科技部補助專題研究計畫成果報告
期末報告

第三型基質金屬蛋白酶組織抑制因子其甲基化程度與蛋白表現在口腔癌致癌過程的研究及調控轉錄因子SP-1的相關性探討

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中 文 摘 要 ： 基質金屬蛋白胺組織抑制因子3 (Tissue inhibitor of metalloproteinase-3 ;TIMP3) 為 TIMPs 家族的一員，當分泌之後會結合到細胞外基質並抑制腫瘤生長、血管新生、爬行及侵襲的能力。由過去文獻發現許多抑癌基因在癌症中都有甲基化的現象，但有關 TIMP3 甲基化在口腔癌的研究卻很少被提到。結果顯示 TIMP3在口腔癌病人組織表現量較相鄰正常組織低。利用去甲基化試劑5-aza 2 ‘-deoxycytidine (5-aza)證實在口腔癌細胞株中 TIMP3的表現會受到甲基化的調控，且此調控會影響轉錄因子 Sp1 與 TIMP3 啟動子結合而抑制 TIMP3 的表現。進一步將 TIMP3 在口腔癌細胞中大量表現，發現在口腔癌細胞與動物模式中，能抑制細胞爬行、侵襲及轉移的能力。此外，TIMP3 能促進上皮型態分子 E-cadherin 與 ZO-1 的表現，並抑制間質型態分子 Snail、Twist、vimentin 及 fibronectin 的表現，來抑制口腔癌細胞的上皮間質型態轉換 (Epithelial-mesenchymal transition; EMT)。綜合以上結果，我們證實 TIMP3 甲基化抑制 TIMP3 的表現會導致口腔癌細胞的轉移。

中 文 關 鍵 詞 ： 第三型基質金屬蛋白酶組織抑制因子; 甲基化; 口腔癌; 轉移; 上皮間質細胞轉化

英 文 摘 要 ： Tissue inhibitor of metalloproteinase-3 (TIMP3), a member of the TIMP family, is the only substance that can bind with the ECM and suppress cancer cell growth, angiogenesis, migration and invasion. However, little is known about whether abnormal expression and promoter methylation of TIMP3 facilitates oral cancer metastasis. The results shown that expression of TIMP3 was decreased in most oral cancer tissues compared with adjacent normal tissues. Using DNA methylation inhibitor 5-Aza-2´-deoxycytidine demonstrates that TIMP3 expression was regulated by DNA methylation. Furthermore, suppression of TIMP3 transcription by DNA methylation involves inhibition of transcription factor SP1 binding to the TIMP3 promoter. Functional analyses revealed that overexpression of TIMP3 reduced the migration and invasion in oral cancer cells and inhibited lymph node metastasis in vivo. Moreover, TIMP3 regulated epithelial–mesenchymal transition (EMT) by increasing expression levels of the epithelial markers E-cadherin and decreasing expression levels of the mesenchymal markers (snail, twist, vimentin and fibronectin). In conclusion, these results suggest that suppression of TIMP3 by DNA methylation contributes to oral cancer metastasis.

英 文 關 鍵 詞 ： TIMP3; methylation; oral cancer; metastasis; epithelial-to-mesenchymal transition

Loss of TIMP3 by promoter hypermethylation induces epithelial-to-mesenchymal transition and promotes oral cancer cell migration

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Abstract

Tissue inhibitor of metalloproteinase-3 (TIMP3), a member of the TIMP family, is the only substance that can bind with the ECM and suppress cancer cell growth, angiogenesis, migration and invasion. However, little is known about whether abnormal expression and promoter methylation of TIMP3 facilitates oral cancer metastasis. The results shown that expression of TIMP3 was decreased in most oral cancer tissues compared with adjacent normal tissues. Using DNA methylation inhibitor 5-Aza-2´-deoxycytidine demonstrates that TIMP3 expression was regulated by DNA methylation. Furthermore, suppression of TIMP3 transcription by DNA methylation involves inhibition of transcription factor SP1 binding to the TIMP3 promoter. Functional analyses revealed that overexpression of TIMP3 reduced the migration and invasion in oral cancer cells and inhibited lymph node metastasis in vivo. Moreover, TIMP3 regulated epithelial–mesenchymal transition (EMT) by increasing expression levels of the epithelial markers E-cadherin and decreasing expression levels of the mesenchymal markers (snail, twist, vimentin and fibronectin). In conclusion, these results suggest that suppression of TIMP3 by DNA methylation contributes to oral cancer metastasis.

