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ADAM23 is downregulated in side population and suppresses lung metastasis of lung carcinoma cells

Masahide Ota,[1,2] Satsuki Mochizuki,[1] Masayuki Shimoda,[1] Hitoshi Abe,[1] Yuka Miyamae,[1] Ken Ishii,[3] Hiroshi Kimura[2] and Yasunori Okada[1,4]

[1]Department of Pathology, Keio University School of Medicine, Tokyo; [2]Second Department of Internal Medicine and Respiratory Medicine, Nara Medical University, Kashihara; [3]Department of Orthopedic Surgery, Keio University School of Medicine, Tokyo; [4]Department of Pathophysiology for Locomotive and Neoplastic Diseases, Juntendo University Graduate School of Medicine, Tokyo, Japan

Key words

ADAM23, colony formation, metastasis, non-small cell lung carcinoma cells, side population

Correspondence

Yasunori Okada, Department of Pathophysiology for Locomotive and Neoplastic Diseases, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan.
Tel: +81-3-5800-7531; Fax: +81-3-5800-7532;
E-mail: ya-okada@juntendo.ac.jp

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Cancer cells contain a small population of cancer stem cells or cancer initiating cells, which can be enriched in the side population (SP) after fluorescence activated cell sorting. To examine the members of the ADAM, ADAMTS and MMP gene families related to phenotypes of the SP and the main population (MP), we screened the expression of all the members in the propagated SP and MP of A549 lung adenocarcinoma cells, and found that the relative expression ratio of ADAM23 in the MP to the SP is most highly increased, but none of them are increased in the SP. A similar result on the ADAM23 expression was obtained with another cell line, Calu-3 cells. Overexpression of ADAM23 inhibited colony formation, cell adhesion and migration, and knockdown of ADAM23 by shRNA showed the reverse effects. ADAM23-mediated suppression of colony formation, cell adhesion and migration was greatly reduced by treatment with neutralizing anti-ADAM23 antibody, anti-αvβ3 integrin antibody and/or ADAM23 disintegrin peptide. Expression of cancer stem cell-related genes, including AKRC1/2, TM4SF1 and NR0B1, was increased by knockdown of ADAM23. In addition, lung metastasis of A549 transfectants with different levels of ADAM23 expression was negatively regulated by the ADAM23 expression levels. Our data provide evidence that ADAM23 plays a role in suppression of cancer cell progression through interaction with αvβ3 integrin, and suggest that downregulation of ADAM23 in SP cells may contribute toward providing a cancer stem cell phenotype by facilitating the activity of integrin αvβ3.

Accumulated lines of evidence have indicated that malignant neoplastic cells contain a small sub-population of cells with properties of tumor initiation, self-renewal, resistance to chemotherapy and metastatic potential, which are called cancer stem cells or tumor initiating cells.[1–3] Besides the method utilizing cell surface markers, side population (SP) technology has been used for isolation of cell subsets enriched for cancer stem cells in a variety of cancer tissues and cancer cell lines.[4–8] SP cells are characterized by their capability to efflux Hoechst 33342 dye through ATP-binding cassette (ABC) transporter.[9,10] Previous studies have demonstrated that the SP cells exist in human lung carcinoma cell lines and are enriched with cancer stem-like cells.[8,11–13]

ADAM (a disintegrin and metalloproteinase), ADAMTS (ADAM with thrombospondin motifs) and MMP (matrix metalloproteinase) are the gene families belonging to the metzincin superfamily of zinc-based metalloproteinases.[14,15] Members of the ADAMTS and MMP gene families digest extracellular matrix macromolecules and other molecules such as growth factors, cytokines and chemokines.[15–17] In contrast, the ADAM family is composed of proteolytic and non-proteolytic ADAM species, which are involved in various biological events such as cell adhesion, fusion, migration, membrane protein shedding and proteolysis.[15,18,19] The proteolytic ADAM members such as ADAM10, ADAM12, ADAM15, ADAM17 and ADAM28 are overexpressed in human cancers and associated with tumor growth and progression.[18,20] Non-proteolytic ADAM species such as ADAM22 and ADAM23 are also involved in tumor cell proliferation and progression in glioma and breast carcinoma cells.[21–23] However, the expression patterns and biological functions of the family members in SP and MP cells remain elusive.

In the present study, we examined expression of the ADAM, ADAMTS and MMP gene families in propagated SP and MP cells of A549 lung adenocarcinoma cells, and found that the ADAM23 expression is most significantly decreased in the SP. These results led us to investigate the effects of ADAM23 on cell behaviors, including colony formation, cell adhesion and migration. We also examined how lung metastasis of A549 transfectants is influenced by the ADAM23 expression levels.

Materials and Methods

Cell culture and flow cytometry. Lung adenocarcinoma cell lines A549, Calu-3 and SK-LU-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and PC-9 cells from Immuno-Biological Laboratories (Gunma,

Japan). They were cultured in RPMI1640 supplemented with 10% FBS and antibiotics (Sigma-Aldrich, St Louis, MO, USA). Detached cells were labeled with Hoechst 33342 dye (1.2–5 μg/mL; Invitrogen, Carlsbad, CA, USA) in the presence or absence of verapamil, an ABC transporter inhibitor (100 μM; Sigma-Aldrich), and 2 μg/mL propidium iodide (Sigma-Aldrich) was added to gate viable cells. SP and MP cells, which excluded and included Hoechst 33342 dye, respectively, were obtained by FACS using a Moflo flow cytometer (Beckman, Brea, CA, USA).

Cell proliferation and colony formation assays. Cell proliferation was measured by cell count, using BrdU labeling methods (Roche Molecular Biochemicals, Basel, Switzerland) or by applying the real-time, label-free cellular analysis xCELLigence system (Roche Diagnostics, Mannheim, Germany). For colony formation assay, the cells were seeded on six-well plates (100 cells/well) (Sigma-Aldrich), and cultured in RPMI1640 containing 10% FBS and antibiotics for 5–7 days. After staining with 0.1% crystal violet, colonies of more than 50 cells were counted. For inhibition studies, A549 cells transfected with ADAM23 expression vectors or empty vectors were treated with rabbit anti-human ADAM23 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human αvβ3 integrin antibody (Millipore, Billerica, MA, USA), non-immune rabbit IgG (Dako Cytomation, Carpinteria, CA, USA) or mouse IgG (Sigma-Aldrich) (5 μg/mL each).

Cell adhesion assay. Cells were adhered on fibronectin-coated 96-well plates (2×10^4 cells/well) (Corning Incorporated, Corning, NY, USA) by incubation for 60 min at 37°C in 10-mM HEPES buffer, pH 7.4 containing 140 mM NaCl, 5.56 mM glucose, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$,[24] and adherent cells were counted. Inhibition studies were performed by transfection of ADAM23 expression vectors or incubation with anti-human ADAM23 antibody (Santa Cruz Biotechnology) and anti-human αvβ3 integrin antibody (Millipore). They were also treated with the synthetic peptide corresponding to the disintegrin loop of ADAM23 (AVNEDCDIT; 40 μg/mL; Sigma-Aldrich) or scrambled peptide (DCVTNIAE; 40 μg/mL; Sigma-Aldrich) prior to adhesion assay.[25]

Migration assay. Cells were grown to confluence on six-well plates (BD Biosciences, Bedford, MA, USA) in RPMI1640 containing 10% FBS and antibiotics, and scratch-wounded with a blue pipette tip.[26] They were allowed to migrate in the medium containing 5 mM hydroxyurea (Sigma-Aldrich). Cell migration areas were determined using NIH imageJ software (http://imagej.nih.gov/ij/). Migration was also measured by xCELLigence system.

