Cell Cycle Regulator ING4 Is a Suppressor of Melanoma Angiogenesis That Is Regulated by the Metastasis Suppressor BRMS1

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Abstract

ING4 has been previously shown to play important roles in regulating apoptosis, cell cycle progress, cell migration, and invasion. In this study, we investigated the impact of ING4 on melanoma angiogenesis. ING4 overexpression strongly suppressed the growth of human umbilical vein endothelial cells (HUVEC) and their ability to form tubular structure in vitro. We also found that ING4 inhibits interleukin-6 (IL-6) at both mRNA and protein levels through suppressing NF-κB activity. Knockdown of endogenous ING4 resulted in enhanced HUVEC growth and IL-6 expression. Our in vivo studies using nude mice confirmed that ING4 inhibited blood vessel formation and the recruitment of CD31-positive cells in matrigel plugs. Furthermore, we found that expression of ING4 was induced by BRMS1, a metastasis suppressor that inhibits melanoma angiogenesis through inhibiting NF-κB activity and IL-6 level as well. Further experiments showed that ING4 knockdown abrogated the suppressive effect of BRMS1 on HUVEC growth, whereas ING4 overexpression inhibited BRMS1 knockdown-induced angiogenesis, indicating that ING4 is a downstream target of BRMS1 in regulating tumor angiogenesis. Collectively, our findings indicate that ING4 is induced by BRMS1 and that it inhibits melanoma angiogenesis by suppressing NF-κB activity and IL-6 expression. Restoration of ING4 function offers a potential new strategy for the treatment of human melanoma.

Introduction

Melanoma is the most lethal form of skin cancer. The incidence of melanoma has drastically increased over the past several decades in Western countries (1, 2). In United States, the number of new melanoma cases in 2009 was 68,720 and 8,650 patients died from melanoma (3). Metastasis is the major cause for melanoma patient death. Melanoma can metastasize quickly and once metastasis occurs, the 5-year survival rate drops to less than 5% (4).

The underlying mechanisms that regulate melanoma progression and metastasis are still poorly understood. Angiogenesis is required by many solid tumors to grow beyond a certain size (5, 6) and is closely related to metastasis of solid tumors, including melanoma (7-9). Genetic alterations in genes regulating tumor angiogenesis might impact the tumor metastasis and patient survival.

Materials and Methods

Cell culture

Human melanoma MMRU and MMAN cell lines are kind gifts from Dr. H.R. Byers (Boston University). SK-mel-110 cell line is kindly provided by Dr. A.P. Albino (Memorial Sloan-Kettering Cancer Center). All melanoma cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum (Invitrogen). Human umbilical vein...
endothelial cells (HUVEC) were cultured in Kaighn’s Modified Ham’s F-12K medium (Mediatech) supplemented with endothelial cell growth supplement (BD Biosciences) and 10% fetal bovine serum. All cells were maintained in 5% CO₂ atmosphere at 37°C.

Transfection and infection
A recombinant adenoviral vector expressing human ING4 gene was kindly provided by Dr. J. Yang (Soochow University). cDNA of ING4 and BRMS1 was cloned into HA- or Flag-tagged vectors to generate HA-ING4 and Flag-BRMS1 plasmids, respectively. The pcDNA EmGFP-miR vector (Invitrogen) was used to develop microRNA-like structures for ING4 and BRMS1 knockdown, and 2 sequences were used for both ING4 and BRMS1 (Supplementary Table S1). For adenoviral infection, melanoma cells were cultured until approximately 70% confluency and infected with control adenoviral vector (adv) or adenoviral ING4 (adING4) at multiplicity of infection (MOI) of 100 as described previously (14). For plasmid transfection, melanoma cells were grown to approximately 50% confluency and then transiently transfected with HA-ING4 or Flag-BRMS1 plasmid, or miING4 or miBRMS1 with Effectene reagent (Qiagen), according to the manufacturer’s instructions. Twelve hours after transfection, the medium containing transfection reagents was removed. The cells were rinsed with PBS and then incubated in fresh medium.

