

# Galectin-3 increases the motility of mouse melanoma cells by regulating matrix metalloproteinase-1 expression

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Abbreviations: ChIP, chromatin immunoprecipitation; IP, immunoprecipitation; MMP-1, matrix metalloproteinase-1

## Abstract

**Although mounting evidence indicates the involvement of galectin-3 in cancer progression and metastasis, the underlying molecular mechanisms remain largely unknown. In this study, we investigated the effect and possible mechanism of galectin-3 on the migration and invasion of B16F10, a metastatic melanoma cell line, in which galectin-3 and matrix metalloproteinase-1 (MMP-1) were both found to be highly expressed. Knockdown of galectin-3 with specific siRNA reduced migration and invasion, which was associated with reduced expression of MMP-1. To further investigate the underlying mechanism, we examined the effect of galectin-3 knockdown on the activity of AP-1, a transcriptional factor regulating MMP-1**

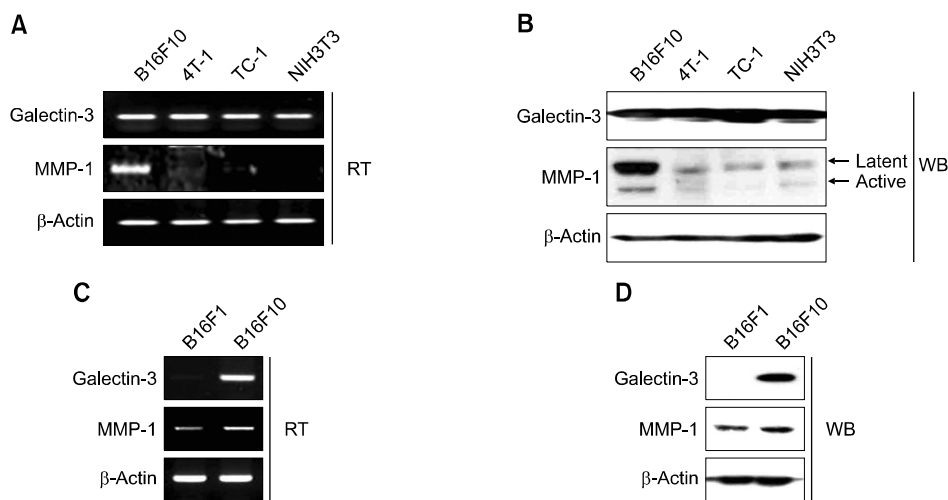
**expression. We found that galectin-3 directly interacted with AP-1 and facilitated the binding of this complex to the MMP-1 promoter that drives MMP-1 transcription. Moreover, silencing of galectin-3 inhibited binding of fra-1 and c-Jun to promoter sites of MMP-1 gene. Consistent with these *in vitro* findings, our *in vivo* study demonstrated that galectin-3 shRNA treatment significantly reduced the total number of mouse lung metastatic nodules. Taken together, galectin-3 facilitates cell migration and invasion in melanoma *in vitro* and can induce metastasis *in vivo*, in part through, regulating the transcription activity of AP-1 and thereby up-regulating MMP-1 expression.**

**Keywords:** galectin 3; matrix metalloproteinase 1; melanoma; neoplasm metastasis; RNA, small interfering; transcription factor AP-1

## Introduction

Galectin-3, a chimera-type of 31-kDa galactose-binding protein, is highly expressed in a variety of primary and metastatic tumors (Raz *et al.*, 1990). Galectin-3 is associated with enhanced tumor progression, invasion and metastasis in various cancer cells, e.g., lung and gastric cancer cells (Hood and Cheresh, 2002; Liu and Rabinovich, 2005). Changes in circulating galectin-3 levels in cancer patients may reflect the metastatic spread of disseminating cancer cells (Yu, 2010). Thus, inhibition of galectin-3 may be a novel therapeutic approach for preventing metastasis and reducing cancer-associated mortality (Kim *et al.*, 2010). However, the mechanisms underlying the role of galectin-3 in tumor progression and metastasis remain largely unclear.