PCR array. The expression profiles of the ADAM, ADAMTS and MMP gene family members and ABC transporters (ABCG2 and ABCC2) were examined by the RT^2 Profiler PCR Array (CAPH-0858; SABiosciences, QIAGEN, Valencia, CA, USA). Relative changes in gene expression were calculated by ΔΔCt method for all the genes by normalizing the values with the average Ct value of the four housekeeping genes, hypoxanthine phosphoribosyl transferase 1, ribosomal protein L13a, GAPDH and β-actin. The expression of ADAMDEC1 in the SP and the MP was examined by quantitative real-time PCR (qPCR).

RT-PCR and qPCR. cDNA were prepared from isolated total RNA, and subjected to RT-PCR for the expression of ADAM23 and GAPDH using the primers for ADAM23 5′-CCACTCGATTCCAAGGGTAA-3′ (forward), 5′-ACCAGCGATGGAGCCTATTA-3′ (reverse) and for GAPDH 5′-CCACCCATGGCAAATTCCATGGCA-3′ (forward), 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (reverse).[27] The relative expression levels of ADAM23 to GAPDH were measured by a SYBR Green real-time PCR assay (Invitrogen).[28] The ADAMDEC1 expression was also examined by qPCR using the primers of 5′-AGCAAACACCTGAATTAACGCTC-3′ (forward) and 5′-CTCAGGTTTCGTGGTAATTTCC-3′ (reverse). In addition, the expression of ABCG2, ABCC2, AKRC1/2, TM4SF1 and NR0B1 was analyzed by qPCR using the corresponding primers (Table S1).

Overexpression and knockdown of ADAM23. ADAM23 expression vectors and empty vectors were purchased from Origene (Rockville, MD, USA) and transfected using FuGENE6 (Roche Diagnostics). For knockdown of ADAM23, the five different lentiviral vectors for ADAM23 shRNA and non-targeting shRNA vector were purchased from Sigma-Aldrich. Because two of them (sh1 and sh5) efficiently suppressed the expression, they were used for the experiments. The coding sequences of the shRNA were as follows: sh1, 5′-CCGGGCAGTGTTACTGGAACTATTACTCGAGTAATAGTTCCAGTAACACTGCTTTTTG-3′; sh5, 5′-CCGGCCACTCGATTCCAAGGGTAAACTCGAGTTTACCCTTGGAATCGAGTGGTTTTTG-3′. The stable transfectants were selected as described previously.[29]

Immunoblotting. The homogenate supernatants of A549 cells and their transfectants were immunoprecipitated with rabbit anti-human ADAM23 antibody (Abcam, Cambridge, UK) or nonimmune rabbit IgG (Dako Cytomation),[30] and then subjected to immunoblotting with rabbit anti-human ADAM23 antibody (Abcam) and horseradish peroxidase-conjugated secondary antibody (DAKO A/S, Glostrup, Denmark), followed by reaction with ECL western blotting reagents (GE Healthcare Bio-Sciences, Uppsala, Sweden).

Evaluation of tumorigenicity and lung metastasis. Lentiviral expression vectors encoding the fusion protein of firefly luciferase and a circularly permuted variant of Venus, named ffLuc-cp156, were infected to A549 cells, and the ffLuc-cp156-positive cells ($A549^{ffLuc\text{-}cp156}$ cells) were sorted according to our methods as we described previously.[29] They were transfected with empty vectors, ADAM23 expression vectors, non-targeting shRNA vectors or ADAM23 shRNA vectors (sh5). For monitoring tumorigenicity of ADAM23-knockdown cells, $A549^{ffLuc\text{-}cp156}$ mock and shRNA transfectants (sh5) (1×10^2, 1×10^3, 1×10^4, 5×10^4 and 1×10^5 cells/site) were subcutaneously injected into male BALB/c-nu/nu mice (Charles River Laboratories International, Wilmington, MA, USA). Lung metastasis was examined by using 6-week-old male NOD/SCID mice (Charles River Laboratories International), which were injected with the $A549^{ffLuc\text{-}cp156}$ transfectants (1×10^6 cells) into the tail vein. Tumorigenicity and lung metastasis were monitored by the In Vivo Imaging System-100 camera system (Xenogen, Alameda, CA, USA). At 2 weeks, the mice were killed, and the lungs were fixed with 4% buffered formalin. Paraffin sections of the whole lungs were stained with H&E. Whole-slide images were acquired using the NanoZoomer 2.0HT (Hamamatsu Photonics K.K., Hamamatsu, Japan), and numbers of metastatic nodules/mm^2 were evaluated. Housing and maintenance of the mice and all procedures were performed according to the guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine.

Statistical analyses. Data were reported as mean ± SD. Results between the two independent groups were determined by Student's *t*-test. Comparisons among more than three

