

***SIP1* promotes invasion and migration
in human oral squamous cell carcinoma
by *ITGB1* and *MMP7***

(*SIP1* は *ITGB1* と *MMP7* により口腔扁平上皮癌の浸潤や遊走を促進する)

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ABSTRACT

Signal-induced proliferation-associated protein 1 (SIPA1) is known to be a GTPase activating protein. Overexpressed SIPA1 is related to metastatic progression in breast and prostate cancers; however, the relevance of SIPA1 in oral squamous cell carcinoma (OSCC) is still unknown. The aim of this study was to examine SIPA1 expression and its functional mechanisms in OSCC. SIPA1 mRNA and protein expressions were analyzed by quantitative reverse transcriptase-polymerase chain reaction, Western blot, and immunohistochemistry. The expressions of SIPA1 were up-regulated significantly *in vitro* and *in vivo*. Moreover, SIPA1 expression was correlated with regional lymph node metastasis. We next assessed the cellular functions associated with tumoral metastasis using SIPA1 knockdown (shSIPA1) cells and analyzed the downstream molecules of SIPA1, i.e., bromodomain containing protein 4, integrin beta1 (ITGB1), and matrix metalloproteinase 7 (MMP7). The shSIPA1 cells showed decreased invasiveness and migratory activities, however cellular adhesion ability was maintained at a high level. In addition, ITGB1 expression was greater in shSIPA1 cells, whereas MMP7 expression was lower than in control cells. This research is the first to establish that SIPA1 promotes cancer metastasis by regulating the ITGB1 and MMP7. Therefore, SIPA1 might be a novel therapeutic target for patients with lymph node metastasis of OSCC.

1. Introduction

Signal-induced proliferation associated protein 1 (SIPA1), a GTPase activating protein, was discovered in proliferating lymphocytes as mitogen-induced nuclear protein [1]. SIPA1 promotes catalyzation of hydrolyze Rap1GTP/GDP and is known to be a negative regulator of Ras-related protein [2], which transduces the signals for various cellular functions, including development, cellular proliferation, and cellular adhesion [3-7].

SIPA1 and Rap1 interaction is related to leukemia. In addition, the interaction controls cellular adhesion in cancer metastasis [5]. SIPA1 links the metastatic predictive gene expression and signaling for cellular adhesion [5]. Overexpression of SIPA1 was found in several types of cancers, such as colon, prostate, and breast cancers [2, 8, 9]. Knockdown experiments have shown that SIPA1 is related closely to cellular adhesion and controls ECM-related genes [9]. In contrast, SIPA1-deficient mice developed myeloid disorders that resembled chronic myelogenous leukemia [10]. Therefore, SIPA1 plays pivotal roles in cancer development and progression. Cancer metastasis induces a recurrence and decreases the survival rates of the patients all over the world. The exploring of cancer metastasis mechanism is a crucial role to improve biomedical treatment and develop novel therapeutics.

This is a first study that we demonstrate the mechanism of cancer progression and metastasis in OSCC. In this experiment, we showed that SIPA1 promotes regional lymph node metastasis of OSCC by controlling cellular invasion and migration. Therefore, SIPA1 might be a novel biomarker of metastasis of OSCCs.

2. Materials and methods

2.1. The statement of ethics

The ethical committee of Chiba University (protocol number, 236) approved the protocol of the present study. Prior participating in our study, all patients were provided informed consent.

2.2. Cells and clinical OSCC tissue samples

The nine cell lines from human OSCC , HSC-2, HSC-3, HSC-4, Sa3, Ca9-22, SAS, KOSC-2, Ho-1-u-1, and Ho-1-N-1, were purchased from RIKEN BioResource Center (Ibaraki, Japan) and JCRB Cell Bank (Osaka Japan). We obtained human normal oral keratinocytes (HNOKs) from young healthy patients and cultured as described previously [11, 12]. In this research, HNOKs was used as a normal control [11-14].

105 clinical OSCC tissue samples and normal oral mucosa samples (patient-matched) were obtained at the Department of Dentistry and Oral-Maxillofacial Surgery, Chiba University Hospital. Tissue samples were formalin-fixed for pathological diagnosis and immunohistochemistry (IHC). Each OSCC sample was performed histopathological diagnosis according to the WHO criteria at the Department of Pathology of Chiba University Hospital [15]. The clinical stages of the OSCCs were determined based on the TNM classification [16].