Matrix metalloproteinases (MMPs) facilitate tumor progression and metastasis (Khokha, 1994; Ahonen *et al.*, 1998; Valente *et al.*, 1998). Among these, MMP-1, MMP-2, MMP-9 and MT1-MMP are involved in human melanoma invasion (Walker and Woolley, 1999; Hofmann *et al.*, 2005). MMP-1, which is capable of degrading type I, II and III collagens, is the only MMP subtype able to degrade triple helical collagens (Pardo and Selman, 2005). Recent



**Figure 1.** Expression levels of galectin-3 and MMP-1 in several mouse cell lines. (A) mRNA and (B) protein expression levels of galectin-3 and MMP-1 in B16F10, 4T-1, TC-1 and NIH3T3 cell lines, determined by RT-PCR and western blotting.  $\beta$ -actin was used as a loading control. (C) mRNA and (D) protein expression levels of galectin-3 and MMP-1 in B16F1 and B16F10 cell lines were determined by RT-PCR and western blotting.  $\beta$ -actin was used as normalization control.

studies showed that MMP-1 knockdown cells reduce melanoma invasion into matrigel, whereas its activation promoted melanoma cell invasion into three dimensional type I collagen (Durko *et al.*, 1997; Benbow *et al.*, 1999). Clinical studies have also provided evidence that MMP-1 is positively correlated with malignancy of melanoma cells and poor prognosis of patients (Nikkola *et al.*, 2002).

MMP-1 expression is controlled by mitogen activated protein kinase (MAPK) pathway(s) and the upstream transcription factor, activation of activator protein-1 (AP-1) (Tower *et al.*, 2003; Jung *et al.*, 2008). Although increasing attention has been paid to the roles of galectin-3 and MMP-1 in cancer metastasis, and a recent study showed a significant role of galectin-3 in MMP-1 expression in gastric cancer cells (Cheong *et al.*, 2010; Cho *et al.*, 2011; Kim *et al.*, 2011), it is unclear whether similar regulation occurs in melanoma.

In this study, we demonstrated that galectin-3 regulated MMP-1 expression and melanoma cell metastasis using B16F10 cell line, as a highly expression of both galectin-3 and MMP-1 in a mouse melanoma cell line. The B16F10 cell line is well established in *in vivo* mouse lung metastasis systems (Nguyen *et al.*, 2007). We demonstrated that knockdown of galectin-3 reduced MMP-1 expression and melanoma metastasis both *in vitro* and *in vivo*, in part by modulating AP-1 activity.

## Results

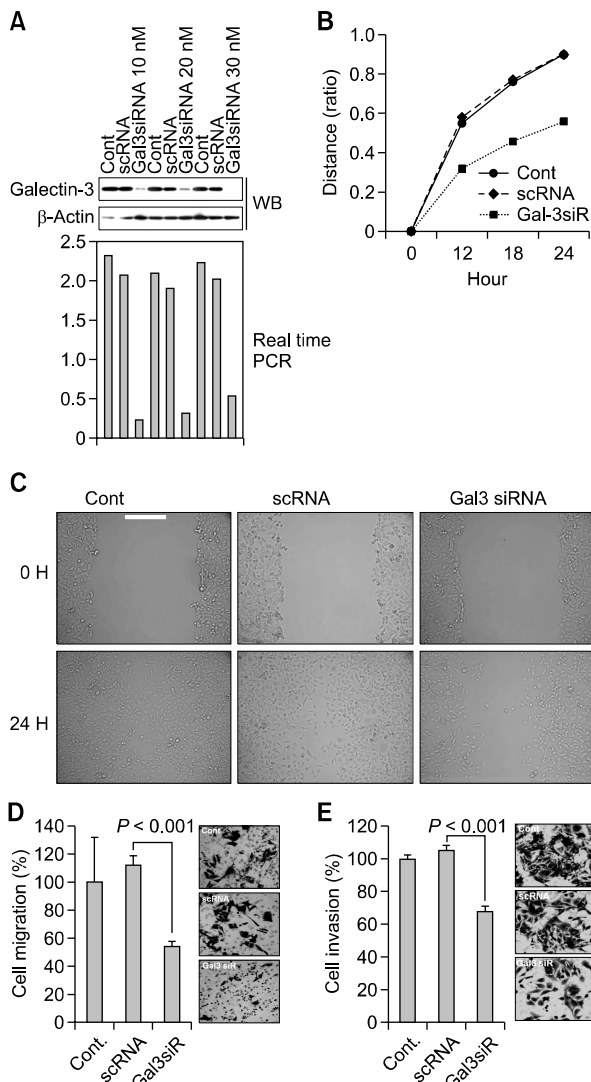
### Expression levels of galectin-3 and MMP-1 in mouse cancer cell lines