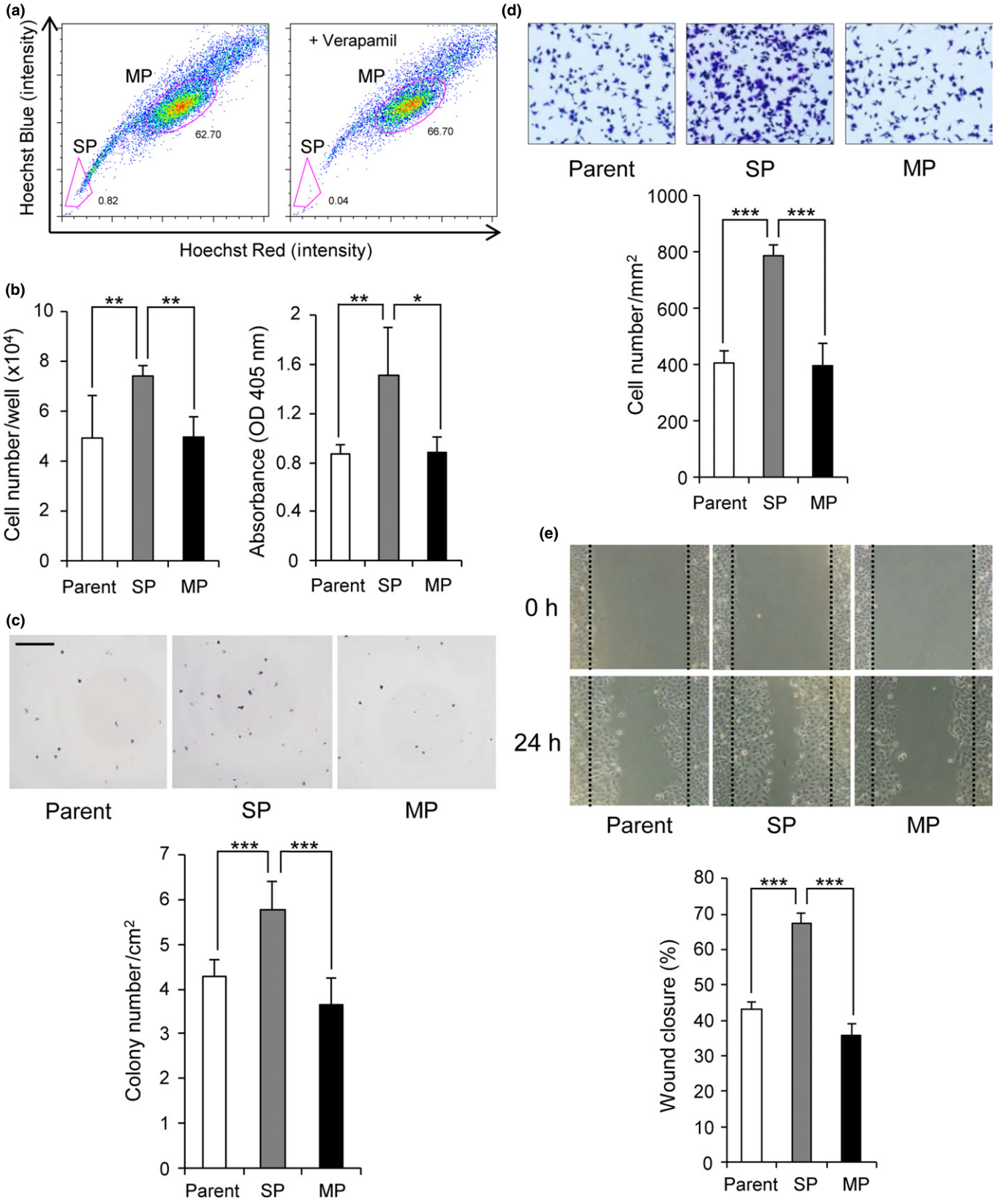


Fig. 1. Characteristics of A549-derived side population (SP) and main population (MP) cells. (a) SP and MP in the absence (left) or presence of verapamil (right) are outlined as a percentage of the total cell population. (b) Proliferation of parent, SP and MP cells was measured by cell counting (left) and BrdU labeling methods (right) at 3 days ($n = 6$). (c) Photos of colonies formed by parent, SP and MP cells (upper panel) and numbers of colonies/cm^2 ($n = 6$). Scale bar = 5 mm. (d) Adherent cells were counted and results are expressed as number of cells/mm^2 ($n = 4$). (e) Migration activity at 24 h was determined and results are expressed as percentage of wound closure ($n = 4$). Bars, mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

groups were adjusted with Bonferroni correction. *P*-values less than 0.05 were considered significant.

Results

Isolation of side population and main population from A549 cells and their characteristics. FACS showed that SP cells, which are diminished to less than 0.05% in the presence of verapamil, occupy approximately 0.8% of the total cells (Fig. 1a). SP cells grew faster than parent and MP cells, and the rate of BrdU labeling was significantly higher in SP cells than in parent and MP cells ($P < 0.05$ or $P < 0.01$) (Fig. 1b). When parent, SP and MP cells were subjected to colony formation assay, the number of colonies was significantly higher in SP cells compared to parent and MP cells ($P < 0.001$) (Fig. 1c). Adhesion assay showed that SP cells adhere approximately twofold more efficiently than parent or MP cells (Fig. 1d). Migration activity of SP cells was significantly higher than that of parent and MP cells ($P < 0.001$) (Fig. 1e).

Propagation of side population fraction and PCR array for expression of the ADAM, ADAMTS and MMP family members. Successive rounds of FACS analysis for SP cells were performed up to nine times by applying the A549-derived fractions of SP and MP; that is, SP(1) and MP(1). As shown in Figure 2a, the percentage of SP cells in the serially nine-time propagated SP(9) cells (8.57 ± 0.12%) was significantly (tenfold) higher than the SP(1) cells (0.81 ± 0.01%) ($P < 0.001$),

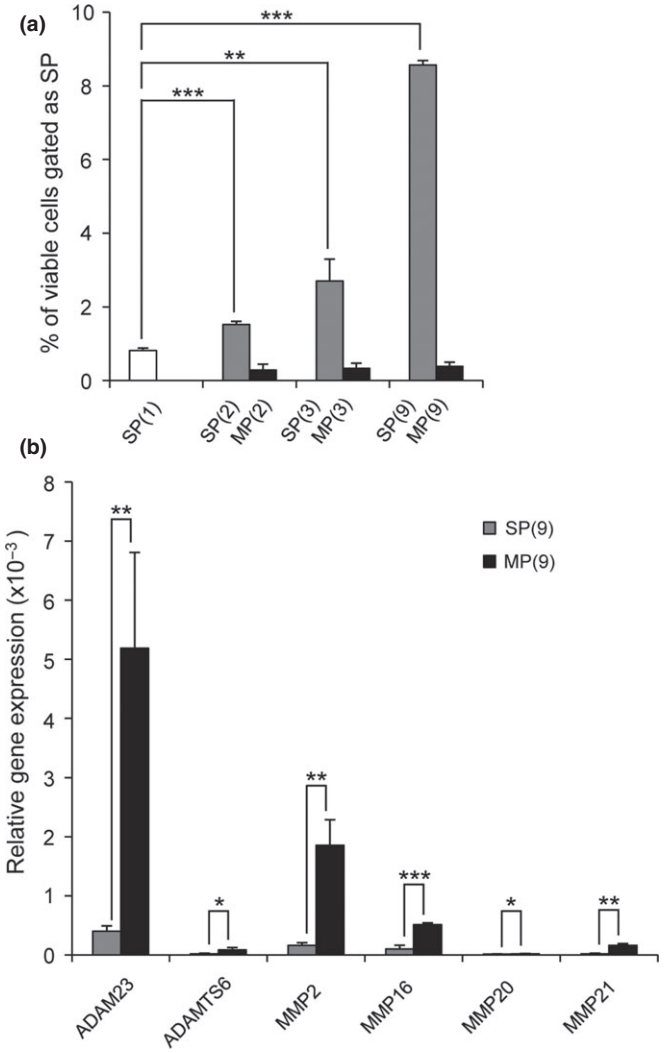


Fig. 2. Propagation of A549-derived side population (SP) cells by successive rounds of FACS and relative gene expression of the ADAM, ADAMTS and MMP family members in main population (MP) and SP. (a) SP cell fraction was sequentially sorted up to nine times. Percentage of SP cells re-sorted from SP(2), MP(2), SP(3), MP(3), SP(9) and MP (9) is shown ($n = 3$). (b) The ADAM, ADAMTS and MMP family members with significantly increased expression in MP(9) compared to SP (9) by the PCR array ($n = 3$). Bars, mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

