EXPERIMENTAL CELL RESEARCH 317 (2011) 445–451

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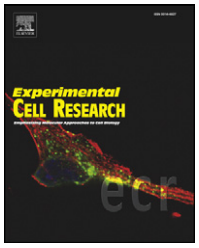

Research Article

Let-7b-mediated suppression of basigin expression and metastasis in mouse melanoma cells

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ARTICLE INFORMATION

Article Chronology:
Received 30 May 2010
Revised version received
20 October 2010
Accepted 4 November 2010
Available online 16 November 2010

Keywords:
Basigin
Let-7b
Matrix metalloproteinase
miRNA

ABSTRACT

Basigin (Bsg), also called extracellular matrix metalloproteinase inducer (EMMPRIN), is highly expressed on the surface of tumor cells and stimulates adjacent fibroblasts or tumor cells to produce matrix metalloproteinases (mmps). It has been shown that Bsg plays an important role in growth, development, cell differentiation, and tumor progression. MicroRNAs (miRNAs) are a class of short endogenous non-protein coding RNAs of 20–25 nucleotides (nt) that function as post-transcriptional regulators of gene expression by base-pairing to their target mRNAs and thereby mediate cleavage of target mRNAs or translational repression. In this study, let-7b, one of the let-7 family members, was investigated for its effect on the growth and invasiveness of the mouse melanoma cell line B16-F10. We have shown that let-7b can suppress the expression of Bsg in B16-F10 cells and also provided evidence that this suppression could result in the indirect suppression of mmp-9. The ability of B16-F10 cells transfected with let-7b to invade or migrate was significantly reduced. In addition, let-7b transfected B16-F10 cells displayed an inhibition of both cellular proliferation and colony formation. Furthermore, it was shown that the overexpression of let-7b in B16-F10 cells could reduce lung metastasis. Taken together, the present study identifies let-7b as a tumor suppressor that represses cancer cell proliferation and migration as well as tumor metastasis in mouse melanoma cells.

Introduction

The endogenous non-protein coding microRNAs (miRNAs) of 18–25 nucleotides (nt) have been shown to be involved in a wide variety of cellular processes and function as post-transcriptional regulators by binding to the 3′ untranslated region (3′UTR) of target mRNAs to repress the translation or to cleave mRNAs [1]. Changes in the expression of miRNAs, as a consequence of up- or down-regulation, have been observed in an array of human tumors [2]. The let-7 miRNA was originally identified in *C. elegans* as a regulator of developmental timing and cell proliferation [3]. In humans and mice, the expression of the let-7 family is barely detectable during stages of embryonic development but increases in differentiated tissues, just as in *C. elegans*. Let-7 family members have been implicated as tumor suppressors in breast cancer [4], malignant melanoma [5], lung [6,7] and colon cancers [8]. Consistent with its tumor suppressor role, the

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doi:10.1016/j.yexcr.2010.11.004

overexpression of let-7 in cancer cells is associated with reduced levels of cell cycle regulators including cyclin-dependent kinases, cyclins, Ras and Myc [9].

Basigin (Bsg), also referred to as extracellular matrix metalloproteinase inducer (EMMPRIN), is highly expressed on the surface of tumor cells and stimulates adjacent fibroblasts or tumor cells to produce matrix metalloproteinases (mmps). Mmps are enzymes that are able to degrade most components of the extracellular matrix (ECM), such as collagen, laminins, fibronectins, elastins, and the protein core of proteoglycans. To date, more than 20 different mmps have been identified and classified [10]. Previous studies have indicated that Bsg is capable of inducing the expression of a range of mmps, including mmp-1, -2, -3, -9 and -11 [11]. It is well known that tumor invasion is dependent on the degradation of the ECM that surrounds the tumor, which is catalyzed by mmps [12]. However, it is unclear if Bsg takes part in the tumor suppression effect of let-7. In this study, we provide the first evidence that Bsg is a target of let-7b. Additionally, it was demonstrated that the down-regulation of mmp-9 could indirectly result from a reduction in Bsg caused by let-7b. Furthermore, we revealed that let-7b overexpression led to suppression of cellular proliferation, invasion or migration and colony formation in mouse melanoma cells *in vitro* and inhibition of metastasis in a mouse model.