The mRNA and protein expression of galectin-3 and MMP-1 were assessed in the following 4 cell types: highly metastatic melanoma cancer cell (B16F10),

highly metastatic breast cancer cell (4T-1), HPV positive and invasive mouse cervical cancer cells (TC-1), and mouse fibroblast cells (NIH3T3). As shown in Figures 1A and 1B, galectin-3 was highly expressed in all four cell lines, whereas the expression of MMP-1 was obviously higher in the B16F10 cell line than in others. Compared to the non-metastatic cell line B16F1, B16F10 cells express a higher level of both galectin-3 and MMP-1, in both mRNA and protein levels, respectively (Figures 1C and 1D).

### Galectin-3 knockdown inhibited B16F10 melanoma cell migration and invasion

To examine the effects of galectin-3 knockdown on B16F10 cell migration and invasion, cells were transfected with various concentrations of galectin-3 siRNA, and galectin-3 expression was assessed using real time PCR and western blotting respectively. As shown in Figure 2A, all three concentrations of galectin-3 siRNA significantly reduced protein expression, with no significant differences in potency. Thus, 20 nM of galectin-3 was chosen for the following experiments. After transfection of B16F10 cells with 20 nM galectin-3 siRNA for 24 h, the living cells were collected and subjected to wound healing, invasion and migration assays. As shown in Figure 2B, cells transfected with galectin-3 siRNA migrated significantly slower than those without transfection or transfected with a scRNA in wound healing assays. Representative images are shown in Figure 2C. In addition, our trans-well migration and invasion assays also demonstrated approximately 50% and 65% decrease in cell migration and invasion, respectively, in the galectin-3 siRNA transfected cells compared to the control cells (Figures 2D and 2E).



**Figure 2.** Effect of galectin-3 silencing on the migration and invasion of B16F10 melanoma cells. (A) mRNA and protein levels of galectin-3 after transfection of B16F10 melanoma cells with different concentrations (10, 20, 30 nmol/L) of scrambled siRNA (scRNA, negative control) and galectin-3 siRNA. Total RNA and protein were obtained after transfection for 48 h, and cells were harvested and analyzed by real time-PCR and western blotting, respectively.  $\beta$ -actin was used as a loading control. (B-C) A wound healing assay of B16F10 cells was performed with scRNA or galectin-3 siRNA at 0, 12, 18 and 24h after scratching. (B) Quantification of cell migration as described in the Methods section and (C) the micrographs of B16F10 cells were obtained according to the width change of the area lacking cells (Bar = 100  $\mu$ m). (D-E) Cell migration and invasion activities were measured respectively by a Transwell assay after transfection of melanoma cells with scRNA or galectin-3 siRNA for 24 h. All experiments were performed individually in triplicate ( $P < 0.001$  vs. control group).