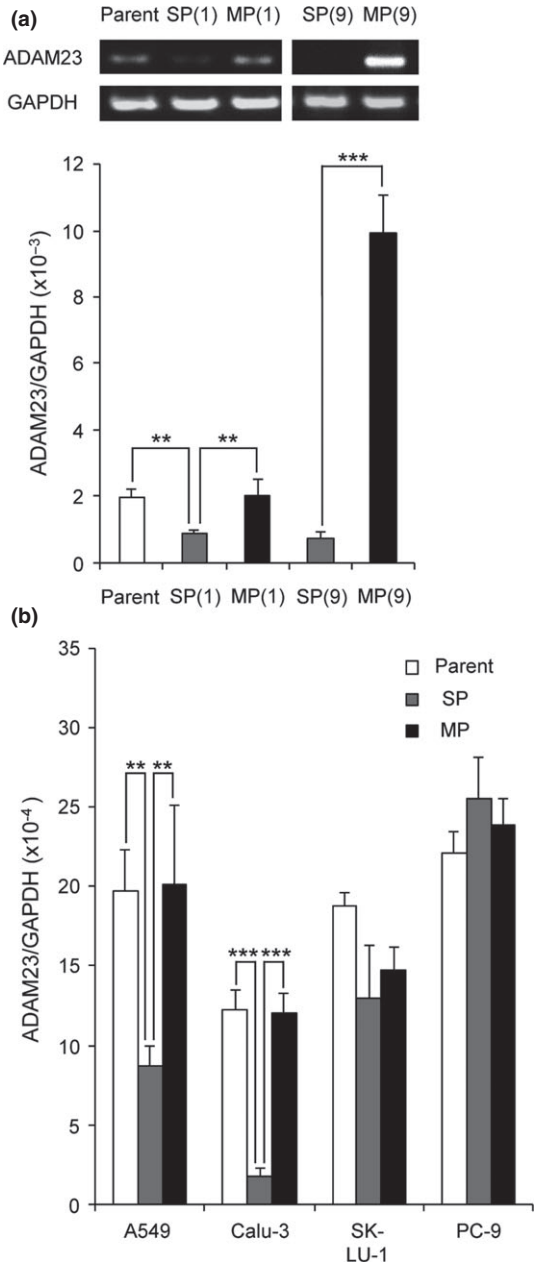


Fig. 3. ADAM23 expression in A549-derived side population (SP) and main population (MP) cells and other lung adenocarcinoma cell lines. (a) mRNA expression of ADAM23 in A549 parent, SP(1), MP(1), SP(9) and MP(9) cells by RT-PCR (upper panel) and qPCR (lower panel) ($n = 4$). (b) mRNA expression of ADAM23 in parent, SP and MP cells derived from lung adenocarcinoma cell lines measured by qPCR ($n = 3$). Bars, mean ± SD. $^{**}P < 0.01$; $^{***}P < 0.001$.

whereas SP sorted from each MP, such as MP(2), MP(3) and MP(9), showed no increase in ratios of SP cells. Using the propagated SP(9) and MP(9) cells, we examined relative gene expression ratios of the ADAM, ADAMTS and MMP family members between SP and MP. Among the 63 members examined, none exhibited significant overexpression in SP compared to MP. However, six members, including ADAM23, ADAMTS6, MMP2, MMP16, MMP20 and MMP21, showed significant increases in MP compared to SP (Tables S2–S4). When the relative expression ratios of these genes were compared, ADAM23 was most strongly fluctuated (Fig. 2b). Thus, we focused on ADAM23 for further studies.

Relative expression of ADAM23 between side population and main population cells in lung adenocarcinoma cell lines. RT-PCR showed the expression of ADAM23 in the A549 parent, MP(1) and MP(9) cells, but only faint or negligible expression in the SP(1) and SP(9) cells (Fig. 3a). qPCR demonstrated that the expression is increased in the MP(1) cells compared to the SP (1) cells ($P < 0.01$) (Fig. 3a). The difference was more conspicuous between the MP(9) and SP(9) cells ($P < 0.001$) (Fig. 3a). Similar significant increase of the ADAM23 expression in MP compared to SP was observed with Calu-3 cells ($P < 0.001$), while SK-LU-1 and PC-9 cells showed no such differences (Fig. 3b).

Effects of ADAM23 on colony formation. Overexpression and knockdown of ADAM23 by transfection of the expression vectors and shRNA in A549 cells were confirmed by RT-PCR and immunoblotting (Fig. 4a). ADAM23-overexpressing cells and ADAM23-knockdown cells showed no difference in cell proliferation or DNA fragmentation as compared to their corresponding mock transfectants (data not shown). When colony formation was examined, number of the colonies decreased more in the ADAM23-overexpressing cells than in the empty vector transfectants ($P < 0.05$), and increased more in the ADAM23-knockdown cells (sh1 and sh5) than in the mock transfectants ($P < 0.01$ and $P < 0.001$) (Fig. 4b). The involvement of ADAM23 was further demonstrated by the result that suppression of colony formation in ADAM23-overespressing cells is restored by treatment with anti-ADAM23 antibody ($P < 0.05$) (Fig. 4c). ADAM23 has been reported to negatively modulate αvβ3 integrin activation.[21] As expected, treatment of control empty vector transfectants with anti-αvβ3 integrin antibody significantly reduced colony formation ($P < 0.001$) (Fig. 4c).

Effects of ADAM23 on cell adhesion and migration. Cell adhesion was significantly reduced by overexpression of ADAM23 ($P < 0.001$) and increased by knockdown of ADAM23 ($P < 0.05$ and $P < 0.001$) (Fig. 5a). The suppression of cell adhesion by ADAM23 overexpression was recovered by treatment with anti-ADAM23 antibody ($P < 0.001$) (Fig. 5b). Because the disintegrin domain of ADAM23 is known to bind to αvβ3 integrin,[25] we examined whether the interaction between the disintegrin domain and αvβ3 integrin is involved in cell adhesion. As shown in Figure 5b, the decreased adhesion of ADAM23 transfectants was restored by treatment with the disintegrin peptide ($P < 0.001$), whereas the scrambled peptide showed no effect. In addition, anti-αvβ3 integrin antibody remarkably inhibited the cell adhesion of disintegrin peptide-treated cells ($P < 0.001$) and empty vector transfectants ($P < 0.001$) (Fig. 5b). We also showed that cell migration is significantly reduced by overexpression of ADAM23 ($P < 0.001$) and enhanced by knockdown of ADAM23

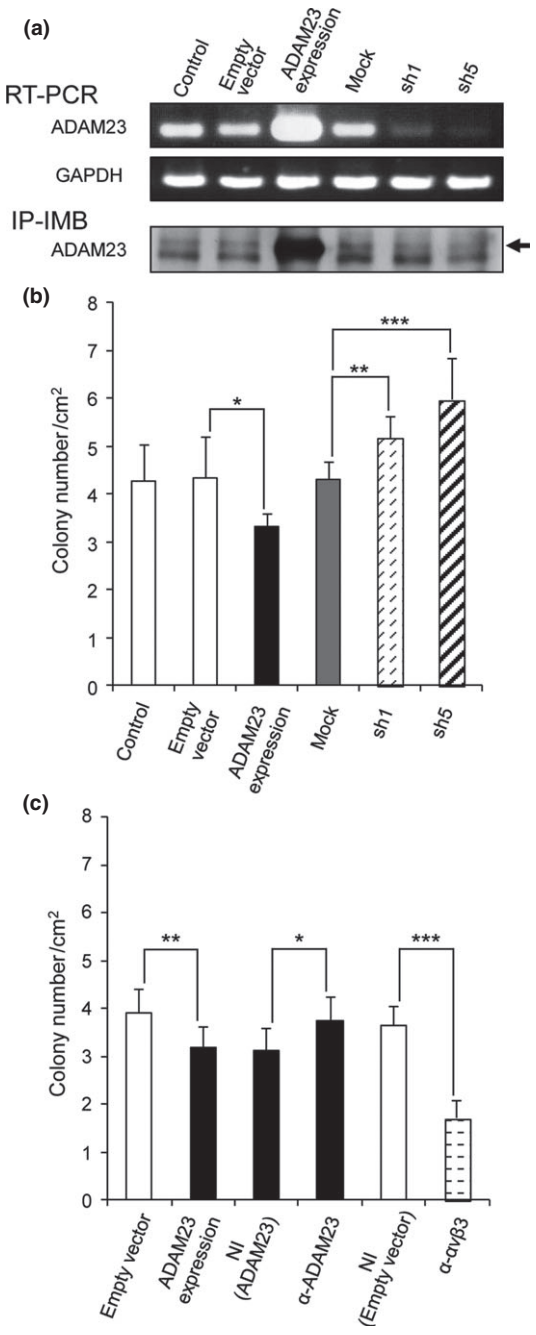


Fig. 4. Effects of ADAM23 expression, anti-ADAM23 antibody and anti-αvβ3 integrin antibody on colony formation. (a) Expression of ADAM23 in A549 cells (control) or A549 transfectants with empty vectors, ADAM23 expression vectors, non-targeting mock vectors or ADAM23-targeting lentiviral vectors (sh1 and sh5) by RT-PCR and by immunoblotting of the immunoprecipitates (IP-IMB). Arrow indicates ADAM23 and the lower band is a non-specific protein band. (b) Colony formation by control A549 cells (control) and A549 cells transfected with empty vectors, ADAM23 expression vectors, non-targeting vectors (mock) or ADAM23-targeting vectors (sh1 and sh5) was assayed ($n = 6$). (c) Colony formation by A549 cells transfected with empty vectors or ADAM23 expression vectors, ADAM23 transfectants treated with non-immune IgG (NI, ADAM23) or anti-ADAM23 antibody (α-ADAM23), and empty vector transfectants treated with non-immune IgG (NI, empty vector) or anti-αvβ3 integrin antibody (α-αvβ3) was determined ($n = 6$). Bars, mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