HUVEC growth and tube formation assay
Melanoma cells were cultured in 60-mm plates with fresh complete medium for 24 hours, and 2 mL of conditioned medium was collected. For HUVEC growth assay, the endothelial cells were seeded in 24-well plate at 2 × 10⁴ cells/well and cultured in fresh F-12K medium for 24 hours, and then in 0.5 mL of conditioned medium for another 24 hours before sulforhodamine B assay was conducted as previously described (15). For IL-6 rescue experiments, we added 0.8 ng/mL recombinant IL-6 (eBioscience) in the conditioned medium from ING4-overexpressing melanoma cells and then conducted the HUVEC growth assay. For IL-6 blocking assay, we added 320 ng/mL of IL-6 antibody (eBioscience) in the conditioned medium from melanoma cells with ING4 knockdown treatment and then collected the conditioned medium for HUVEC growth assay. For tube formation assay, 96-well plate was coated with Matrigel (BD Biosciences) and kept in 37°C for 2 hours. Then, 2 × 10⁴ HUVECs were suspended in 100 μL of conditioned medium and applied to the precoated 96-well plate. After incubation at 37°C for another 24 hours, photographs were taken under microscope and the tubular structures formed in the matrigel were counted in 5 random fields.

Real-time PCR, immunoprecipitation, Western blot, and ELISA
Total RNA was extracted using TRIzol (Invitrogen). Real-time PCR reactions were conducted in triplicate with SYBR Green PCR Master Mix using a 7900HT qPCR system thermal cycler (Applied Biosystems). Primer sequences are listed in supplementary Table S1. For immunoprecipitation and Western blot analysis, nuclear extracts were prepared, and immunoprecipitation was conducted as previously described (16, 17) by incubating 500 μg of nuclear extract with either monoclonal mouse anti-Flag antibody (Applied Biological Materials) or polyclonal rabbit anti-HA antibody (ImmuneChem Pharmaceuticals) and then were separated on 12% SDS-PAGE gels and electrophoresed onto polyvinylidene difluoride membranes (Bio-Rad). Membranes were probed with the following primary antibodies: monoclonal mouse anti-BRMS1 (kind gift from Dr. D.R. Welch, University of Alabama at Birmingham) (18), polyclonal rabbit anti-ING4 (Invitrogen), and monoclonal anti-Actin (Sigma-Aldrich, Oakville). Signals were detected using an Odyssey infrared imaging system (LI-COR Biosciences). For ELISA, the secreted IL-6 protein in the conditioned medium was measured with a human IL-6 ELISA kit (eBioscience) according to the manufacturer’s instructions.

Electrophoretic mobility shift assay
Nuclear extracts were prepared from MMRU cells overexpressing ING4, BRMS1, or ING4 plus BRMS1 and the control sample. Then we incubated the nuclear extract with oligonucleotide probe bearing NF-kB binding sequence on IL-6 promoter (sequences are listed in Supplementary Table S1). The probe was labeled with a γ-32P phosphate at its 5’ end and another oligonucleotide with the same sequence but without labeling was used as a competitive sequence at 100- or 500-fold concentration. Binding reaction and detection procedure were carried out as described previously (19).

In vivo angiogenesis assay and immunofluorescent staining
MMRU human melanoma cells (1 × 10⁶) were supported by 300 μL Matrigel and implanted subcutaneously into the flanks of 6-week-old male nude mice. Ten days later, the mice were sacrificed and the implanted matrigel plugs were excised, photographed, and immediately embedded in Tissue-Tek O.C.T. compound (Sakura Finetek USA). After frozen at −80°C for 1 hour, 5-μm sections were cut from the embedded tissues using a CM1850 cryostat (Leica Microsystems) and applied to glass slides. Immunofluorescent staining for CD31 or vWF was conducted using CD31 or vWF antibody expression was then conducted using rat anti-mouse CD31 antibody and FITC-labeled goat anti-rat secondary antibody (Santa Cruz Biotechnology), or anti-vWF antibody (Dako Diagnostics) plus CY3-conjugated goat anti-mouse antisemur (Jackson ImmunoResearch). Sections were counterstained with propidium iodide (PI; Sigma-Aldrich) or Hoechst 33258. Photographs were taken with a cooled mono 12-bit Retiga-Ex camera equipped with Northern Eclipse imaging software. The number of CD31 or vWF positive cells was counted in 5 random fields for both vector control and ING4-overexpressing groups.