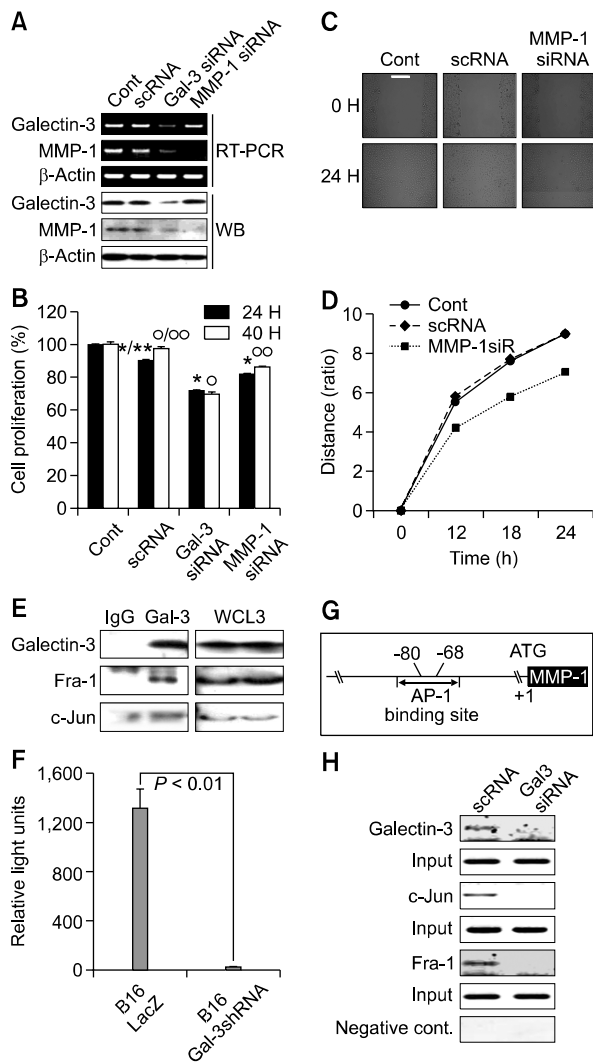
### Galectin-3 modulated MMP-1 expression through interacting with AP-1

Given the expressions of both galectin-3 and MMP-1 in the mouse B16F10 cell line, we examined

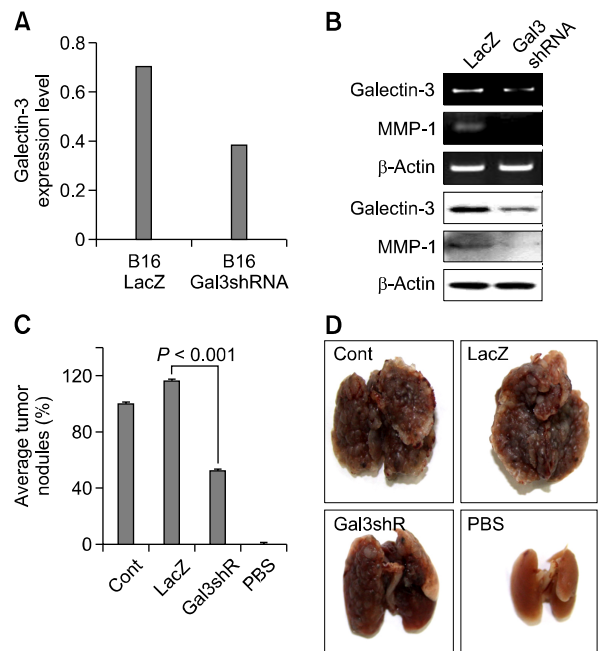
their possible interactions and the effect of MMP-1 on cell motility. MMP-1 knockdown had no effect on the expression of galectin-3, but galectin-3 knockdown resulted a marked reduction of MMP-1 expression, indicating that galectin-3 is an upstream regulator of MMP-1 (Figure 3A). A cell proliferation assay demonstrated that transfection of gal-3 siRNA or MMP-1 siRNA slightly decreased cell proliferation compared to the scrambled siRNA transfected cells (Figure 3B). In contrast, MMP-1 knockdown dramatically decreased cell motility (Figures 3C and 3D). To further explore how galectin-3 increases MMP-1 expression in melanoma cells, we focused on the transcription factor, activator protein-1 (AP-1), which is a dimer composed of Jun and Fos-like region antigen (Fra-1) (Angel and Karin, 1991). The direct interaction between galectin-3 and members of AP-1, c-Jun and Fra-1 was demonstrated by immunoprecipitation (Figure 3E). Next, we assessed transcription activity of AP-1 using a luciferase assay. Compared to the control cells, transfection of the cells with galectin-3 shRNA significantly reduced AP-1 transcription activity (Figure 3F). Finally, we examined DNA binding activity of AP-1 with or without galectin-3. Figure 3G shows the binding site of the AP-1 complex on the MMP-1 promoter region. Our ChIP assay demonstrated that c-jun and fra-1 bound to the promoter regions of MMP-1 in the presence, but not in the absence, of galectin-3 (Figure 3H).