2.3. cDNA synthesis and protein extraction

RNA and protein were isolated as described previously [11]. We isolated cytoplasmic and nuclear fractions by NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Fisher Scientific, Rockford, IL, USA).

2.4. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

qRT-PCR was conducted to evaluate the expression levels of target mRNAs using the FastStart Essential DNA Probes Master (Roche Diagnostics, Mannheim, Germany) on the LightCycler 480 Instrument II (Roche Diagnostics, Switzerland). Primers and universal probes (designed by Universal Probe Library) were used as follows: *SIPA1* forward, 5'-ACCTTGCTCTG CGGAAC TC-3' and reverse, 5'-GTCCTC CAG GGTCCCACT-3' (probe #18), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) forward, 5' -AGCCACATCGCTCAGACAC-3' and reverse, 5' -GCCCAATACGACCAAATCC-3' (probe #60), *integrin beta1 (ITGB1)* forward, 5'-CGATGCCATCATGCAAGT-3' and reverse, 5'-ACACCAGCAGCCGTGTAAC-3' (probe #65), and *matrix metalloproteinase 7 (MMP7)* forward, 5'-TGGACGGATGGTAGCAGTCT-3' and reverse, 5'- TCTCCATTTCCATAGGTTGGAT-3' (probe #6). The amounts of target and control transcripts were estimated from the respective standard curves. The values were normalized according to the level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), the endogenous control.

2.5. Western blot analysis

Western blot analysis was performed as described previously [11]. The membranes were incubated with anti-SIPA1 rabbit monoclonal antibody (dilute concentration 1:3000, Abcam, Cambridge, UK, ab189929), anti-GAPDH mouse monoclonal antibody (dilute concentration 1:200, Santa Cruz Biotechnology, Dallas, TX, USA, sc-32233), anti-bromodomain containing protein 4 (BRD4) rabbit monoclonal antibody (dilute concentration 1:1000, Abcam, ab128874), anti-lamin A/C rabbit polyclonal antibody (dilute concentration 1:200, Santa Cruz Biotechnology, sc-20681), anti-ITGβ1 rabbit

monoclonal antibody (dilute concentration 1:10000, Abcam, ab52971), and anti-MMP7 rabbit monoclonal antibody (dilute concentration 1:1000, Abcam, ab205525).

2.6. Immunohistochemistry (IHC)

IHC was carried out as described previously [17-20]. We used the free software, IHC Profiler for IHC scoring (<https://sourceforge.net/projects/ihcprofiler/>) [21]. The SIPA1 IHC scores were calculated combining the proportion of SIPA1 stained cells and the staining intensity. The intensity of the stained cells was classified four levels: 0 (none staining); 1 (weak staining); 2 (moderate staining); and 3 (strong staining). In order to determine the cutoff points of the SIPA1 IHC scores for each clinical classification, we analyzed all scores of 105 OSCC patients using receiver operating characteristic (ROC) curve analysis. Independent pathologists who belong to Chiba University Hospital evaluated the degree of immunostaining.

2.7. Transfection of shRNA plasmid

SIPA1 shRNA plasmid (shSIPA1) (Santa Cruz Biotechnology, sc-45418-SH) or control shRNA plasmid (shMock) (Santa Cruz Biotechnology, sc-108060) were transfected into two OSCC cells (HSC-3 and HSC-4) using Lipofectamine 3000 (Invitrogen) as described previously [14]. The stable transfected cells were isolated in a culture medium containing puromycin (1 µg/ml) (Santa Cruz Biotechnology). To evaluate the knockdown efficiency of SIPA1, we conducted qRT-PCR and Western blot analyses.

2.8. Cellular proliferation assay

To examine the effect of SIPA1 knockdown on cellular proliferation, we performed the cell proliferation assay as described previously [19].

2.9. Cellular invasion assay

To examine the effect of SIPA1 knockdown on cellular invasion, the cells (2.5×10^5) were seeded on Corning® BioCoat™ Matrigel® invasion chamber with 8.0µm PET Membrane 6 well plates (Corning, NY, USA, product #354481). The cellular invasion assay was performed, as described previously [22-25].

2.10. Cellular migration assay

To investigate the effect of SIPA1 knockdown on cellular migration, cells were plated in 6 well culture plates in DMEM containing 10% FBS and cultured until a confluent. The cell migration assay was performed as described previously [22-25]. The wound area was monitored at every 12 hours for 24 hours.