Results
Expression of ING4 in melanoma cells suppressed growth and tube formation of HUVECs
To test the role of ING4 on melanoma angiogenesis, we first overexpressed ING4 in MMRU and SK-mel-110 cells or
knocked down ING4 in MMRU and MMAN cells. The expression of ING4 was then confirmed by Western blot (Fig. 1A). For ING4 knockdown, we tested 2 miING4 sequences and both showed significant inhibition on ING4 expression by qRT-PCR and Western blot (Supplementary Fig. S1). The sequence 2 was used for further experiments, as it showed relatively better knockdown effect than the sequence 1. Then conditioned medium was collected from melanoma cells and applied to either HUVEC growth assay or tube formation assay. Compared with the corresponding control, overexpression of ING4 in MMRU and SK-mel-110 cells resulted in decreased HUVEC growth by 71% and 46%, respectively. In contrast, ING4 knockdown in MMRU and MMAN cells led to 2- and 1.5-fold increase of HUVEC growth, respectively (Fig. 1B). The average number of complete tubular structures formed by HUVECs was also decreased by 73% in conditioned medium from ING4-overexpressing SK-mel-110 cells, whereas increased by 1.6-fold in conditioned medium from ING4 knockdown MMRU cells, when compared with respective controls (Fig. 1C and D).

**ING4 mediates HUVEC growth by suppressing IL-6 expression**

Previously, we reported that NF-κB p50 subunit enhanced melanoma angiogenesis through upregulating IL-6 at both transcriptional and protein level (20). Therefore, we next tested whether ING4 inhibits melanoma angiogenesis by suppressing NF-κB/IL-6 pathway. Our real-time RT-PCR data revealed that ING4 overexpression in MMRU and SK-mel-110 cells downregulated IL-6 mRNA expression by 49.5% and 47.2% compared with the vector control (Fig. 2A, left). ING4 knockdown in MMRU and MMAN cells elevated IL-6 mRNA by 1.9- and 2.4-fold, respectively (Fig. 2A, right). We then conducted ELISA assay and found that ING4 overexpression in MMRU and SK-mel-110 cells decreased IL-6 protein in conditioned medium from 1.7 and 1.6 ng/mL/10⁵ cells to 0.9 and 0.7 ng/mL/10⁵ cells, respectively (Fig. 2B, AB).
left). In contrast, IL-6 protein was increased by 3.4- and 2.9-fold in conditioned medium collected from ING4 knockdown MMRU and MMAN cells, respectively (Fig. 2B, right). The role of IL-6 in ING4-regulated melanoma angiogenesis was then confirmed by IL-6 rescue and IL-6 blocking assays. The addition of 0.8 ng/mL recombinant IL-6 to ING4-overexpressing MMRU and SK-mel-110 cells rescued the HUVEC growth to the similar level of the corresponding vector control cells (Fig. 2C), whereas the application of sufficient IL-6 antibody abrogated the elevated HUVEC growth by ING4 knockdown in MMRU and MMAN cells (Fig. 2D).

**ING4 expression in melanoma cells inhibited angiogenesis in vivo**

We conducted the in vivo matrigel plug assay to investigate whether ING4 expression in melanoma cells could inhibit the neovessel formation in a mouse model. Visual examination revealed obviously less vascularization in matrigel plugs containing ING4-overexpressing MMRU cells than the control plugs (Fig. 3A). CD31 staining showed that the control plugs had much denser neovessels with 3-fold CD31-positive cells than with ING4-overexpressing plugs (Fig. 3B and C). We then investigated the level of tumor-derived IL-6 in those matrigel plugs. qRT-PCR data showed that the IL-6 mRNA level was significantly decreased by 51% in matrigel plugs containing ING4-overexpressing MMRU cells compared with the control plugs (Fig. 3D). Immunofluorescent staining was also conducted with another endothelial cell specific marker vWF and similar result was observed, supporting our hypothesis that ING4 overexpression inhibits melanoma angiogenesis in vivo (Supplementary Fig. S2).