### Galectin-3 knockdown decreased B16F10 metastasis in lung metastasis mouse models

We constructed lentiviral vectors containing lacZ (negative control) or galectin-3 shRNA, and infected B16F10 cells with the lentivirus. As shown in Figure 4A, transfection of the B16F10 cells with galectin-3 siRNA resulted in approximately 53% reduction of the mRNA level of the target protein. As expected, transfection of B16F10 cells with galectin-3 markedly reduced the expression of MMP-1 (Figure 4B). To examine the effect of galectin-3 knockdown on melanoma metastasis *in vivo*, we applied the lentiviral infected cells to a mouse pulmonary metastasis model. Metastasis was assessed in C57BL/6 mice four weeks after tail vein injection of B16F10 parent cells, B16F10 cells infected with lentiviral lacZ, or gal-3shRNA. As shown in Figures 4C and 4D, the total numbers of lung metastatic nodule were reduced by approximately 50% in the animals injected with the B16F10 cells containing lentiviral gal-3 shRNA compared to the animals injected with B16F10 parent cells or lacZ expressing B16F10 cells.



**Figure 3.** Effect of galectin-3 on MMP-1 expression through binding with AP-1 complex and effect of MMP-1 silencing on the motility of B16F10 melanoma cells. (A) mRNA and protein expression levels of galectin-3 or MMP-1 were detected by RT-PCR and western blotting respectively after transfection of B16F10 melanoma cells with galectin-3 or MMP-1 siRNA. scRNA was used as negative control and  $\beta$ -actin was used as a loading control. (B) Cell proliferation was detected after transfection of B16F10 cells with scrambled siRNA, galectin-3 siRNA or MMP-1 siRNA for 24 and 40 h by MTT assay as described in the Methods section ( $*P < 0.001$ ,  $**P < 0.001$ ,  $\circ P < 0.001$ ,  $\circ\circ P < 0.001$ ). (C, D) Wound healing assays utilizing B16F10 melanoma cells were performed by scRNA or MMP-1 siRNA transfected cells at 0, 12, 18 and 24 h after creating a scratch. (C) The phase micrographs of B16F10 cells were obtained according to the data. (bar=100  $\mu$ m) and (D) quantification of cell migration using the monolayer wound healing assay was performed as described in the Methods section. All experiments were performed individually in triplicate. (E) Interaction between galectin-3 and AP-1 (Fra-1 and c-Jun) was measured by immunoprecipitation as previously described in "Methods", and then expression of galectin-3, Fra-1 and c-Jun was detected by western blot. Whole-cell lysates (WCLs) were used as a positive control. (F) Luciferase activity of AP-1 in LacZ and galectin-3 knock down cells. Luciferase assay was performed using an AP-1 expression luciferase vector transfection to lacZ and galectin-3 knock down cells ( $P < 0.01$ , vs. Cont). (G) The proximal AP-1 binding site which is located between -80 and -68 from the initiation codon of the MMP-1 gene. (H) A ChIP assay was performed using antibodies against galectin-3, c-Jun and Fra-1 in melanoma cells transfected with scRNA and galectin-3 siRNA. In the input lane, total genomic DNA was used as a control for the PCR reaction.