2.11. Cellular adhesion assay

The adhesion assay was performed as described previously [26], to assess the cellular adhesiveness of shSIPA1 cell and shMock cells. The numbers of stained adherent cells were observed and counted by a light microscope at $\times 200$ magnification in five random fields.

2.12. Statistical analysis

Statistical differences were analysed by the χ^2 test, Student's t-test, Fisher's exact test, and Mann-Whitney U-test. Statistical significant was indicated with asterisk on the figures. All data are expressed as the mean \pm the standard error of the mean.

3. Results

3.1. Up-regulation of SIPA1 in OSCC cells

To assess the expression status of SIPA1, we conducted qRT-PCR and Western blot analyses using nine OSCC cells and the HNOKs. The both analyses showed that SIPA1 expressions were increased significantly in all nine OSCC cells compared with the HNOKs ($P<0.05$) (Fig. 1A, 1B).

3.2. Evaluation of SIPA1 expression in primary OSCCs

Representative IHC data of SIPA1 in adjacent normal oral tissue and primary OSCC tissue were shown in Fig. 2A and B. We found that SIPA1 expression was markedly up-regulated in the cytosol of the OSCC specimens. However, normal tissues were seen almost negative immunostaining. Using the IHC scoring system, the SIPA1 IHC scores in the OSCCs ranged from 60 to 290 (median, 120) and adjacent normal oral tissues ranged from 13 to 105 (median, 60), a difference that reached significance ($P<0.05$) (Fig. 2C). The area under the curve (AUC) of the ROC curve analysis was 0.713, and the cutoff value was 110.0 in the analysis of the regional lymph node metastasis (Fig. 2D). Representative IHC data of SIPA1 protein in the regional lymph node metastasis of primary OSCCs were shown in Fig. 2E.

3.3. Establishment of SIPA1 knockdown cells

To investigate SIPA1 expression levels in shSIPA1 cells, qRT-PCR and Western blot analyses were conducted (Fig. 3A, B, respectively). The both analyses showed that SIPA1 expression was markedly ($P<0.05$) down-regulated in shSIPA1 cells compared with control cells (shMock) (Fig. 3A, 3B).

3.4. Cellular functional analyses of SIPA1 knockdown cells

In order to assess the cellular functions of SIPA1 knockdown cells, we examined cellular proliferation, invasion, migration, and adhesion assays. Regarding cellular proliferation, there was no significant difference of cell growth ability between shSIPA1 cells and control cells (shMock) (Fig. 4A). The number of penetrating shSIPA1 cells clearly decreased compared with shMock cells ($P<0.05$) (Fig. 4B). Regarding the migration assay, the area which was wounded by a micropipette tip decreased obviously in shMock cells after 24 hours ($P<0.05$). In contrast, the gap was still remained in shSIPA1 cells over 24 hours (Fig. 4C). The number of adhering cells markedly increased in shSIPA1 cells compared with shMock cells ($P<0.05$) (Fig. 4D).

3.5. BRD4 expression in SIPA1 knockdown cells

Because a previous study showed that SIPA1 interacts directly with a chromatin adaptor, BRD4 [27], we performed Western blot analysis to examine BRD4 expression of the nuclear and cytosolic fractions in shSIPA1 and shMock cells. Abundant BRD4 protein expression in the nuclear fraction of the shSIPA1 cells was seen compared with shMock cells ($P<0.05$) (Fig. 5).

3.6. ITGB1 and MMP7 expression levels in SIPA1 knockdown cells

BRD4 regulates a series of ECM-related gene expression in mammary tumoral cells [28]. When we investigated ITGB1 and MMP7, the expressions of ITGB1 in shSIPA1 cells were markedly higher than in shMock cells ($P<0.05$) (Fig. 6A, B), whereas the expression of MMP7 in shSIPA1 cells was significantly lower than in control cells ($P<0.05$) (Fig. 6C, D).

3.7. Correlations between SIPA1 expressions and clinicopathological characteristics

The correlations between the status of SIPA1 protein expressions and the clinicopathological characteristics of the patients with OSCC using the IHC scoring system were exhibited in Table 1. Among the clinical classifications, the SIPA1-positive OSCCs were correlated with regional lymph node metastasis. ($P < 0.05$)

4. Discussion

Our study found that SIPA1 was up-regulated in OSCC cells and primary OSCCs compared with the normal counterparts. Moreover, higher SIPA1 expression was markedly ($P<0.05$) related to regional lymph node metastasis (Table 1). Therefore, SIPA1 overexpression might be linked to OSCC invasiveness and migration and have a crucial role in OSCC metastasis.