Materials and methods

Animals

Sexually mature female C57BL/6 (8 weeks old) mice were supplied by BioLASCO Taiwan Co. Ltd. The animals were maintained in an individually ventilated cage (IVC) system under lighting conditions of 14 h of light (07:00–21:00) and 10 h of dark. Food and water were provided *ad libitum*. The use of animals and the procedures for animal handling and treatments were approved by the Institutional Animal Use and Care Committee (IACUC) at the National Chung Hsing University.

Cell culture

The mouse skin melanoma B16-F10 cell line was purchased from Bioresource Collection and Research Center (Taiwan) and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Biological Industries Ltd.; Kibbutz Beit Haemek, Israel), at 37 °C in a humidified 5% CO_2 incubator.

Transfection and luciferase assay

Luciferase reporter plasmids were constructed by cloning the Bsg 3′UTR (NM_009768) into the pRL4-SV40 vector (Promega, Madison, WI, USA), using the *Xba*I site that was located immediately downstream of the luciferase gene. The 3′UTR of Bsg was amplified by polymerase chain reaction (PCR) from mouse genomic DNA using a forward primer (5′-GTGGTGGGGCAGGC-3′) and reverse primer (5′-GACACACACATGGTGGGGTA-3′). Additionally, the recognition sequence of the restriction enzyme *Xba*I was added at the 5′ ends of both primers. This construct was designated as the pRL4-Bsg-3′UTR vector. A total of $2–3\times10^4$ B16-F10 cells were seeded onto 24-well culture plates. After 24 h of culture, transfection was performed with Lipofectamine 2000 reagent (Invitrogen by Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Cells were co-transfected with 1 μg of either pRL4-Bsg-3′UTR vector or empty pRL4-SV40 vector and 60 ng of pGL3 vector (a kind gift from Dr. Y. L. Shiue), which served as an internal control in the presence and absence of anti-let-7b (2′-methyl-oligonucleotides: 5′-AACCACACAACCUACUACCUCA-3′; IDT, Integrated DNA Technologies, Inc., Coralville, IA, USA). Cells were harvested and lysed at 24 h after transfection. The Dual Luciferase Reporter assay system (Promega) was used to analyze the expression of luciferase in the transfected cells according to the manufacturer's instructions. Cells transfected with 200 nM let-7b precursor (Ambion Applied Biosystems, Austin, TX, USA) were used for further analysis.

Immunoblotting

Cell lysates from transfected cells were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of total protein were denatured in electrophoresis sample buffer (10% SDS; 0.5 M Tris, pH = 6.8; 20% (v/v) glycerol and 0.2% (w/v) bromophenol blue) and boiled at 95 °C for 5 min before running SDS-PAGE. Afterward, proteins were transferred into nitrocellulose (NC) membranes in the transfer buffer (25 mM Tris–HCl, pH = 8.3; 192 mM glycine and 10% methanol) at a constant 300 V for 1 h. The NC membranes were blocked in TTBS (200 mM Tris–HCl; 5 M NaCl and 0.05% Tween-20, pH = 7.5) containing 2.5% skim milk (w/v) for 1 h and probed with antibodies for EMMPRIN (sc-9757, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:500 dilution) or mmp-9 (sc-6841, Santa Cruz Biotechnology; 1:500 dilution) in TTBS at room temperature for 2 h. After several thorough washes with TTBS, the NC membranes were incubated with the secondary antibodies conjugated to horseradish peroxidase (sc-2020, Santa Cruz Biotechnology; 1:2500 dilution) in TTBS at room temperature for 1 h. After washing with TTBS, the NC membranes were visualized using Chemiluminescence ECL kit (Thermo, Waltham, MA, USA), according to the manufacturer's instructions. The expression signals of Bsg or mmp-9 were normalized to β-actin as detected in each lane by an antibody (sc-1616, Santa Cruz Biotechnology; 1:1000 dilution).

Invasion and migration assay

At 48 h after transfection of the let-7b precursor, B16-F10 cells were trypsinized and seeded onto Millicell cell culture plate inserts (Millipore, Billerica, MA, USA) that had been coated with 30 μl of ECMatrix™ gel solution (Millipore; 1:10 dilution) in the 24-well culture plates. Cells were incubated in the inserts for 24 h. Those cells that did not migrate or invade the membrane pores of the inserts were removed with a cotton swab, whereas cells on the other side of the insert membrane were stained with Coomassie blue R250, and five random fields per well were counted under an inverted microscope at 400× magnification.