**ING4 expression was induced by BRMS1**

As we previously showed that BRMS1 inhibited melanoma angiogenesis through decreasing NF-κB activity and IL-6 level (21), we next investigated whether these 2 molecules are related in expression and/or function. We first studied the expression of ING4 and BRMS1 in 173 melanocytic lesions using tissue microarray. Among all these biopsies, 63 showed weak ING4 expression whereas the other 110 had strong ING4 staining. The percentage of strong BRMS1 staining increased from 47% in weak ING4 group to 68% in strong ING4 group (P = 0.011, χ² test, Fig. 4A). We next conducted RT-PCR to examine the expression of ING4 and BRMS1 in 7 melanoma cell lines. Expression of ING4 and BRMS1 was also compared in 7 melanoma cell lines by Western blot. Both ING4 and BRMS1 showed high expression in MMAN and MeWo cells, deficient expression in SK-mel-110 cells, and moderate expression in the other cell lines, exhibiting the similar expression trend in different melanoma cell lines (Fig. 4B). We next examined whether the manipulation of BRMS1 expression regulated ING4 level, or vice versa. Our Western blot result showed that when we overexpressed BRMS1 in MMRU and SK-mel-110 cells, ING4 expression was increased by 2.2- and
1.9-fold, respectively (Fig. 4C, left). Although relatively higher overexpression of BRMS1 was seen in MMRU (10.5 folds) than that in SK-mel-110 (4.5 folds), similar extent of increased ING4 expression was observed, which may be due to the saturation effect of ectopic expression of BRMS1 on ING4. Moreover, BRMS1 knockdown in MMRU and MMAN cells resulted in decreased ING4 expression by 60% and 70% in MMRU and MMAN, respectively (Fig. 4C, right). In contrast, neither overexpression of ING4 in MMRU and SK-mel-110 cells nor knockdown of ING4 in MMRU and MMAN cells altered the endogenous expression of BRMS1 in these cell lines (Fig. 4D). This induction of ING4 by BRMS1 was also confirmed by qRT-PCR. Knockdown of ING4 in MMRU and MMAN did not change BRMS1 mRNA expression (Supplementary Fig. S4A), whereas knockdown of BRMS1 in MMRU and MMAN cells resulted in 70.5% and 63.3% decrease of ING4 mRNA expression, respectively (Supplementary Fig. S4B). Immunoprecipitation analysis of BRMS1 and ING4 indicated that these 2 molecules are not in the same complex (Supplementary Fig. S5).

**ING4 is the downstream target of BRMS1 in melanoma angiogenesis inhibition**

We next examined whether ING4 and BRMS1 are functionally related. We overexpressed either ING4 or BRMS1 alone, or coexpressed both ING4 and BRMS1, and then tested their impact on HUVEC growth. We found that coexpression of ING4 and BRMS1 in MMRU cells did not further suppress HUVEC growth, but to the similar extent as overexpression of either ING4 or BRMS1 (Fig. 5A). We also showed that concomitant overexpression of ING4 and BRMS1 inhibited the tubular structure formation to the similar level as overexpression of either ING4 or BRMS1 (Fig. 5B). We repeated HUVEC growth assay and tube formation assay in another melanoma cell SK-mel-110 and confirmed the finding that combination of ING4 and BRMS1 did not produce additional impact on angiogenesis (Supplementary Fig. S6). Further experiments showed that ING4 knockdown abolished BRMS1-induced suppression of HUVEC growth, suggesting that ING4 is the downstream target of BRMS1 in regulating angiogenesis (Fig. 5C). In a parallel experiment, both ING4 overexpression alone and ING4 overexpression plus BRMS1 knockdown inhibited HUVEC growth to the similar level, suggesting that BRMS1 knockdown did not interfere the effect of ectopic expression of ING4 on HUVEC growth (Fig. 5D). As both ING4 and BRMS1 inhibited melanoma angiogenesis through decreasing IL-6 expression, we next examined whether coexpression of ING4 and BRMS1 exert synergistic suppression on IL-6. qRT-PCR showed that overexpression of ING4 plus BRMS1 in MMRU and SK-mel-110 cells decreased IL-6 mRNA expression to the similar level as individual overexpression of either one (Fig. 6A). This result was supported by ELISA assay data, showing that overexpression of both ING4 and BRMS1 only decreased IL-6 protein in the conditioned medium to the similar level as individual overexpression (Fig. 6B). Finally, we conducted electrophoretic mobility shift assay (EMSA) to study the effect of ING4 and BRMS1 on the DNA binding activity of NF-kB to IL-6 promoter. Our data revealed that overexpression of ING4 or BRMS1 alone inhibited the binding of NF-kB to IL-6 promoter, whereas co-overexpression of both genes showed the inhibitory effect to the similar extent (Fig. 6C).
Discussion