Preventing metastasis of cancer is an important factor to improve biomedical treatment all over the world. The prognosis in advanced OSCC is poor and the 5-year survival rate in patients with OSCC is about 50% [29, 30]. The 5-year survival rate of OSCC is 90% in patients without regional lymph node metastasis but less than 40% in patients with regional lymph node metastasis, suggesting that metastasis is one of the most important prognostic factors [31-36]. In addition to the current study, IHC from the patients with prostate cancers and SIPA1 overexpression or knockdown experiments found a positive correlation between SIPA1 expression and tumor metastasis [9].

SIPA1 interacts with chromatin-binding protein, BRD4, to modulate the enzymatic activity against Rap1GTP, which mediates the ECM-related gene expression and affects cancer metastasis but not cellular proliferation rates [9]. Since SIPA1 promoted metastasis of several cancer cells by inhibiting the adhesion ability and down-regulated the expression of ECM-related genes [9], we speculated that the downstream molecules of the SIPA1/BRD4 interaction have crucial roles in OSCC metastasis. Some reports including our previous study showed that integrin and MMPs regulated tumoral metastasis in several types of cancers [37-41]. Consistent with this hypothesis, we found that SIPA1 knockdown cells showed high ITGB1 expression and low MMP7 expression in OSCC (Fig. 6), indicating that these two molecules might modulate oral cancer metastasis.

MMPs (MMP2, 7, and 9) have been implicated in tumoral invasion and metastasis and tumoral initiation and growth [42]. Of them, MMP7 expression at the invasive front was detected in 50% of cancer tissues and was associated with the depth of invasiveness [37]. There were no evident differences of MMP2 and MMP9 between shSIPA1 cells and control cells in the current study (data not shown), suggesting that the SIPA1/BRD4 interaction in OSCC might directly control MMP7 expression not MMP2 and 9. Therefore, more researches are needed to better understand the target molecules of the SIPA1/BRD4 interaction.

In conclusion, this study is the first to establish that SIPA1 plays a crucial role in metastatic progression in OSCC by controlled expression of ITGB1 and MMP7 through the SIPA1/BRD4 interaction. These altered expressions promoted environmental changes in cellular invasion, migratory, and adhesion abilities. Thus, our data suggested that SIPA1 might be a potential biomarker of cancer metastasis and a novel therapeutic target in OSCC.

Conflict of Interest Disclosures

The authors have declared that no competing interests exist.

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Legends

Fig. 1. Up-regulation of SIPA1 expression in OSCC-derived cell lines. (A) Quantification of *SIPA1* mRNA expression in OSCC-derived cell lines by qRT-PCR analysis. Significant ($*P<0.05$, Student's t-test) up-regulation of *SIPA1* mRNA is seen in nine OSCC-derived cell lines compared with the HNOKs. Data are expressed as the mean \pm SEM of triplicate results. (B) Western blot analysis of SIPA1 protein in OSCC-derived cell lines and HNOKs. SIPA1 protein expression is up-regulated in OSCC-derived cell lines compared with the HNOKs. Densitometric SIPA1 protein data are normalized to the GAPDH protein levels. The values are expressed as a percentage of the HNOKs.

Fig. 2. Evaluation of SIPA1 expression in primary OSCCs. (A) Representative IHC results of SIPA1 protein in normal oral tissue and (B) primary OSCC (original magnification, $\times 100$. Scale bars, 100 μm). (C) The status of SIPA1 protein expression in primary OSCCs ($n=105$) and normal counterparts based on the IHC scoring system. The SIPA1 IHC scores of OSCCs and normal oral tissues range from 60 to 290 (median, 120) and 13 to 105 (median, 60), respectively. SIPA1 protein expression levels in OSCCs are markedly ($*P<0.05$, Student's t-test) higher than in normal oral tissues. (D) ROC curve analysis showed the optimal cutoff point, 110.0 (AUC, 0.713) in analysis of the regional lymph node. (E) Representative IHC results of SIPA1 protein in the regional lymph nodes of primary OSCCs (original magnification, $\times 100$ and $\times 200$. Scale bars, 100 μm).