Cell proliferation assay

The B16-F10 cells were seeded in 24-well culture plates and transfected with let-7b precursor. After 24 h, the cells were trypsinized and the number of cells was counted with a

hemocytometer under a microscope. In addition, 72 h after transfection, 100 μl of thiazolyl blue tetrazolium bromide (5 mg/ml, MTT, Sigma-Aldrich Corp., St. Louis, MO, USA) was added for a further 3-h culture in a humidified atmosphere containing 5% CO_2 at 37 °C. Medium was discarded and the cells were harvested in 500 μl of DMSO (Sigma-Aldrich) and shaken for 15 min. Cell lysate was analyzed for MTT activity to indicate cell proliferation by measuring the absorbance at 595 nm.

Colony formation assay

At 48 h after transfection, 5000 B16-F10 cells mixed with agarose for a final concentration of 0.7% containing 20% FBS and 2× DMEM were plated onto a 3.5 cm culture dish over a base layer of 1% agar and allowed to gel. Colonies were counted in 8 random fields at 40× magnification by microscopy after 10 days of incubation at 37 °C in a humidified 5% CO_2 incubator.

Cell cycle analysis

Following transfection with let-7b, 48 h later B16-F10 cells were fixed with cold 70% ethanol and stained with propidium iodide (PI, 10 μg/ml) containing 0.1% (v/v) Triton X-100 and 0.2 mg/ml of RNase A. Cell populations in G0-G1, S, and G2-M phases were measured by Cytomics™ FC 500 flow cytometry (Beckman Coulter), and the data was processed by CXP analysis software.

Tumor metastasis in vivo

A total of eight C57BL/6 mice at 8 weeks old were used for this study. Four mice were intravenously injected with 2×10^6 let-7b-transfected or non-transfected B16-F10 cells. Mice were sacrificed 3 weeks after injection, at which time the lungs were harvested for the observation of tumor nodules.

Statistical analysis

All data were subjected to analysis by *t*-test using the general linear models (GLMs) in a statistical analysis system program (SAS version 9.1; SAS Institute, Cary, NC, USA), to determine statistical differences among experimental groups. All experiments were repeated at least 3 times. A *P* value of less than 0.05 was considered statistically significant.

Results

Let-7b targets the 3′UTR of Bsg

By computer-based sequence analysis, we found that the seed regions of the let-7 family all matched the 3′UTR of Bsg, and that let-7b had the most complementary sequence to the Bsg 3′UTR among the let-7 family members (Fig. 1A). To confirm whether let-7b directly binds to the 3′UTR of Bsg, we constructed a reporter plasmid named pRL4-Bsg-3′UTR, in which the Bsg-3′UTR was located immediately downstream of the luciferase gene from the plasmid of pRL4-SV40. After transfecting pRL4-Bsg-3′UTR into mouse B16-F10 melanoma cells, which express let-7b endogenously, the luciferase activity was found to be significantly lower than that in cells transfected with pRL4-SV40 alone. Importantly, the repression