ING4 was first identified in 2003 by searching for homologous sequences with ING1, the founding member of ING family proteins, followed by PCR and rapid amplification of cDNA ends (RACE) of placenta cDNA library (10). We have previously shown that ING4 expression was decreased in human cutaneous melanoma compared with dysplastic nevi, indicating its tumor suppressor role in melanoma pathogenesis (11). We also reported that ING4 expression inhibited melanoma cell migration and invasion (22). In this study, we investigated the role of ING4 in melanoma angiogenesis. Our data for the first time showed that ING4 inhibited melanoma angiogenesis in vitro and in vivo, through inhibiting NF-κB activity and IL-6 expression. Moreover, we showed that ING4 is a downstream target of BRMS1 in the regulation of melanoma angiogenesis. To our knowledge, this is the first study showing that ING4 expression was controlled by BRMS1.

Angiogenesis is a multistep process, which includes endothelial cell proliferation, migration, and the formation of blood vessels (23, 24). In this study, we found that both the growth and tube formation of HUVECs were inhibited in conditioned medium collected from ING4-overexpressing melanoma cells. This effect was confirmed by ING4 knockdown experiments as silencing ING4 in melanoma cells resulted in enhanced endothelial cell growth and tube formation ability (Fig. 1B–D). Furthermore, we showed that ING4 overexpression in melanoma cells inhibited the supportive vasculature in vivo (Fig. 3). Inhibition of melanoma angiogenesis by ING4 expression described in this study is consistent with and partially explains our previous finding that ING4 expression was decreased in human melanoma when compared with dysplastic nevi, and this reduced ING4 staining was correlated with melanoma thickness and patient survival (11). Tumor growth can be separated into 2 stages, avascular and vascular (25). The invasive tumor growth can only be achieved when angiogenesis is "switched on" and new capillaries penetrate into the tumor (26). Besides invasiveness, angiogenesis is also widely believed to be associated with tumor metastasis. A series of studies indicated that angiogenesis was closely related to metastasis in various tumors, including breast, lung, prostate, head and neck, as well as melanoma (7–9). Combined with the fact that metastasis is the major cause of melanoma patient death, it is not surprising to see that reduced ING4 is associated with tumor invasion, angiogenesis, metastasis, and eventually poorer outcome of melanoma patients.

We previously reported that p50 subunit of NF-κB promoted melanoma angiogenesis by upregulating IL-6 level (20). It is also known that ING4 inhibits NF-κB activity and thus its downstream gene expression (12, 27, 28). Therefore, we...
hypothesized that ING4 may suppress melanoma angiogenesis through inhibiting NF-κB target gene IL-6 expression. Our data showed that ING4 overexpression in melanoma cells decreased whereas ING4 knockdown increased IL-6 mRNA expression and protein in conditioned medium (Fig. 2A and B). These data were consistent with the finding that IL-6 can be secreted by melanoma cells (29), and the expression of IL-6, as a NF-κB target gene, can be inhibited by ING4 (27, 28). Furthermore, the role of IL-6 in ING4-mediated melanoma angiogenesis was confirmed by IL-6 rescue and blocking experiments. The inhibited HUVEC growth by ING4 overexpression can be rescued by adding recombinant IL-6 to conditioned medium, and the application of IL-6 antibody in conditioned medium abrogated ING4 knockdown-induced HUVEC growth (Fig. 2C and D). Collectively, these data indicated that ING4 regulated HUVEC growth through decreasing IL-6 level.