Fig. 3. Establishment of SIPA1 knockdown cells. (A) Expression of *SIPA1* mRNA in shMock and shSIPA1 cells (HSC-3 and HSC-4 derived transfectants). *SIPA1* mRNA expression in shSIPA1 cells is significantly ($*P<0.05$, Student's t-test) lower than in the

shMock cells. (B) Immunoblotting analysis shows that the SIPA1 protein levels in the shSIPA1 cells also are decreased markedly compared with the shMock cells.

Fig. 4. Cellular functional analyses with SIPA1 knockdown cells (A) A cellular proliferation assay of shMock and shSIPA1 cells (HSC-3 and HSC-4 derived transfectants) was performed to determine the effect of shSIPA1 on cellular proliferation. shMock and shSIPA1 cells are seeded in 6-cm dishes at a density of 1×10^4 viable cells/well. The shSIPA1 cells show similar cellular growth compared with the shMock cells. The results are expressed as the means \pm SEM of values from three assays ($*P < 0.05$, Student's t-test). (B) An invasion assay of the shMock and shSIPA1 cells (HSC-3 and HSC-4 derived transfectants) was examined to evaluate the effect of SIPA1 knockdown cells on invasiveness. The number of shSIPA1 cells penetrating through the micro pores is decreased significantly ($*P < 0.05$, Student's t-test) compared with the control cells (shMock). The mean value was calculated from data obtained from three separate chambers. (C) A migration assay of shMock and shSIPA1 cells (HSC-3 and HSC-4-derived transfectants) was examined to evaluate the effect of SIPA1 knockdown on migration. Uniform wounds are made in confluent cultures of the shSIPA1 and shMock cells and the extent of closure is monitored visually at every 12 hours for 24 hours. The mean value is calculated from data obtained from three separate chambers. The wound area is decreased obviously ($*P < 0.05$, Student's t-test) in the culture of shMock cells after 24 hours, whereas a gap remains in the shSIPA1 cells. (D) An adhesion assay of the shMock and shSIPA1 cells (HSC-3 and HSC-4-derived transfectants) was examined to evaluate the effect of SIPA1 knockdown on adhesion ability. The shSIPA1 and shMock cells are seeded on collagen I-coated 96-well plates at a density of 2×10^4 cells/well and

allowed to adhere for 1 hour. After crystal violet staining, the numbers of adherent stained cells are counted using a light microscope at $\times 100$ magnification. The cellular adhesion of shSIPA1 cells is increased markedly ($*P < 0.05$, Student's t-test) compared with the shMock cells.

Fig. 5. BRD4 expression in SIPA1 knockdown cells. Western blot analysis shows the expression levels of SIPA1 and BRD4 protein in the nuclear and cytosolic fractions of the shMock and shSIPA1 cells. The BRD4 protein expression in the nuclear fraction of the shSIPA1 cells is obviously ($*P < 0.05$, Student's t-test) greater than that in the shMock cells.

Fig. 6. ITGB1 and MMP7 expression levels in SIPA1 knockdown cells. qRT-PCR and Western blot analysis show the expression levels of ITGB1 and MMP7 in the shMock and shSIPA1 cells (HSC-3 and HSC-4-derived transfectants). (A) The expression of *ITGB1* mRNA in shSIPA1 cells is markedly ($*P < 0.05$, Student's t-test) higher than that in the shMock cells. The data are expressed as the mean \pm SEM of triplicate results. (B) ITGB1 protein levels in the shSIPA1 cells also are markedly increased compared with the shMock cells by Western blot analysis. (C) Analysis of the expression of *MMP7* mRNA in the shMock and shSIPA1 cells (HSC-3 and HSC-4 derived transfectants) shows that there is significantly ($*P < 0.05$, Student's t-test) less *MMP7* mRNA in the shSIPA1 cells compared with that in the shMock cells. (D) In the shSIPA1 cells, the expression of MMP7 protein is clearly decreased compared with the shMock cells.

Table 1. Correlation between SIPA1 expression and clinical classification in OSCCs.