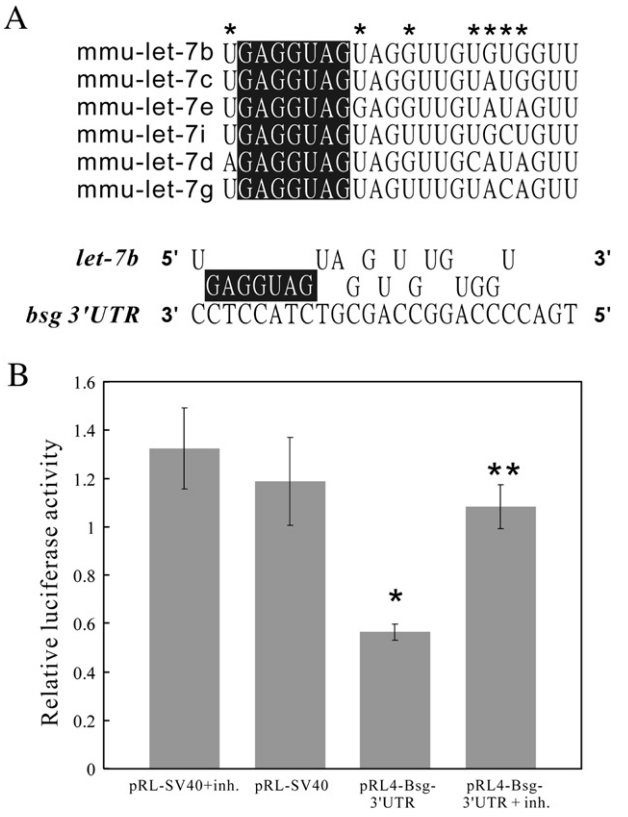

Fig. 1 – Let-7b targets the 3′UTR of Bsg. (A) Upper panel: the ranking of the predicted targeting of the mouse let-7 family to the 3′UTR of Bsg, based on the scores in TargetScan (http://www.targetscan.org/). The GAGGUAG (shaded block) is the seed region of let-7 family. Sequence differences among family members are labeled by single asterisks. Lower panel: the complementarity of mouse let-7b with the binding site at the 3′UTR of Bsg. (B) Luciferase activity in cells transfected with either pRL4-SV40/pRL4-Bsg-3′UTR alone, or combined with anti-let-7b (inh.). *, $P<0.05$, related to pRL4-SV40. **, $P<0.05$, related to pRL4-Bsg-3′UTR. Data are represented as the mean ± standard deviation from three replicates.

of luciferase activity was restored after co-transfection of pRL4-Bsg-3′UTR with anti-let-7b (Fig. 1B). In addition, immunoblotting results demonstrated that Bsg levels were markedly suppressed in B16-F10 cells transfected with 200 nM of let-7b for 72 h (Fig. 2), indicating that let-7b is a negative regulator of Bsg. Taken together, these results suggest that Bsg is a target of let-7b in B16-F10 melanoma cells.

Suppression of Bsg and mmp-9 by overexpression of let-7b

Previous studies indicated that the down-regulation of Bsg could lead to reduced secretion of mmps [13]. To examine the effect of let-7b-depressed Bsg expression on the level of mmps, the expression profile of mmp-9 in B16-F10 melanoma cells that had been transfected with let-7b in serum-free medium was analyzed. At 6 h after transfection, cells were changed to culture medium containing 10% FBS for 18 h, and the culture medium was subsequently replaced with serum-free medium and cultured for

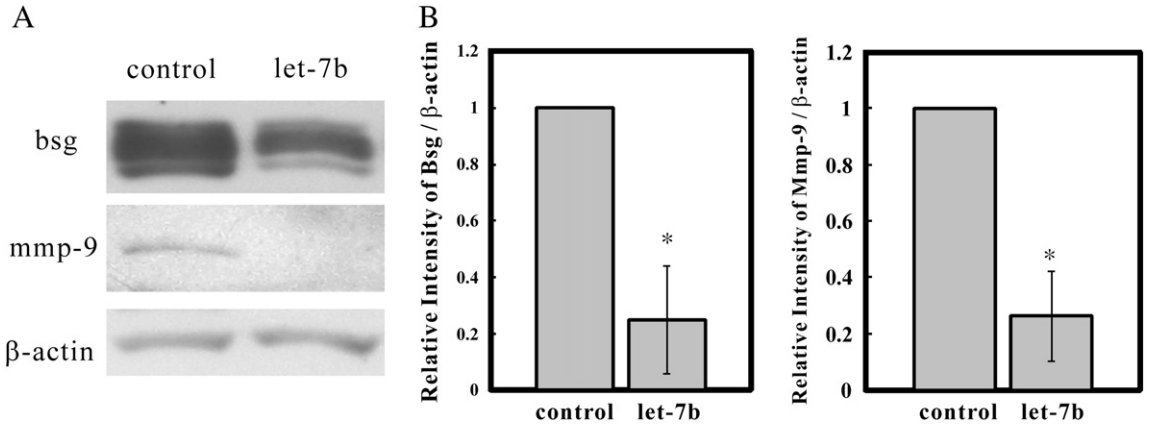

Fig. 2 – Reduction of mmp-9 expression by let-7b-induced suppression of Bsg. B16-F10 melanoma cells were transfected with 200 nM of let-7b precursor. After 6 h, the culture medium was changed to one containing 10% FBS. Eighteen hours later, the culture medium was replaced with serum-free medium and the cells were cultured for another 48 h. At the end of culture, the cell lysate was subjected to Western blotting for Bsg and mmp-9 (A). The results were analyzed by Total Lab software and the expression of Bsg and mmp-9 was normalized to β-actin (B). Asterisks indicated significance ($P<0.05$). Data represent the mean ± standard deviation from three replicates.