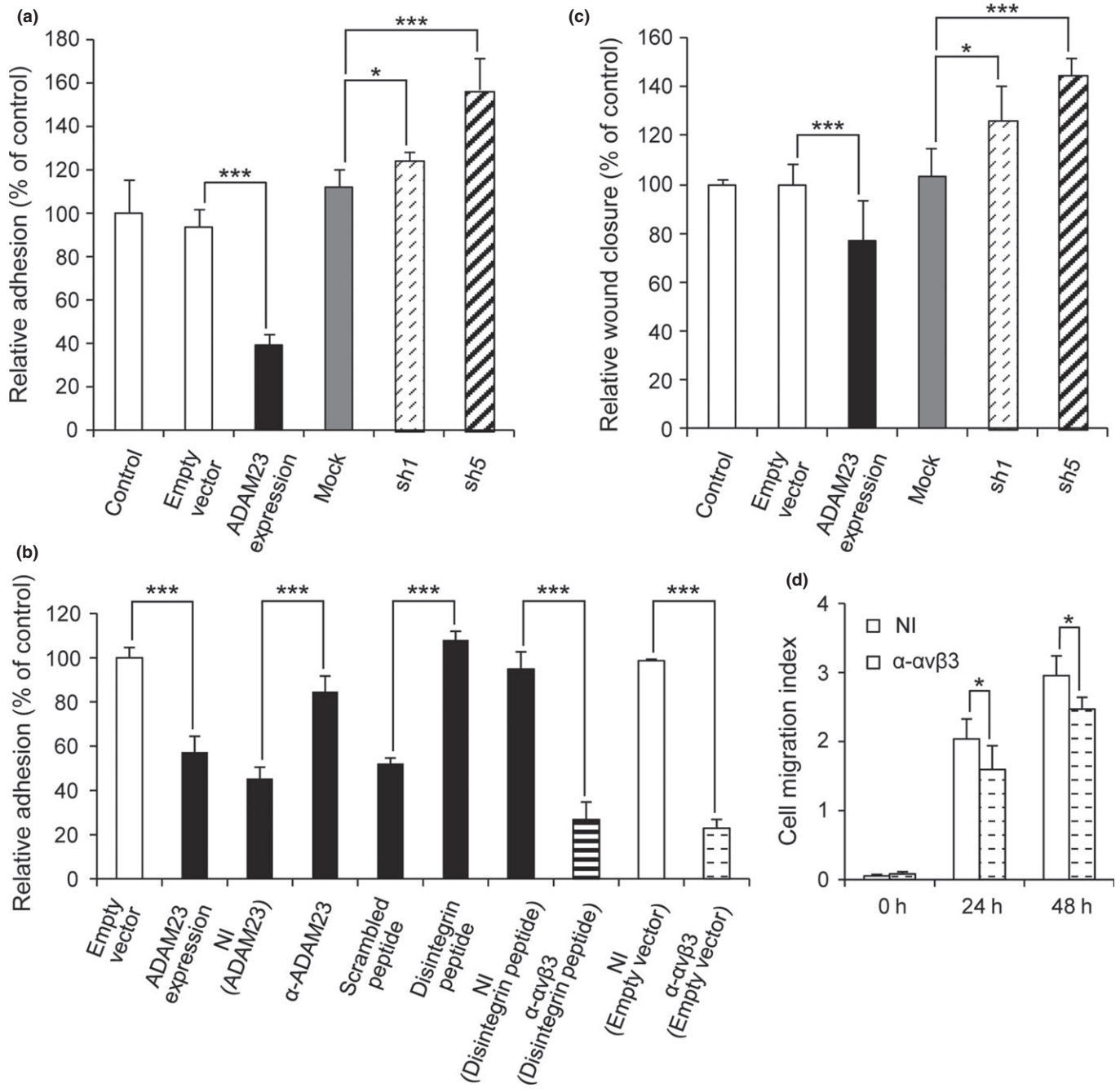
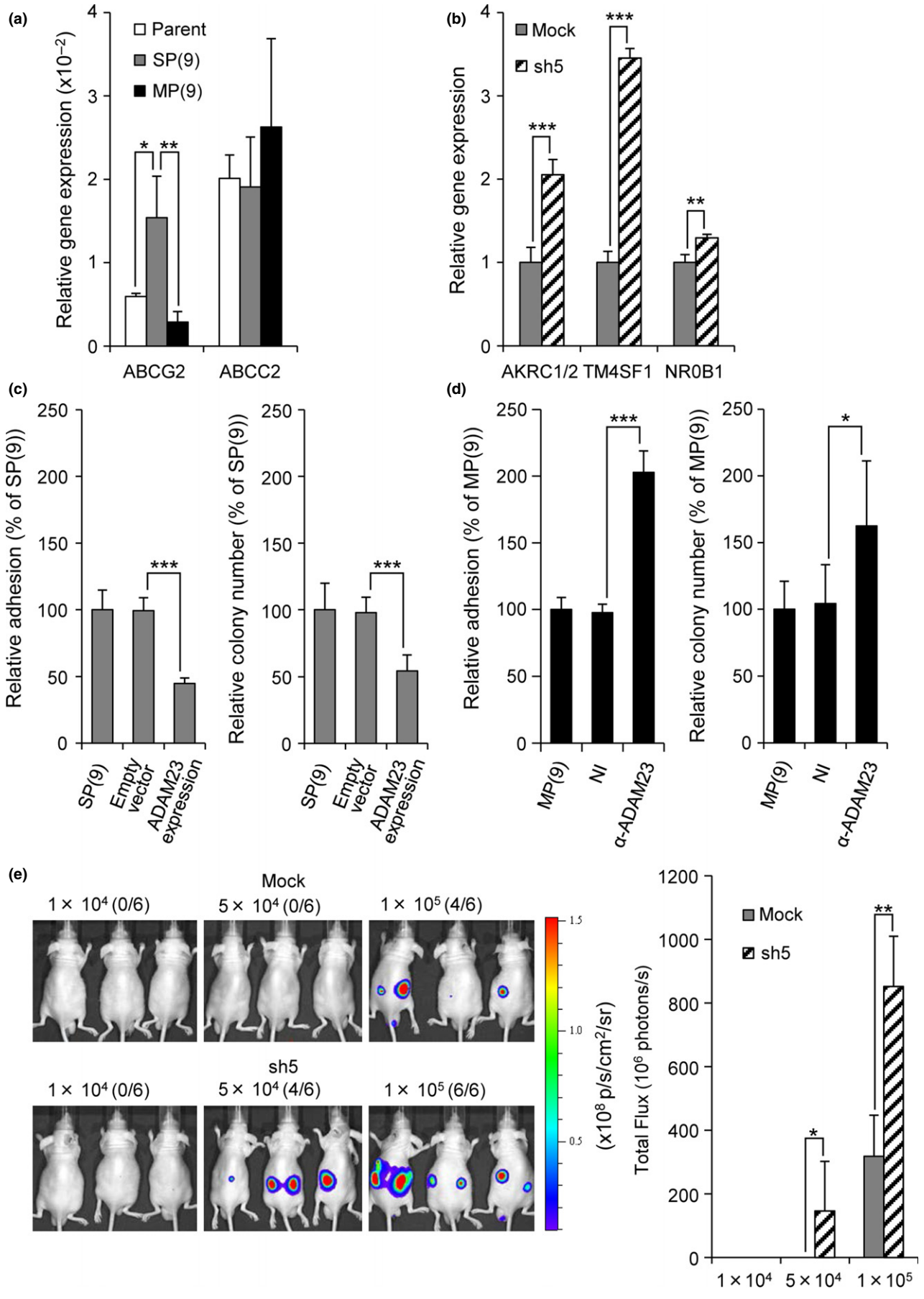