Clinical classification	Results of immunostaining No. patients			P value
	Total	SIPA1 negative	SIPA1 positive	
Age at surgery (years)				
< 70	61	19	42	0.08 ^b
≥70	44	20	24	
Gender				
Male	63	20	43	0.16 ^b
Female	42	19	23	
T-primary tumor				
T1+T2	56	22	34	0.193 ^b
T3+T4	49	17	32	
N-regional lymph node				
Negative	65	29	36	0.043 ^{b*}
Positive	40	10	30	
Stage				
I+ II	43	18	25	0.193 ^b
III+ IV	62	21	41	
Vascular invasion				
Negative	88	36	52	0.069 ^b
Positive	17	3	14	
Histopathologic type				
Well	69	23	46	0.512 ^c
Moderately	33	15	18	
Poorly	3	1	2	
Tumoral site				
Gingiva	42	13	29	0.53 ^a
Tongue	35	15	20	
Buccal mucosa	19	6	13	
Oral floor	3	1	2	
Soft palate	6	4	2	

(*)Asterisks indicate significance.

^a χ^2 test. ^bFisher's exact test. ^cMann-Whitney U-test.

Figure 1

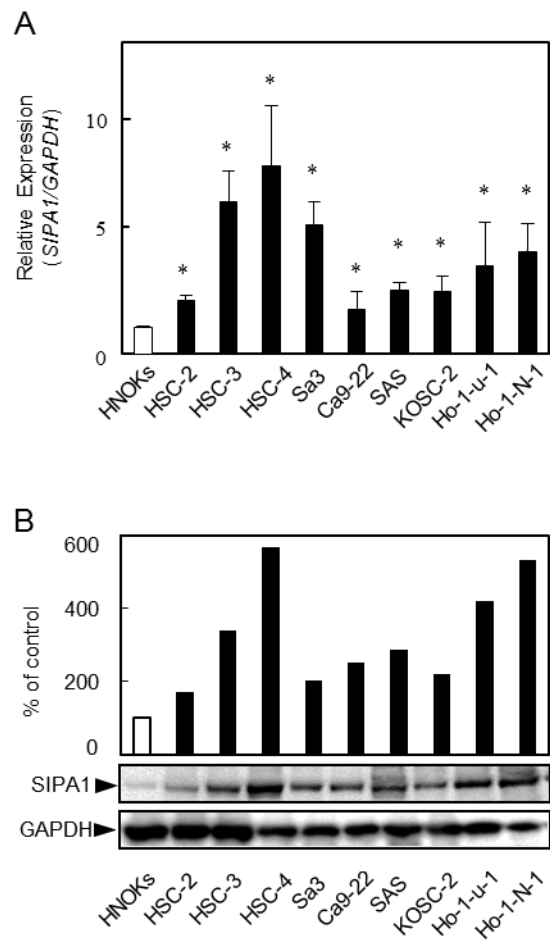


Figure 2

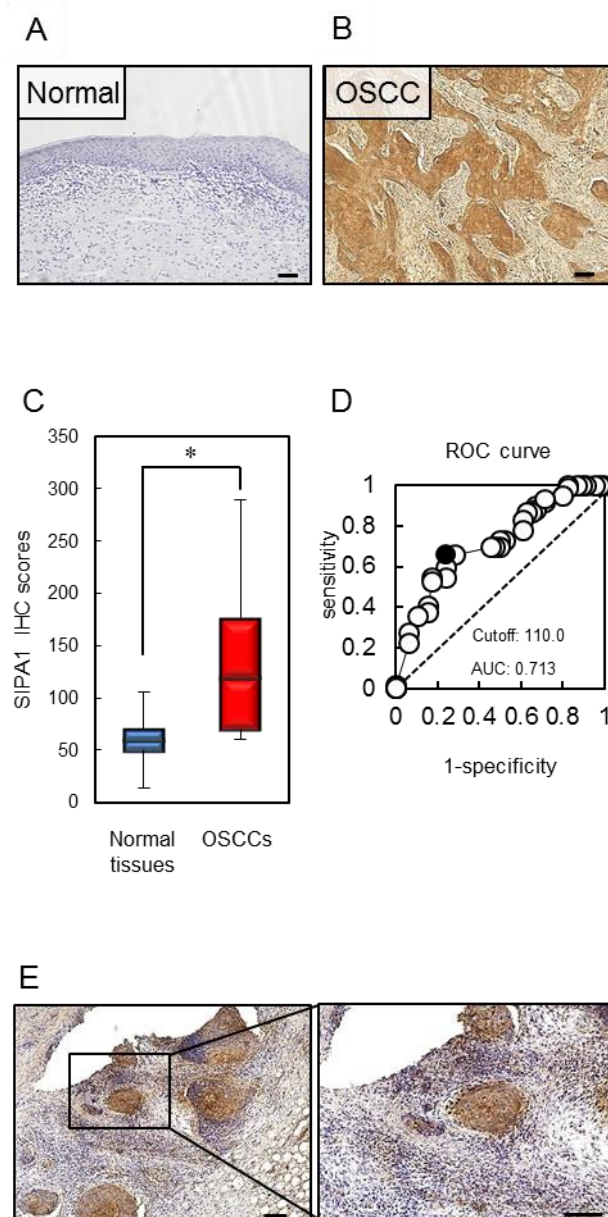


Figure 3

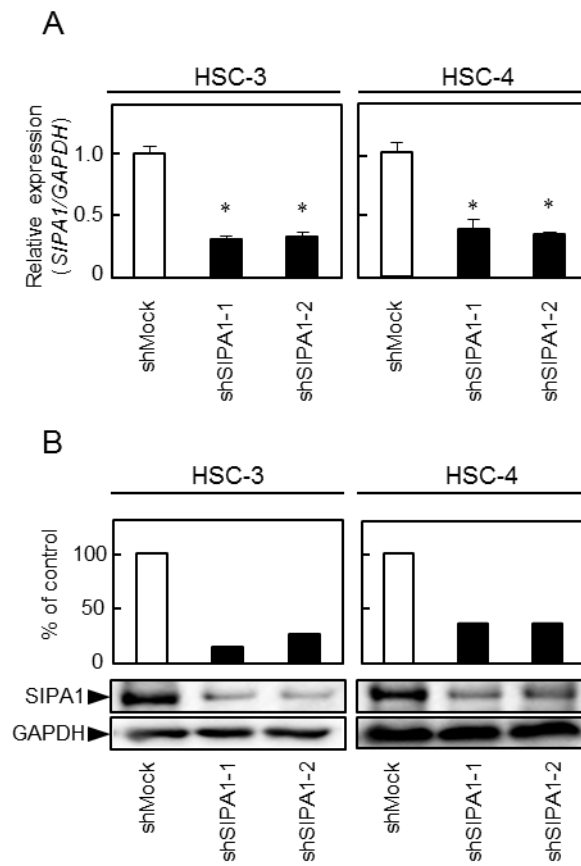


Figure 4

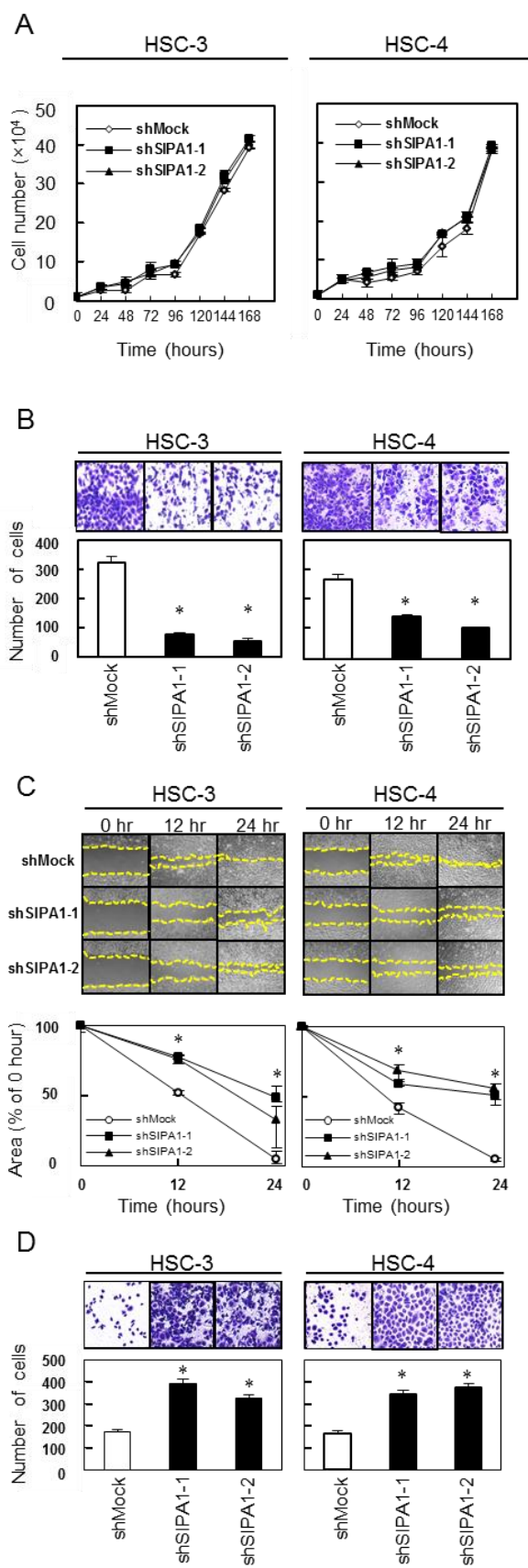


Figure 5

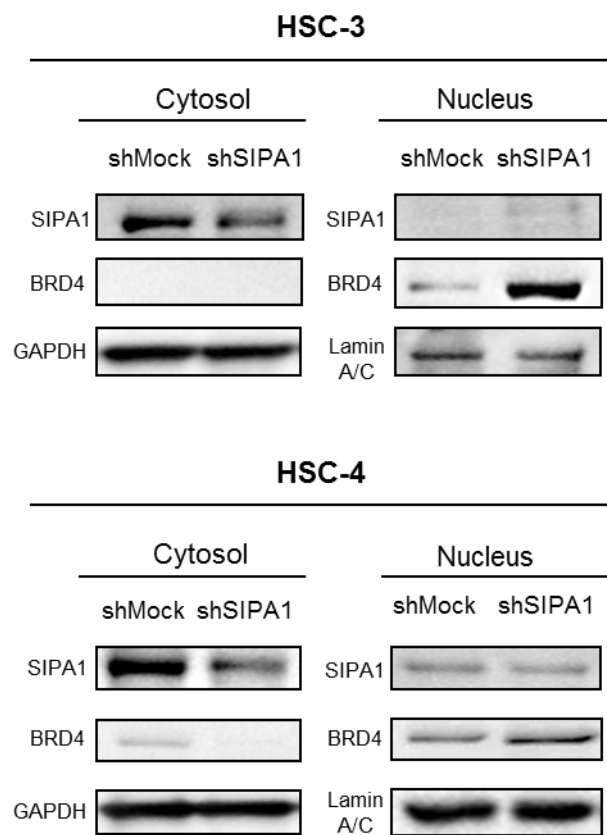
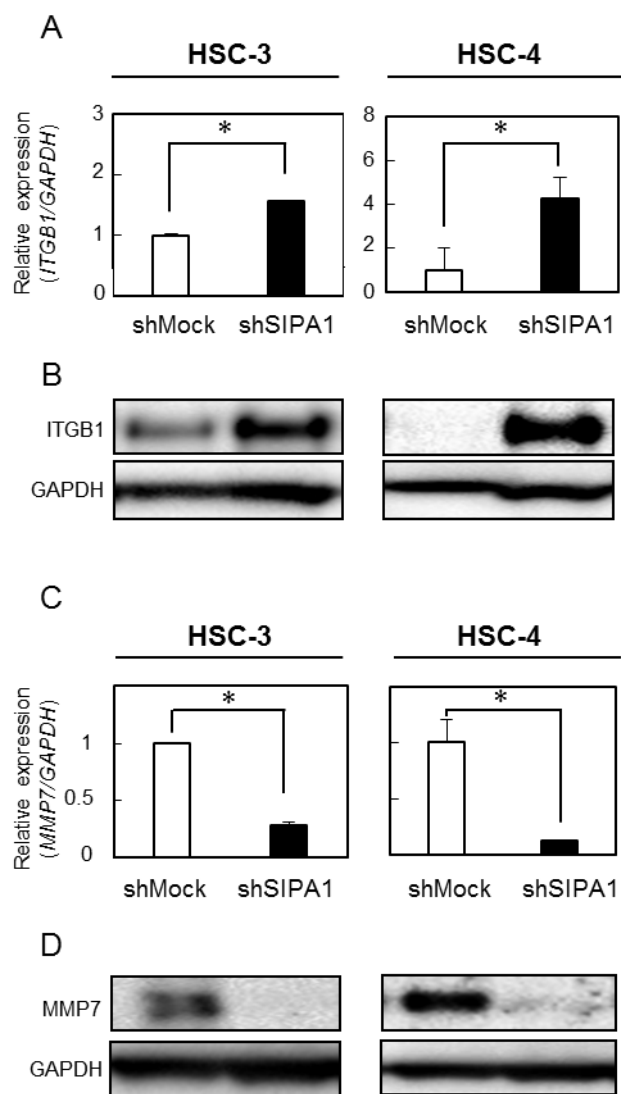


Figure 6



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