an additional 48 h. Expression of Bsg and mmp-9 in the transfected B16-F10 melanoma cells was analyzed by immunoblotting. It was found that not only the level of Bsg but also the level of mmp-9 was significantly down-regulated 72 h after let-7b transfection (Fig. 2). These results clearly demonstrated that let-7b functions as a suppressor of mmp-9 expression, possibly through its silencing effect on Bsg.

Let-7b negatively regulates cell invasion or migration in vitro

Since the levels of mmp-9 were significantly reduced by let-7, and mmps are known to be highly important for tumor migration and invasion, we investigated the effects of let-7b on the invasiveness of melanoma cells. At 48 h after transfection, B16-F10 melanoma cells were placed on ECMatrix™ gel-coated filters and cultured for 24 h. Cells maintained on the filters were stained with Coomassie blue and counted (Fig. 3). The results showed that significantly more cells invaded or migrated in the control group than those in the let-7b-transfected group (88.6 ± 5.83 vs. 26.8 ± 8.67, $P<0.05$), and the invasiveness of cells transfected with let-7b decreased by about 70%. This dramatic decrease in the invasiveness of let-7b-transfected cells likely resulted from let-7b-induced Bsg down-regulation. Similarly, it has been previously reported that the invasion ability of PC-3 cells (prostate carcinoma cells) was significantly lowered after Bsg expression was reduced by siRNA [13].

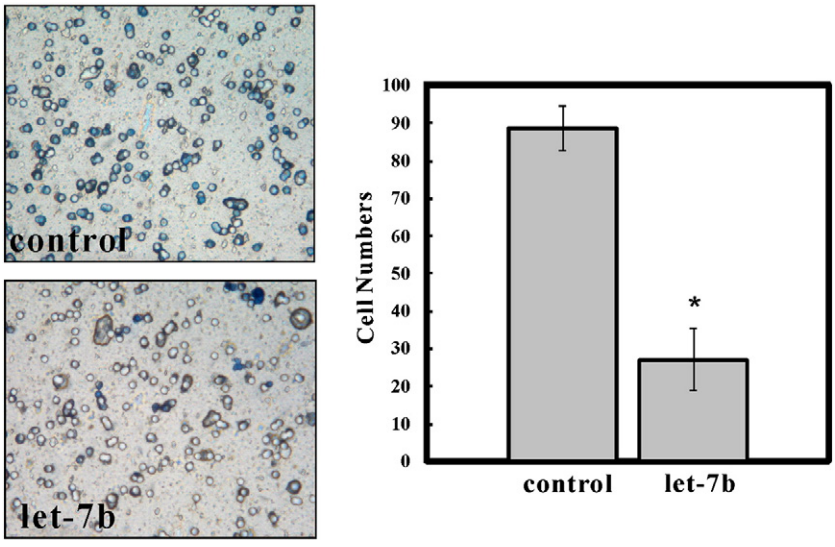

Fig. 3 – Negative regulation of let-7b on cell invasion or migration in vitro. B16-F10 melanoma cells were seeded onto ECMatrix™ gel-coated filters 48 h after the transfection of let-7b precursor or Lipofectamine alone (control). The filters were stained with Coomassie blue R250 and counted by microscopy (left panels). The numbers of cells that invaded or migrated were counted (right panel). The asterisk indicates statistical significance ($P<0.05$). Data are represented as the mean ± standard deviation from three replicates.