**Figure 4.** Suppressive effects of galectin-3 silencing on metastasis of B16F10 melanoma cells in lung metastasis mouse models. (A) Expression levels of galectin-3 were detected by real time-PCR analysis in B16F10 cells infected with lenti-virus expressing LacZ or galectin-3 shRNA (gal-3shRNA). The preparation and infection of these lenti-viruses were described in the Methods section. (B) Expression levels of galectin-3 and MMP-1 were detected by RT-PCR and western blot analysis in B16F10 melanoma cells transfected stably with lacZ and gal-3shRNA respectively.  $\beta$ -actin was used as a loading control. (C, D) Four weeks after tail vein injection of PBS (negative control), parent B16F10 melanoma cells, lacZ expressing B16F10 cells or gal-3shRNA expressing cells into C57BL/6 mice, the formation of lung colonies was counted and illustrated as a histogram ( $P < 0.01$  vs. lacZ group) (C) and photographs (D). The details are described in the Methods section.

## Discussion

This study was performed to better understand the mechanisms underlying the role of galectin-3 in melanoma invasion and metastasis. We have previously shown that after silencing of galectin-3, some genes such as MMP-1 and MMP-3 are down-regulated, while other genes, such as serine protease inhibitor and tissue inhibitors of MMP-2 were up-regulated (data not shown) (Cheong *et al.*, 2010). Among these genes, MMP-1 was down-regulated by galectin-3 to a greater extent than others, which was further confirmed by the following findings. We confirmed the extensive expression of galectin-3 in different mouse cell lines including B16F1, B16F10 (melanoma), 4T-1 (breast carcinoma), TC-1 (lung cancer) and NIH3T3 (embryonic fibroblast), which may imply its conservation and significance in many types of cancer. However, much higher expression of MMP-1 was detected in the melanoma cell line B16F10 than in the 4T-1,

TC-1 and NIH3T3 cell lines. We also assessed galectin-3 and MMP-1 expression in the B16F1 and B16F10 melanoma cell lines and found that the non-metastatic cell line (B16F1) did not express galectin-3 while the metastatic cell line (B16F10) did. A previous report indicated that galectin-3 regulates the metastasis of various types of cancer *via* different mechanisms (Liu and Rabinovich, 2005). However, the mechanisms underlying the involvement of galectin-3 in mouse cancer cell motility remain largely unknown. Our findings seem to indicate that one possible mechanism of melanoma cell motility in mice involves a putative relationship between galectin-3 and MMP-1 expression.

We observed that galectin-3 knockdown dramatically suppressed the motility, migration, and invasion of melanoma cells. In parallel, MMP-1 knockdown also significantly reduced the cell motility. Knockdown of galectin-3 reduced the expression of MMP-1 but not vice versa, and it is suggested that galectin-3 is an upstream regulator of MMP-1. This finding sheds new light on the mysterious relationships between galectin-3 and MMP-1. To understand how galectin-3 regulates MMP-1 expression, we determined the interaction between galectin-3 and the AP-1 complex (Song *et al.*, 2005). The AP-1 family of transcription factors is composed of the homodimers or heterodimers of the Jun (JunB, JunD, c-Jun) and Fos (c-Fos, Fos B, fra-1, fra-2) onco-proteins (Lee *et al.*, 1987). Among them, c-Jun plays important roles in regulating cancer invasion and metastasis. For instance, the c-Jun dominant-negative mutant, TAM67, inhibits the effects of AP-1 on the growth of breast cancer (Moore-Carrasco *et al.*, 2006), as well as on the invasion and metastasis of murine osteosarcomas (Leaner *et al.*, 2009). Fra-1, another component of the AP-1 family, plays a key role in MMP-1 transcription in A2058 melanoma cells (Tower *et al.*, 2003). Here, we provide evidence for the association of galectin-3 with c-Jun and fra-1 and regulation of AP-1 transcription activity. Interestingly, c-Jun and fra-1 bound to the promoter regions of MMP-1 in the presence of galectin-3, but not in its absence. This is the first case reported in which galectin-3 contributes to MMP-1 promoter activity by forming a complex with c-Jun and fra-1 in melanoma cells. The role of galectin-3 in melanoma metastasis was also supported by the *in vivo* experimental results. In a mouse pulmonary metastasis model, animals treated with galectin-3 shRNA-transfected B16F10 melanoma cells showed considerable inhibition of lung metastasis compared with the untreated control group.