Fig. 5. Involvement of ADAM23 and αvβ3 integrin in cell adhesion and migration. (a) Cell adhesion of control A549 cells (control) and A549 cells transfected with empty vectors, ADAM23 expression vectors, non-targeting vectors (mock) or ADAM23-targeting vectors (sh1 and sh5) was assayed ($n = 4$). (b) Cell adhesion of A549 cells transfected with empty vectors or ADAM23 expression vectors, ADAM23 transfectants treated with non-immune IgG (NI, ADAM23) or anti-ADAM23 antibody (α-ADAM23), ADAM23 transfectants treated with scrambled peptide or disintegrin peptide, ADAM23 transfectants treated with disintegrin peptide and non-immune IgG (NI, disintegrin peptide) or disintegrin peptide and anti-αvβ3 antibody (α-αvβ3, disintegrin peptide), and empty vector transfectants treated with non-immune IgG (NI, empty vector) or anti-αvβ3 integrin antibody (α-αvβ3, empty vector) was determined ($n = 4$). (c) Migration of control A549 cells (control) and A549 cells transfected with empty vectors, ADAM23 expression vectors, non-targeting vectors (mock) or ADAM23-targeting vectors (sh1 and sh5) was measured by migration assay ($n = 4$). (d) Migration of A549 cells treated with non-immune IgG (NI) or anti-αvβ3 integrin antibody (α-αvβ3) was measured using the xCELLigence system ($n = 4$). Bars, mean ± SD. $^*P < 0.05$; $^{***}P < 0.001$.

Fig. 6. Relationship between ADAM23 expression and stem cell functions. (a) Relative expression levels of ABCG2 and ABCC2 to GAPDH in SP (9) and MP(9) were measured by qPCR ($n = 3$). (b) Relative expression levels of AKRC1/2, TM4SF1 and NR0B1 in ADAM23-knockdown (sh5) cells compared to A549 (mock) cells were measured by qPCR ($n = 3$). (c,d) Effects of ADAM23 overexpression in SP(9) cells or treatment of MP(9) cells with anti-ADAM23 antibody on cell adhesion ($n = 4$) and colony formation ($n = 6$) were examined. (e) Tumorigenicity of A549$^{ffLuc\text{-}cp156}$ (mock) and ADAM23-knockdown (sh5) cells was monitored at 2 weeks by bioluminescence imaging ($n = 6$ per group; bilateral sites of 3 mice), and photon counts were analyzed by LIVING IMAGE 3.0 software. Bars, mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.



($P < 0.05$ and $P < 0.001$) (Fig. 5c). Cell migration was slightly inhibited by treatment with anti-αvβ3 integrin antibody (Fig. 5d).

Negative correlation of ADAM23 expression with stem cell functions. Because PCR array analysis suggested overexpression of ABCG2 and ABCC2 in SP(9) compared to MP(9) (data not shown), we examined the expression levels by qPCR and confirmed that ABCG2 is overexpressed in SP(9) cells (Fig. 6a). Examination of the cancer stem cell-related genes of A549 cells (AKRC1/2, TM4SF1 and NR0B1) showed that the expression levels are significantly higher in ADAM23-knockdown cells than in mock transfectants (Fig. 6b). Cell adhesion and colony formation were decreased by transfection of the ADAM23 expression vectors to SP(9) cells as compared to the empty vector transfectants (Fig. 6c), and increased by treatment of MP(9) cells with anti-ADAM23 antibody compared to non-immune IgG treatment (Fig. 6d). To test whether ADAM23 expression affects tumorigenicity, we injected $A549^{ffLuc\text{-}cp156}$ mock or ADAM23-knockdown cells into bilateral subcutaneous tissues of mouse back and monitored for tumor development. As shown in Figure 6e, ADAM23-knockdown cells (sh5) formed xenografts at 5×10^4 cells in four of six sites and at 1×10^5 cells in all six sites, whereas mock transfectants showed tumors in none of six sites at 5×10^4 cells and four of six sites at 1×10^5 cells. The tumor generated by ADAM23-knockdown cells was significantly larger than that of mock transfectants (Fig. 6e). No tumor formation was observed by inoculation of both transfectants less than 1×10^4 cells. All these data suggest that ADAM23 negatively modulates cancer stem cell-like functions.

Negative role of ADAM23 in lung metastasis. We next examined the effect of ADAM23 on lung metastasis of $A549^{ffLuc\text{-}cp156}$ cells, which were transfected with empty vectors, ADAM23 expression vectors, mock-shRNA vectors or ADAM23 shRNA vectors. When lung metastasis was monitored, there was no difference in photon counts 1 h after the i.v. injection, but the counts at 1 day were, albeit generally reduced in all groups, significantly decreased in the group of ADAM23-overexpressing cells ($P < 0.05$) and increased in that of ADAM23-knockdown cells ($P < 0.05$) (Fig. 7a,b). Lung metastasis at 2 weeks was significantly inhibited in the group of ADAM23-overexpressing cells ($P < 0.01$) and enhanced in that of ADAM23-knockdown cells ($P < 0.05$) (Fig. 7a,b). Microscopic examination of lung metastasis at 2 weeks showed that numbers of the metastatic nodules are significantly increased in the group of ADAM23-knockdown cells (12.9 ± 0.8 per mm^2) compared to that of mock transfectants (5.8 ± 3.1 per mm^2) ($P < 0.01$) (Fig. 7c,d). Metastasis tended to be decreased in the group of ADAM23-overexpressing cells (0.7 ± 0.6 per mm^2) compared with that of empty vector transfectants (5.0 ± 4.6 per mm^2), although the difference was not statistically significant (Fig. 7d).

Discussion

The SP technology is a useful method to isolate subsets enriched for cancer stem cells, and SP cells demonstrate characteristics of cancer stem cells as indicated by tumor-initiating capability, drug resistance and invasive potential in comparison with MP cells.[4–8] Previous studies on differential gene expression profiles performed in SP and MP cells from lung carcinoma cell lines showed a number of cancer stem cell-related genes overexpressed in SP cells, which include AKRC1/C2, TM4SF1 and NR0B1.[12,13] However, these studies provided no information about the expression profiles of the ADAM, ADAMTS and MMP gene family members, many of which are implicated for cancer cell proliferation and progression.[18] In the present study, by screening changes in the gene expression levels of all the members of the families in propagated SP and MP cells of A549 lung adenocarcinoma cells, we showed that ADAM23 is the most significantly downregulated gene in the SP compared with the MP between the two populations.