Reduction of cell proliferation and colony formation by let-7b in vitro

It has been previously reported that members of the let-7 family are able to suppress cancer cell growth [14,15]. Accordingly, we examined the effect of let-7b on the growth of B16-F10 cells and found that, compared to controls, the cell numbers were significantly decreased in the group of cells transfected with let-7b after 24 h (61260 ± 2613.5 vs. 44760 ± 1459.0, $P<0.05$; left panel on Fig. 4A). By MTT assay, we also showed that the cellular proliferation of the let-7b-transfected cells was dramatically reduced by about 50% (right panel on Fig. 4A). Additionally, the influence of let-7b on colony formation in B16-F10 melanoma cells was observed by conducting soft agar assays. As shown in Fig. 4B, a significant reduction in colony number compared to controls was observed in cells with overexpression of let-7b after 10 days of culture (42.5 ± 1.23 vs. 20.1 ± 0.92, $P<0.05$). Collectively, our results revealed the inhibitory effect of let-7b on the proliferation and colony formation potential of the B16-F10 melanoma cells.

Cell cycle analysis

Based on the results provided in the present study, let-7b could inhibit the proliferation of melanoma cells. To further understand whether cell cycle progression could be affected by let-7b, flow cytometry was used to analyze B16-F10 cells after transfection with let-7b. As shown in Fig. 4C, compared with the other two groups (no treatment and Lipofectamine transfection groups), the overexpression of let-7b in B16-F10 cells significantly reduced the ratios of G2/M and S stages (G2/M stage: 7.5 ± 0.39 vs. 13.6 ± 1.20, 10.5 ± 3.59; S stage: 17.3 ± 0.55 vs. 24.4 ± 0.75, 24.0 ± 1.85, $P<0.05$) and increase the ratio of cells in the G0/G1 stage (75.2 ± 0.80 vs. 61.9 ± 0.50, 65.6 ± 2.05, $P<0.05$). Moreover, the percentage of cells at the G0/G1 stage was increased by about 10% in comparison to that in the Lipofectamine-treated group. These data imply that the effect of let-7b on the cell cycle could be responsible for the inhibition of cell proliferation.

Reduction of tumor metastasis in the mouse model

It is known that B16-F10 cells are highly metastatic in C57BL/6 mice. Therefore, the possibility of the inhibition of metastasis by let-7b in B16-F10 cells was examined. Let-7b-transfected B16-F10 cells were injected into the tail veins of C57BL/6 mice. At 3 weeks after injection, the number of tumor nodules found in the lung was obviously lower in the group receiving let-7b treatment compared to the control group (Fig. 5). It was difficult to count the number of nodules in the control group as they spread over the entire lung and were connected together. These results suggest that let-7b may be an inhibitory regulator of melanoma cell metastasis.