In conclusion, the promotion of galectin-3 expression resulted in melanoma invasion/metastasis due at

least in part to increased MMP-1 expression *via* the transcriptional activation of AP-1. These findings combined with the recent report by Kim *et al.*, for the involvement of galectin-3 in gastric cancer cell motility (Kim *et al.*, 2011), suggest that galectin-3 plays a role in various cancers' metastasis through a common molecular mechanism.

## Methods

### Cell culture and siRNAs transfection

B16F1, B16F10, 4T-1, TC-1 and NIH3T3 cell lines obtained from the Korea Cell Line Bank were cultured in DMEM or RPMI 1640 medium with 5% fetal bovine serum (FBS) and 1% Antibiotics (Invitrogen). Both galectin-3 and MMP-1 siRNAs were purchased from Invitrogen and transfection was performed with Lipofectamine RNAiMAX reagent (Invitrogen) following the manufacturer's instructions. The sequences of mouse galectin-3 and MMP-1 siRNAs were 5'-AUGAUUGUGAUCAGCAUGCTT-3' and 5'-GCCA GAACTTCCAACCAT-3', respectively.

### RNA isolation and RT-PCR

Total RNA was extracted from mouse melanoma cells with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RT was carried out using a Reverse Transcription system (Promega), and PCR was performed with Ex-taq DNA polymerase (TaKaRa). The sequences of primers were as follows: mMMP-1; 5'-CCTGGAATTGGCAACAAAGT-3' (sense) and 5'-TAGCACGCAAGAATCAGGTG-3' (anti-sense); mGalectin-3; 5'-CAGTGCTCCTGGAGG CTATC-3' (sense) and 5'-AAGGGGAAGGCTGACTGTCT-3' (anti-sense); m $\beta$ -actin: 5'-AGCCTCGCCTTTGCCGA-3' (sense) and 5'-CTG GTGCCTGGGCG-3' (anti-sense).

### Western blot analysis

Western blot analysis was carried out as described previously (Kim *et al.*, 2010). Briefly, cells were lysed in RIPA buffer (Biosesang) containing a protease inhibitor cocktail (Sigma), followed by sonication on ice. The cell lysate was centrifuged and the supernatant was collected. Twenty  $\mu$ g of proteins were subjected to SDS-PAGE and transferred to PVDF transfer membranes (Amersham). After being blocked with 5% skim milk for 1 h, the membrane was incubated with primary antibody dissolved in 5% BSA overnight at 4°C. The membrane was then incubated with secondary antibody for 1 h, followed by detection with an ECL kit (Amersham). The following antibodies were used: anti- $\beta$ -actin (Santa Cruz); anti-galectin-3 (Santa Cruz); anti-MMP-1 (Calbiochem, Santa Cruz).

### Immunoprecipitation assays

Cell lysate containing 750  $\mu$ g of protein was pre-cleared by incubation with 40  $\mu$ l protein-A/G linked agarose beads (Santa Cruz) for 1 h at 4°C. After the beads were spun

down, the supernatant was incubated with 1  $\mu$ g specific antibody (anti-galectin-3, anti-c-Jun and anti-Fra-1, respectively) overnight at 4°C, followed by incubation with 40  $\mu$ l protein-A/G agarose beads for 1 h. Mouse/rabbit IgG (Santa Cruz) was used as the negative control. After the incubation, beads were washed 3 times in RIPA buffer before being dissolved in SDS-PAGE loading buffer. Western blot analysis was performed as described above.

