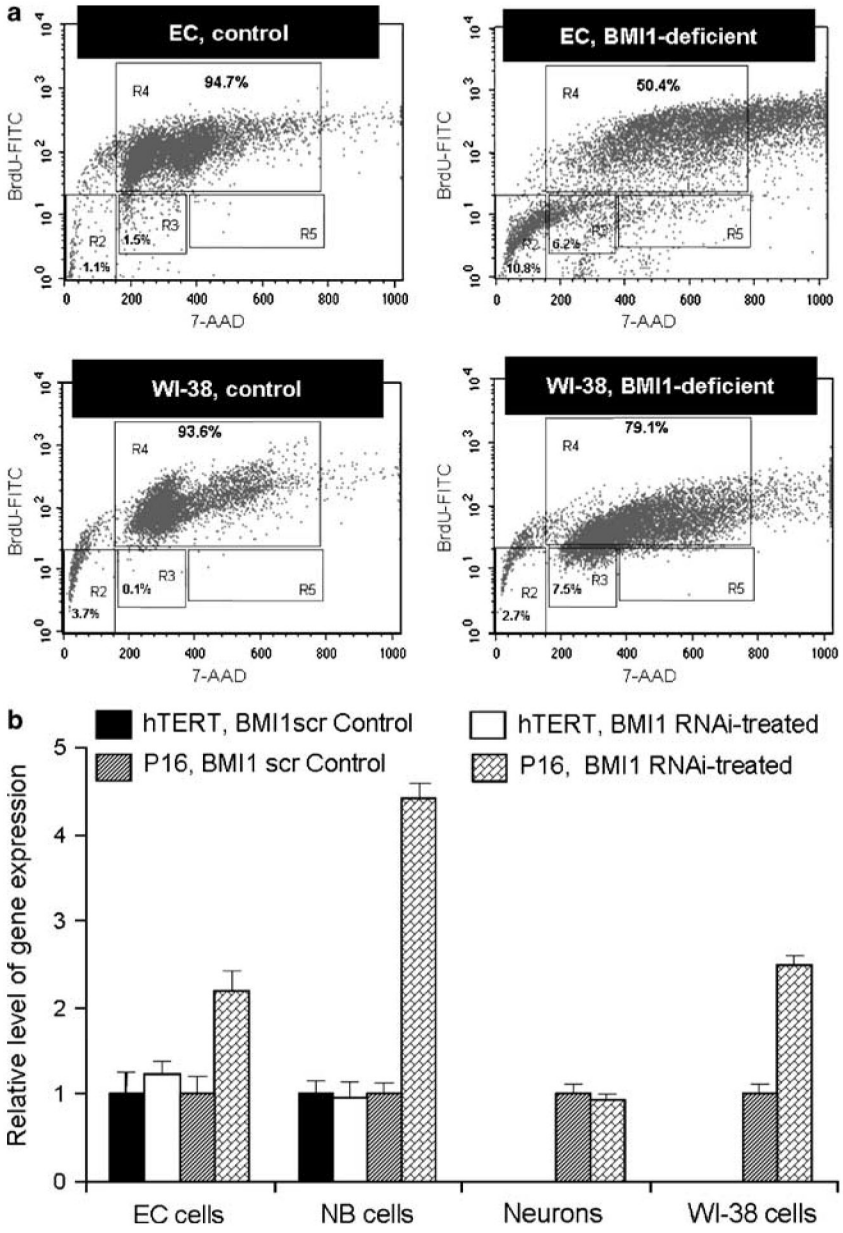

Figure 3.

Effects of RNA interference (RNAi) of BMI1 on cell cycle progression and the expression of INK4A-ARF and hTERT. (a) Cell cycle position and apoptosis of BMI1 RNAi-treated and scrambled (scr)-treated control EC cells (upper panel) and WI-38 lung cells (lower panel) were detected by using a FITC BrdU Flow system following the manufacturer's instructions (BD Pharmingen). For labeling, BrdU was added directly to the cell culture at a final concentration of 100 μ_M 24 h after transfection, and was incubated for another 48 h. Cells were then harvested, fixed, permeabilized, treated with DNase I and stained with FITC-conjugated anti-BrdU antibodies and 7-AAD (to display DNA content) (BD Pharmingen). A Becton Dickinson FACS apparatus was used to acquire and analyse a minimum of 10 000 events using the Cellquest program. The graphs shown are representative of similar results obtained from three independent experiments; (b) Real-time PCR was performed to quantify the relative level of gene expression for *hTERT* and *p16* using the ABI real-time PCR apparatus as reported

previously (Liu et al., 2004). As BMI1 may affect the expression of p16 and p14 differentially in a tissue-specific manner (Bruggeman et al., 2005), we have chosen primer sequences that allowed us to simultaneously assess the expression of both p16 and p14 encoded by the INK4A-ARF locus in the same real-time PCR analysis. The expression levels of both *hTERT* and *INK4A-ARF* in control cells were normalized to a relative value of 1, and the level of gene expression in the corresponding RNAi-treated cells was adjusted accordingly to reflect the fold-changes by comparison with the controls. Vertical bars represent s.d. from at least three sets of experiments.

Table 1

Normal and cancer human cell lines studied for the effect of BMI1-deficiency on cell growth and survival[a]

	Tissue origin	*Growth and survival in the absence of BMI1*
Cancer cell line		
NCCIT	Embryonic carcinoma	Acute cell death and growth inhibition
MCF-7	Mammary epithelial adenocarcinoma	Acute cell death and growth inhibition
MDA-157	Mammary medullary carcinoma	Acute cell death and growth inhibition
SH-SY5Y	Metastatic neuroblastoma	Acute cell death and growth inhibition
OVCAR-3	Ovary adenocarcinoma	Acute cell death and growth inhibition
SKOV-3	Ovary adenocarcinoma	Acute cell death and growth inhibition
Normal cell line		
HES	Embryonic stem cells	Moderate growth inhibition, no significant cell death
WI-38	Embryonic lung cells	Moderate growth inhibition, no significant cell death
HMEC	Mammary epithelial cells	Moderate growth inhibition, partial cell detachment
MRC-5	Lung fibroblasts	Moderate growth inhibition, no significant cell death
MBO	Skeletal muscle cells	Moderate growth inhibition, no significant cell death
HCN-2	Brain cortical neurons	Moderate growth inhibition, no significant cell death

[a]The criteria we used to determine ‘acute’ versus ‘moderate’ growth inhibitory effects were mainly based on the difference in the number of live cells and the presence of dead cells between the RNAi-treated sample and the scr-treated control sample three days following transfection. The effect is considered acute if the number of cells in the RNAi-treated sample is <30% of the scr-control sample in addition to clearly visible cell death in the RNAi-treated culture. The effect is considered moderate if the number of cells in the RNAi-treated sample is >75% of the scr-control sample and there are no dead cells observed.