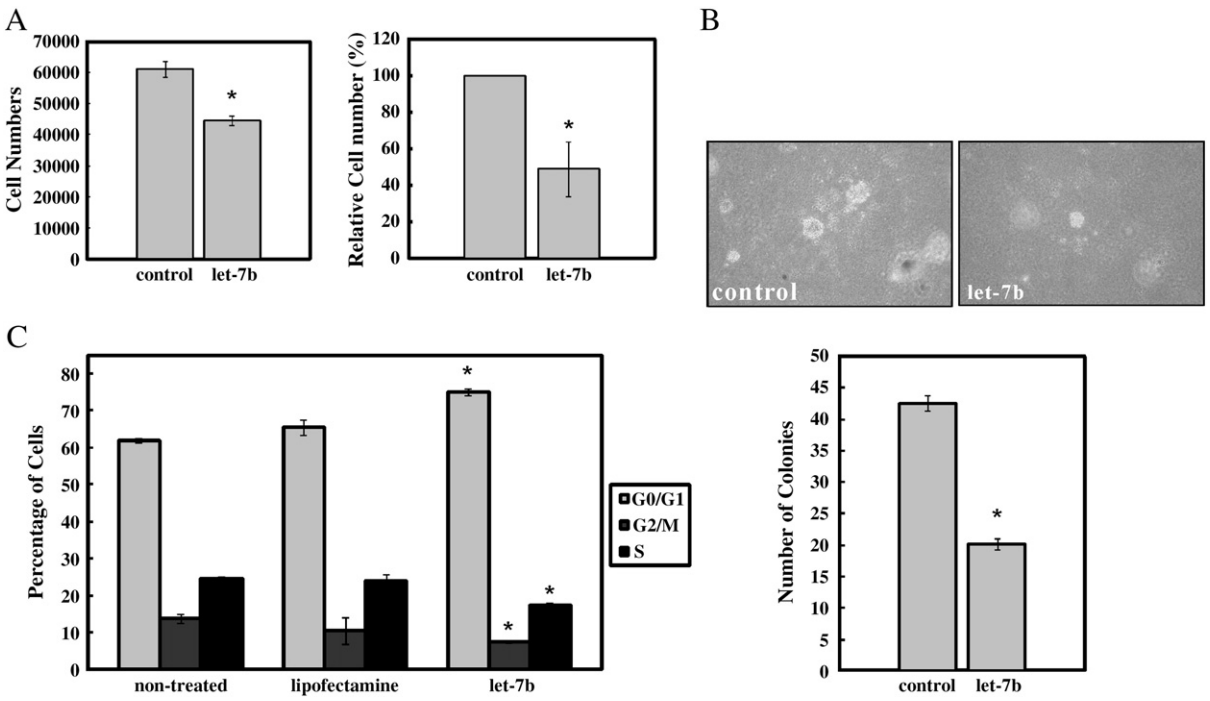

Fig. 4 – Effects of let-7b on cell proliferation, colony formation and cell cycle *in vitro*. The cell numbers were counted using light microscopy 24 h after transfection with let-7b precursor (left panel in A), and the MTT assay was performed after 72 h (right panel in A). The pictures of colonies from one representative experiment at 40× magnification are shown (upper panel in B), and the numbers of colonies were counted and presented as mean ± standard deviation (lower panel in B). The impact of let-7b on the cell cycle in B16-F10 melanoma cells was analyzed. At 48 h after transfection with let-7b precursor, cells were subjected to analysis by flow cytometry. Asterisks indicate statistical significance of the comparisons between the group of let-7b precursor-transfected cells and the groups of non-treated and Lipofectamine-transfected cells (C). Asterisks indicate statistical significance ($P<0.05$). Data are represented as the mean ± standard deviation from at least three replicates.

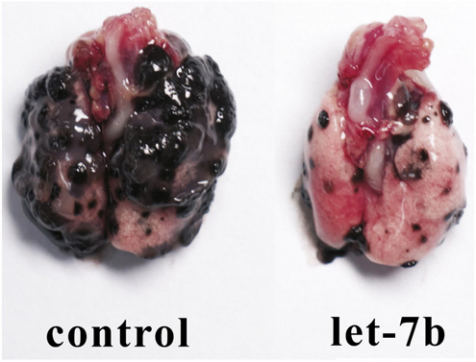

Fig. 5 – Reduction of tumor metastasis in mouse animal model. Let-7b-transfected cells were injected into C57BL/6 mice through the tail vein, and the lungs were collected 3 weeks after injection. The tumor nodules (black spots) were observed. Four mice were included in each group.

Discussion

In this study, we postulated that Bsg was a potential target of let-7b according to a computer analysis performed using the miRGen database (http://www.diana.pcbi.upenn.edu/miRGen/v3/miRGen.html). We have shown that let-7b directly interacts with the 3′UTR of Bsg by luciferase assay. Furthermore, immunoblotting revealed that the protein level of Bsg in the mouse melanoma cell line B16-F10 was down-regulated by let-7b, validating that Bsg is a target of let-7b (Fig. 1). It was known that Bsg is involved in a variety of cellular functions, such as growth, development and differentiation. More importantly, it is highly expressed in malignant melanoma cells and has been implicated to play an important role in tumor invasiveness and metastasis by stimulating the surrounding fibroblasts [16], tumor cells or endothelial cells [17] to produce mmps. In the human breast cancer cell line MDA-MB-436, it was found that increased tumorigenesis and invasiveness was associated with higher expression of mmp-2 and mmp-9 after transfection with a Bsg cDNA [18]. Additionally, the expression of mmps was enhanced in mouse endometrial stromal cells that had been treated with recombinant Bsg [19]. In the present study, reduced expression of Bsg and mmp-9 were shown after the overexpression of let-7b in mouse melanoma cells (Fig. 2). Results from a computer sequence analysis in the miRGen database showed no complementary sequence in the mmp-9-3′UTR to let-7b (data not shown), indicating that reduced expression of mmp-9 most likely results from the suppression of Bsg through let-7b, and that Bsg is an effective factor through which the expression of mmp-9 is regulated in mouse melanoma cells.