The clonogenic ability is known to be higher in SP cells than in MP cells, as shown by the present and previous studies.[4,13,31] Although a previous study using a gastric cancer cell line showed that forced expression of ADAM23 causes inhibition of colony formation, the study provided no data on the role of ADAM23 in colony formation.[32] In the present study, we have shown direct evidence that suppression of clonogenic ability is through the interaction of ADAM23 with αvβ3 integrin. Our study also demonstrated that ADAM23 inhibits cell adhesion by blocking the activity of αvβ3 integrin and also suppresses migration activity. Similar results showing the suppressive effects of ADAM23 on cell adhesion and migration have been reported with MDA-MB435 breast carcinoma cells, in which ADAM23 was knocked down but not overexpressed.[21] Although colonies are formed through a complex process involving various cellular activities such as adhesion, survival, apoptosis, proliferation and migration, the data in our study and previous studies [21] suggest that ADAM23 plays a negative role in colony formation, at least in part, by inhibiting cell adhesion and migration.

One of the important findings of the present study is that pulmonary metastasis is negatively controlled according to the ADAM23 expression level. A previous study showed that knockdown of ADAM23 increases trapping of MDA-MB435 breast carcinoma cells within the pulmonary blood capillaries (i.e. embolization of breast carcinoma cells) by analyzing at 4 h after the i.v. carcinoma cell injection, although the study provided no information about the influence of ADAM23 on cellular activities by overexpression of ADAM23 or how lung metastasis is finally modulated by the ADAM23 expression levels.[21] The data in the present and previous studies[21,25] strongly suggest that ADAM23 in human lung and breast carcinoma cells plays a negative role in cancer cell progression including metastasis by inhibiting the function of integrin αvβ3.

Recent study has indicated that αvβ3 integrin is necessary and sufficient to reprogram non-small cell lung carcinoma cells toward a cancer stem cell phenotype such as tumor initiation, anchorage-independence and self-renewal and EGFR-tyrosine kinase inhibitor resistance.[33] Integrin αvβ3 is shown to perform this function via a non-canonical pathway by recruiting KRAS and RalB to the tumor cell plasma membrane, which induces the activation of TBK-1/NF-κB pathway.[33] Integrin αvβ3 also has been reported to promote Slug activation, anchorage-independent growth and tumor initiation in breast carcinoma cells.[34] In the present study, we have demonstrated that SP cells, which are enriched by cancer stem cells, have no or negligible expression of ADAM23. Because ADAM23 negatively regulates the activity of integrin αvβ3 as shown in the present and previous studies,[21] it is plausible that downregulation of ADAM23 in SP cells may contribute toward providing a phenotype of cancer stem cells by facilitating the activity of integrin αvβ3.

Because our experimental study has suggested that upregulation of ADAM23 suppresses lung metastasis of A549 cells,

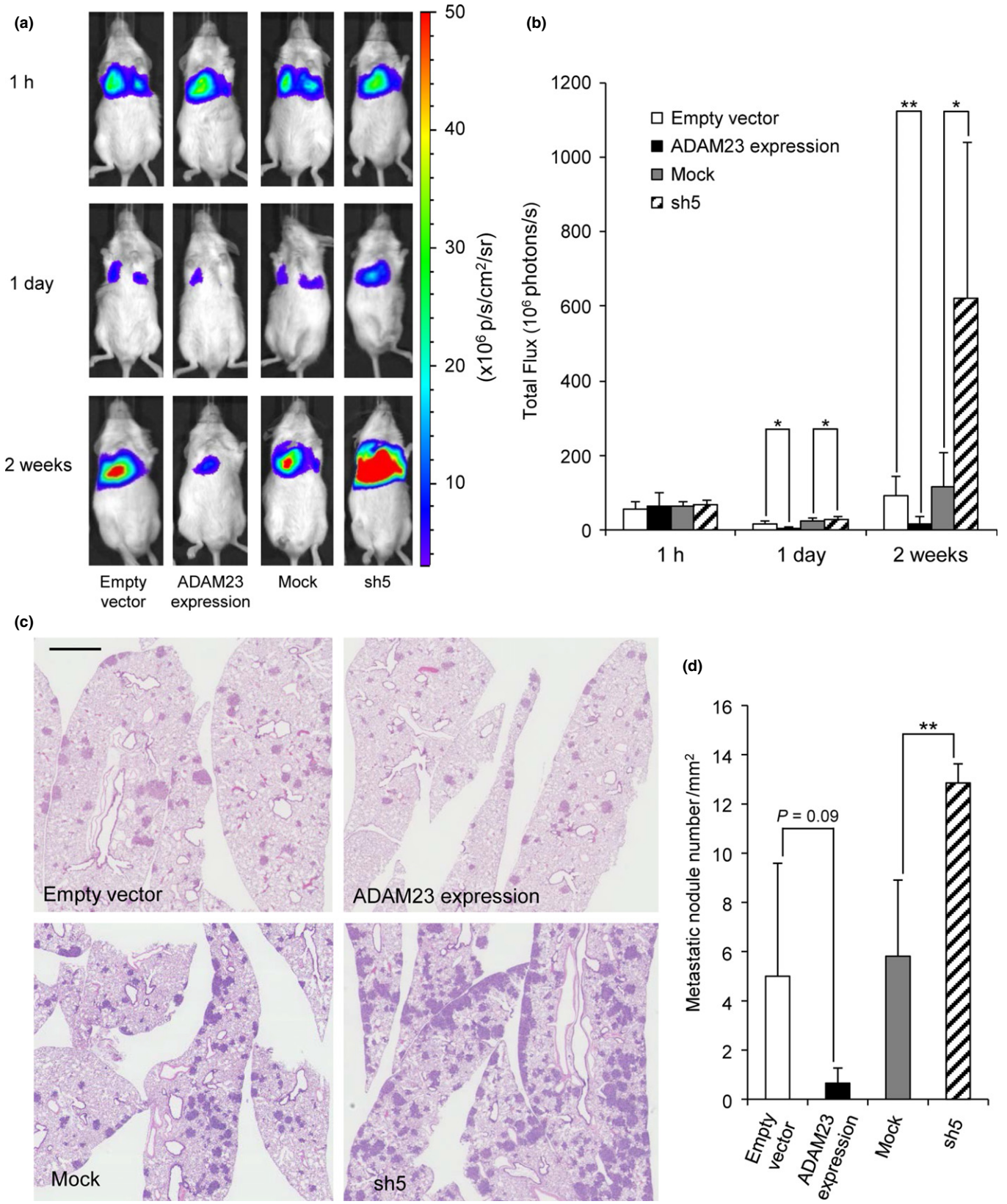


Fig. 7. Lung metastasis in NOD/SCID mice after i.v. injection of A549$^{ffLuc\text{-}cp156}$ transfectants. (a) Lung metastasis of the transfectants with empty vectors, ADAM23 expression vectors, mock vectors or shRNA vectors (sh5) was monitored at 1 h, 1 day and 2 weeks by bioluminescence imaging (n = 6 per group). Representative mice of each group are shown. (b) Photon counts were analyzed (n = 6). (c) Representative microscopic views of the lung sections. Hematoxylin-positive blue-colored nodules indicate metastatic foci. Bar, 1 mm. (d) Evaluation of lung metastasis by calculating the numbers/mm^2 (n = 3). Bars, mean ± SD. *P < 0.05; **P < 0.01.

modulators of the ADAM23 expression may be useful to negatively control lung metastasis of non-small cell lung carcinomas. ADAM23 expression is reportedly downregulated by DNA promoter hypermethylation in gastric carcinoma cells,[32] breast carcinoma cells [21] and non-small cell lung carcinomas,[35] and silencing of the ADAM23 gene by hypermethylation is associated with lower distant metastases-free and disease-specific survival in human breast carcinomas.[21] Because functional analyses of DNA methylation have made great progress and drugs targeting the epigenetic abnormalities have been developed to increase efficacy and stability and to decrease toxicity,[36,37] epigenetic drugs targeting ADAM23 and/or development of upregulators of ADAM23 expression could represent promising and feasible strategies to sensitize human non-small cell lung carcinomas to the effects of chemotherapy and molecular target therapy.