### Transfilter migration and invasion assays

Transfilter migration and invasion assays were carried out with 8.0- $\mu$ m pore inserts in 24-well Transwells (Corning Costar). Briefly, B16F10 cells were transfected with galectin-3 or MMP-1 siRNAs. One day after the transfection, cells were isolated and added to upper Transwell chambers with 0.5 mg/ml collagen type I (BD Bioscience) coated filters for the migration assay, and with 1/15 dilution of matrigel (BD Bioscience) coated filters for the invasion assays. DMEM containing 10% FBS and 1% antibiotics was added to the lower chamber and incubation continued for 20 h. Cells that had migrated or invaded into the lower chamber were quantified after H&E staining. For quantification, cells on were counted at 5 randomly selected areas in each well using a wide-field microscopy. Data were expressed as mean  $\pm$  SD from three independent experiments.

### Wound healing assays

B16F10 cells were seeded into 6-well culture dishes at  $1 \times 10^5$  cells/well, and maintained in DMEM containing 5% FBS for 24 h. The nearly confluent cells were scratched with a pipette tip and cellular debris was removed. The scratched monolayer was then incubated for another 24 h in DMEM. Cells migrating into the gap were visualized and counted under a microscope.

### Lentiviral galectin-3 shRNA construction and stable cell line establishment

Lenti-viral vectors containing galectin-3 shRNA (Gal-3shRNA) was constructed by inserting the double strand gal-3 shRNA into the lentiviral vector pLL3.7 as described previously (Kim *et al.*, 2010). The sequence of the shRNA was as following: gal-3shRNA sense: 5'-TGAACAACAGGAGAGTCATTGTTTCAAGAGAACAATGACTCTCCTGTTGTTCTTTTTC-3'; gal-3shRNA antisense: 5'-TCGAGAAAAA GAACAACAGGAGAGTCATTGTTCTCTTGAACAATGACTCTCCTGTTGTTCA-3'. The lentiviral vector containing lacZ was used as a control. Lentiviral infection of B16F10 melanoma cells was performed as described previously (Kim *et al.*, 2010). Approximately 80% cells were infected and confirmed with FACS analysis.

### Mouse pulmonary metastasis model

Female C57BL/6 mice, purchased from Orient Bio in Korea, were maintained in the animal facility at the National Cancer Center (NCC). B16F10 cells ( $1 \times 10^6$  cells/ml in 100  $\mu$ l PBS) infected with lentiviral lacZ (control) or gal-3shRNA was injected into 6-week-old female C57BL/6

mice (five mice per group) through the lateral tail vein. Four weeks after the injection, mice were sacrificed with an overdose of anesthesia. The number of metastatic nodules on the surface of the lung was counted under the surgical microscope.

### Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay was carried out using a ChIP assay kit (Upstate). Samples were applied to dishes after galectin-3 siRNA treatment and assays were conducted following the manufacturer's instructions. Anti-galectin-3, c-Jun and Fra-1 and normal mouse/rabbit IgG were used to immunoprecipitate DNA containing complexes. Prior to PCR, primers were prepared for AP-1 with MMP-1 promoter binding sites: MMP-1 promoter (-2950) 5'-AAGAAGAAGGTGCCAGGAT-3' and (-2901) 5'-TGCCTTCATTT TCCATTTCC-3'. PCR was performed with Ex Taq (Takara).

### Cell proliferation assay

B16F10 cells, growing in 96-well culture plates at a density of  $3 \times 10^3$  cells/well, were transfected with scrambled siRNA, galectin-3 siRNA, and MMP-1 siRNA respectively. 24 or 40-h post transfection, cell viability was analyzed by MTT assay, as described previously (Cheong *et al.*, 2010).

### Luciferase assays

B16F10 melanoma cell lines were co-transfected with an AP-1 reporter plasmid and galectin-3 siRNA 48 h before the luciferase assay was performed, as described previously (Kim *et al.*, 2010).

### Statistics analysis

All data were obtained from at least three independent experiments and are presented as mean  $\pm$  SD, unless otherwise indicated. Statistical analysis was performed using one-way ANOVA. Data were considered significant if  $P < 0.05$ .

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