We next investigated whether ING4 collaborated with BRMS1 in this angiogenesis modulation, as both ING4 and BRMS1 inhibited melanoma angiogenesis through NF-κB/IL-6 pathway, and both were reported to inhibit NF-κB activity (21, 28, 30, 31). The immunoprecipitation data showed that ING4 and BRMS1 cannot be precipitated together, ruling out the possibility that they functioned in the same complex (Supplementary Fig. S5). Therefore, the possible correlation between ING4 and BRMS1 expression was tested. We previously examined the expression alteration of both ING4 and BRMS1 using the same tissue microarray, which enabled us to compare the expression of these 2 biomarkers in the same group of patients. Our data revealed that higher ING4 expressing-group patients also showed higher BRMS1 expression (Fig. 4A). Endogenous expression of ING4 and BRMS1 also showed similar trend in 7 melanoma cell lines (Fig. 4B). Further experiments revealed that BRMS1 overexpression induced ING4 expression whereas BRMS1 knockdown resulted in decreased ING4 expression. In contrast, manipulation of ING4 expression did not affect BRMS1 expression, indicating that ING4 expression was under the control of BRMS1 (Fig. 4C and D, Supplementary Fig. S4). So far, ING4 has been shown to inhibit the activity of several important transcription factors, including NF-κB (12, 27, 28), HIF-1 (32–34), and p53 (10, 35), which may help explain the functions of ING4 in different aspects of cellular activity. For example, it has been reported that ING4 induces apoptosis of RKO colon cancer cell line in p53-dependent manner (10). But our experiments did not show significant effect of ING4 on melanoma cell apoptosis (unpublished data); therefore, ING4 may regulate melanoma progression through other functions, such as inhibiting angiogenesis. To our knowledge, this is the first study showing that ING4 was regulated by BRMS1 at both mRNA and protein levels, which may help to better understand the complete pathway of ING4 to regulate various biological functions. More experiments are needed to further elucidate the details of BRMS1 regulation on ING4 expression.
We then investigated whether ING4 and BRMS1 are functionally related. Our data showed that coexpression of ING4 and BRMS1 did not show further inhibition on HUVEC growth and blood tube formation than individual expression of ING4 or BRMS1 alone (Fig. 5A and B). We also found that the inhibited HUVEC growth by BRMS1 expression can be abrogated by knockdown of endogenous ING4 whereas BRMS1 knockdown did not affect the inhibitory impact of ING4 expression on HUVEC growth (Fig. 5C and D). These data were consistent with the finding that BRMS1 induced ING4 expression and suggested that they exerted their effect through the same pathway, with BRMS1 being the upstream regulator of ING4 to mediate melanoma angiogenesis. ING4 is under the control of BRMS1 and inhibits the activity of NF-κB either directly by interacting with the RelA subunit of NF-κB or its downstream gene promoter (12, 28) or indirectly by inducing IκB expression (27). Data from qRT-PCR and ELISA also supported that coexpression of ING4 and BRMS1 decreased only IL-6 mRNA or secreted protein to the similar level as single expression of individual genes (Fig. 6A and B). Moreover, EMSA revealed that overexpression of ING4 and BRMS1 alone inhibited the binding activity of NF-κB to IL-6 promoter to the similar level as the coexpression sample (Fig. 6C), suggesting that they inhibited NF-κB activity in the same pathway. All these data suggested that BRMS1 and ING4 inhibited melanoma angiogenesis through suppressing NF-κB activity and thus its target gene, IL-6 expression, in a sequential pattern.

In summary, we showed that ING4 inhibited human melanoma angiogenesis. We identified BRMS1 as the upstream regulator and NF-κB/IL-6 as the downstream targets of ING4 in mediating melanoma angiogenesis. Our data contribute to the better understanding of tumor-suppressive role of ING4 in melanoma and imply that ING4 restoration may be a novel approach for antiangiogenesis therapy for human melanoma.

**Disclosure of Potential Conflicts of Interest**

The authors state no conflict of interest.

**Acknowledgments**

We thank Mr. S. Hendy for technical assistance with EMSA, Dr. J. Yang for adenoviral ING4, and Dr. D.R. Welch for mouse anti-BRMS1 antibody.

**Grant Support**

This work is supported by the Canadian Institutes of Health Research (MOP-84559 and MOP-93810) and Canadian Dermatology Foundation to G. Li. J. Li is a recipient of Postgraduate Scholarship from the Natural Sciences and Engineering Research Council of Canada, CBHR Skin Research Training Centre Trainee Award, and University of British Columbia Graduate Fellowship.

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Received 08/17/2010; revised 09/28/2010; accepted 10/22/2010; published OnlineFirst 11/05/2010.
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Cell Cycle Regulator ING4 Is a Suppressor of Melanoma Angiogenesis That Is Regulated by the Metastasis Suppressor BRMS1

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Cancer Res 2010;70:10445-10453. Published OnlineFirst November 5, 2010.

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