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Disclosure Statement

The authors have no conflict of interest to declare.

References

1 Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001; **414**: 105–11.
2 Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008; **8**: 755–68.
3 Vermeulen L, de Sousa e Melo F, Richel DJ, Medema JP. The developing cancer stem-cell model: clinical challenges and opportunities. *Lancet Oncol* 2012; **13**: e83–9.
4 Loebinger MR, Giangreco A, Groot KR *et al.* Squamous cell cancers contain a side population of stem-like cells that are made chemosensitive by ABC transporter blockade. *Br J Cancer* 2008; **98**: 380–7.
5 Hirschmann-Jax C, Foster AE, Wulf GG *et al.* A distinct "side population" of cells with high drug efflux capacity in human tumor cells. *Proc Natl Acad Sci USA* 2004; **101**: 14228–33.
6 Szotek PP, Pieretti-Vanmarcke R, Masiakos PT *et al.* Ovarian cancer side population defines cells with stem cell-like characteristics and Mullerian inhibiting substance responsiveness. *Proc Natl Acad Sci USA* 2006; **103**: 11154–9.
7 Das B, Tsuchida R, Malkin D, Koren G, Baruchel S, Yeger H. Hypoxia enhances tumor stemness by increasing the invasive and tumorigenic side population fraction. *Stem Cells* 2008; **26**: 1818–30.
8 Ho MM, Ng AV, Lam S, Hung JY. Side population in human lung cancer cell lines and tumors is enriched with stem-like cancer cells. *Cancer Res* 2007; **67**: 4827–33.
9 Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996; **183**: 1797–806.
10 Zhou S, Schuetz JD, Bunting KD *et al.* The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001; **7**: 1028–34.
11 Han H, Bourboulia D, Jensen-Taubman S, Isaac B, Wei B, Stetler-Stevenson WG. An endogenous inhibitor of angiogenesis inversely correlates with side population phenotype and function in human lung cancer cells. *Oncogene* 2014; **33**: 1198–206.
12 Seo DC, Sung JM, Cho HJ *et al.* Gene expression profiling of cancer stem cell in human lung adenocarcinoma A549 cells. *Mol Cancer* 2007; **6**: 75.
13 Oda T, Tian T, Inoue M *et al.* Tumorigenic role of orphan nuclear receptor NR0B1 in lung adenocarcinoma. *Am J Pathol* 2009; **175**: 1235–45.
14 Shiomi T, Lemaitre V, D'Armiento J, Okada Y. Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases. *Pathol Int* 2010; **60**: 477–96.
15 Okada Y. *Proteinases and Matrix Degradation*. In: Firestein GS, Budd RC, Gabriel SE, McInnes IB, O'Dell JR. 9th edn. Philadelphia: Elsevier Saunders, 2013; pp. 97–115
16 Porter S, Clark IM, Kevorkian L, Edwards DR. The ADAMTS metalloproteinases. *Biochem J* 2005; **386**: 15–27.
17 Deryugina EI, Quigley JP. Matrix metalloproteinases and tumor metastasis. *Cancer Metastasis Rev* 2006; **25**: 9–34.
18 Mochizuki S, Okada Y. ADAMs in cancer cell proliferation and progression. *Cancer Sci* 2007; **98**: 621–8.
19 Edwards DR, Handsley MM, Pennington CJ. The ADAM metalloproteinases. *Mol Aspects Med* 2008; **29**: 258–89.
20 Murphy G. The ADAMs: signalling scissors in the tumour microenvironment. *Nat Rev Cancer* 2008; **8**: 929–41.
21 Verbisck NV, Costa ET, Costa FF *et al.* ADAM23 negatively modulates alpha(v)beta(3) integrin activation during metastasis. *Cancer Res* 2009; **69**: 5546–52.
22 McCartan D, Bolger JC, Fagan A *et al.* Global characterization of the SRC-1 transcriptome identifies ADAM22 as an ER-independent mediator of endocrine-resistant breast cancer. *Cancer Res* 2012; **72**: 220–9.
23 D'Abaco GM, Ng K, Paradiso L, Godde NJ, Kaye A, Novak U. ADAM22, expressed in normal brain but not in high-grade gliomas, inhibits cellular proliferation via the disintegrin domain. *Neurosurgery* 2006; **58**: 179–86.
24 Terraube V, Pendu R, Baruch D *et al.* Increased metastatic potential of tumor cells in von Willebrand factor-deficient mice. *J Thromb Haemost* 2006; **4**: 519–26.
25 Cal S, Freije JM, Lopez JM, Takada Y, Lopez-Otin C. ADAM 23/MDC3, a human disintegrin that promotes cell adhesion via interaction with the alphavbeta3 integrin through an RGD-independent mechanism. *Mol Biol Cell* 2000; **11**: 1457–69.
26 Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc* 2007; **2**: 329–33.
27 Mitsui Y, Mochizuki S, Kodama T *et al.* ADAM28 is overexpressed in human breast carcinomas: implications for carcinoma cell proliferation through cleavage of insulin-like growth factor binding protein-3. *Cancer Res* 2006; **66**: 9913–20.
28 Chijiiwa M, Mochizuki S, Kimura T *et al.* CCN1 (Cyr61) is overexpressed in human osteoarthritic cartilage and inhibits ADAMTS-4 (Aggrecanase 1) activity. *Arthritis Rheumatol* 2015; **67**: 1557–67.
29 Mochizuki S, Soejima K, Shimoda M *et al.* Effect of ADAM28 on carcinoma cell metastasis by cleavage of von Willebrand factor. *J Natl Cancer Inst* 2012; **104**: 906–22.
30 Shiomi T, Inoki I, Kataoka F *et al.* Pericellular activation of proMMP-7 (promatrilysin-1) through interaction with CD151. *Lab Invest* 2005; **85**: 1489–506.
31 Mitsutake N, Iwao A, Nagai K *et al.* Characterization of side population in thyroid cancer cell lines: cancer stem-like cells are enriched partly but not exclusively. *Endocrinology* 2007; **148**: 1797–803.
32 Takada H, Imoto I, Tsuda H *et al.* ADAM23, a possible tumor suppressor gene, is frequently silenced in gastric cancers by homozygous deletion or aberrant promoter hypermethylation. *Oncogene* 2005; **24**: 8051–60.
33 Seguin L, Kato S, Franovic A *et al.* An integrin beta(3)-KRAS-RalB complex drives tumour stemness and resistance to EGFR inhibition. *Nat Cell Biol* 2014; **16**: 457–68.
34 Ucar A, Streuli CH. A role for beta3-integrins in linking breast development and cancer. *Dev Cell* 2014; **30**: 251–2.
35 Hu C, Lv H, Pan G *et al.* The expression of ADAM23 and its correlation with promoter methylation in non-small-cell lung carcinoma. *Int J Exp Pathol* 2011; **92**: 333–9.
36 Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012; **13**: 484–92.
37 Baylin SB, Jones PA. A decade of exploring the cancer epigenome – biological and translational implications. *Nat Rev Cancer* 2011; **11**: 726–34.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. The primers for qPCR.

Table S2. Changes in gene expression of the ADAM family members.

Table S3. Changes in gene expression of the ADAMTS family members.

Table S4. Changes in gene expression of the MMP family members.