Approximately 90% of deaths among patients suffering from solid tumors are caused by metastasis. Metastasis is a complex and multistep process in which primary tumor cells invade adjacent tissues and enter the lymphatic and blood vessels. Notably, increasing lines of evidence highlight the integral role of miRNAs in regulating metastasis [20]. For example, miRNA-10b [21] and miRNA-373 [22] were both up-regulated and found to promote metastasis in both human glioblastoma and breast tumor cell lines. Additionally, Zhu et al. [23] indicated that miRNA-21 plays a promoting role not only in tumor growth but also in invasion and tumor metastasis, by targeting multiple tumor suppressor genes. In contrast to these pro-metastatic miRNAs, other groups of miRNAs negatively mediate metastasis. For instance, in some highly metastatic cancer cells, the let-7 family [4,5,7,14,15], miRNA-205 [24], miRNA-335 and miRNA-126 [25] are down-regulated and function as metastasis suppressors. Furthermore, in human glioblastoma U373 cells, miRNA-146b was shown to cause a significant reduction in cell migration and invasion through the targeting of mmp-16 [26]. In our study, we identified another metastasis-suppressive miRNA, let-7b, and revealed that let-7b negatively regulates metastasis, possibly by down-regulation of Bsg, which might in turn lead to a reduction of mmp-9 level and the consequent suppression of invasiveness in B16-F10 cells (Fig. 3). However, we could not rule out the possibility that the reduction of mmp-9 after let-7b overexpression is induced by other mechanism in the present study, as the stimulating effect of Bsg on the production of mmps might be different in various cell types [17].

Notably, although let-7b indirectly suppressed mmp-9, as no complementary sequences between let-7b and mmp-9-3′UTR were found, our computer-based sequence analysis revealed that the let-7 family could possibly target other mmps, including mmp-1a, mmp-8 and mmp-19, by directly binding to their 3′UTR sequences. Thus, further investigation will be required to identify the functional regulation of let-7 on these mmps.

The let-7 miRNA family has been identified as tumor suppressive. This notion is supported by other's findings that the growth of cancer cells was suppressed by let-7 family [15,16] and by the association of the down-regulation of the let-7 family with the overexpression of oncogenes such as Ras, Myc and HMGA2 (High Mobility Group A2). In particular, the 3′ UTR of HMGA2 has seven conserved sites that are complementary to the let-7 miRNA family, and the loss of repressive elements that were recognized by let-7 in the 3′ UTR of HMGA2 was associated with various human tumors [27]. In addition, it has been shown that cdc34, an E2 ubiquitin-conjugating enzyme of the SCF (Skp1/cullin/F-box) complex, is a direct target of let-7b [8]. Since degradation of multiple cell cycle regulators is mediated through ubiquitination by the SCF complex, an increase in let-7b levels that causes down-regulation of cdc34 would stabilize the SCF target, Wee 1 kinase, leading to a reduction in cell number and G2/M cell cycle arrest.

It has been pointed out that glycolysis is enhanced and that the amount of the end product of glycolysis, lactate, is increased in tumor cells. Accordingly, the proliferation, invasiveness, metastasis and angiogenesis of tumor cells might result from the reduction of the extracellular pH value. During the progression of malignant melanoma, the highly expressed Bsg protein interacts with monocarboxylate transporters (MCT) 1 and 4 to promote tumor cell glycolysis. In this study, we showed that let-7b is a suppressor of Bsg and inhibits the proliferation, colony formation and invasiveness of B16-F10 melanoma cells (Fig. 4). Similar results with regard to proliferation and colony formation were also found in a previous report [5]. Thus, it is speculated that knockdown of Bsg by let-7b might reduce the rate of glycolysis and inhibit the decrease in extracellular pH value and/or the production of ATP, as previously described [28].

In conclusion, our findings identified Bsg as a direct target of let7b. Also, our data demonstrated that the reduction of mmp-9

might account for one of the mechanism underlying the anti-metastasis function of let-7b, which was evidenced by the suppression of invasiveness *in vitro* and the repression of lung metastasis *in vivo*. We argue that this study may have significant clinical implications and therefore warrants further investigation.

Acknowledgments

The authors would like to thank BioLASCO Taiwan Co., Ltd. for the supply of experimental animals. Also, the authors are grateful to the National Science Council for the partial financial support (95-2313-B-005-035-MY2).

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