Keywords: TIMP3; methylation; oral cancer; metastasis; epithelial-to-mesenchymal transitio

Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignance of head and neck, which include cancer of the lip, tongue, gingiva, salivary glands, floor of the mouth, buccal mucosa, palate, and other intra-oral locations. The primary risk factors associated with OSCC include alcohol drinking, betel quid chewing, radiation and viral infections [12, 31]. This type of malignance has a high potential for local invasion and lymph node metastasis [7, 26], while metastasis is the most vital cause of death. The metastasis is comprised of multiple events involving cell migration, cell invasion, epithelial-to-mesenchymal transition (EMT), angiogenesis and disruption of extracellular matrix (ECM) [3, 32]. Various proteinases, such as the matrix metalloproteinases (MMPs), cathepsins, and plasminogen activator (PA), are involved in the degradation of the ECM in metastasis [39].

Tissue inhibitor of metalloproteinase-3 (TIMP3) is one of a member from TIMP family. It is a 24-kDa secreted protein and unlike other family members, binds firmly to the extracellular matrix (ECM). In addition, TIMP3 has broad metalloproteinase inhibitory activity against members of the matrix metalloproteinase (MMP), a disintegrin and metalloproteinases (ADAM), and ADAM with thrombospondin domain (ADAM-TS) families [5, 35]. TIMP3 acts as a tumor suppressor gene, and loss of expression of TIMP3 was found in esophageal adenocarcinoma, gastric adenocarcinoma and clear cell renal cell carcinoma [13, 18]. TIMP3 has many anti-cancer properties such as anti-angiogenesis effect by blockage of VEGF binding to VEGF receptor-2 [23]. Other studies also demonstrated that TIMP3 plays an important role to promote apoptosis in prostate cancer cells and inhibit tumor cell migration and invasion ability in thyroid tumor [2, 10]. Loss of expression of one gene can due to

different mechanism including genetic alternation or epigenetic alternation. DNA promoter hypermethylation is one of the epigenetic mechanisms that may cause transcriptional silencing by interfering binding of transcription factors to DNA promoter [12, 28]. Tumor suppressor gene such like RUNX3, RASSF1A and CD44 are often silenced in cancer due to promoter hypermethylation [17, 27, 29]. In oral cancer, many tumour suppressor genes have been tested for DNA methylation, these genes include APC, Survivin, E-cadherin, MGMT, MLH1, p14ARF, p15INK4B, p16INK4A, RARβ and RASSF genes, all have their role in carcinogenesis and have been implicated in other tumor types [12, 14, 25].

In previous studies, TIMP3 have been known as tumor suppressor gene in many type of cancer, but there is still few report about whether abnormal expression and promoter methylation of TIMP3 facilitates oral cancer metastasis. So to investigate the relationship between TIMP3 and oral cancer, we analyze TIMP3 expression in oral cell lines and oral tissue from oral cancer patient. Result show that TIMP3 is downregulated in both oral cancer cell line and oral cancer tissue. To further demonstrate that TIMP3 is regulated by hypermethylation of promoter, we used pyrosequencing and NGS to analyze the methylation status in oral tissue and oral cell line. Last, to know the function of TIMP3 in oral cancer, TIMP3 overexpression vector was transfected to oral cancer cell and investigate the cell growth, migration, invasion and adhesion ability after TIMP3 restoration.

Result

Loss of TIMP3 was frequent in oral cancer

To realize if TIMP3 is down-regulated in oral cancer, 17 pairs of tissue from oral cancer patient including cancer tissue and their corresponding normal tissue were used to analyze TIMP3 expression level. Result show that TIMP3 mRNA level is decrease in cancer tissue compare to normal tissue in 13 patients (figure 1A). In protein level, TIMP3 is also down-regulated in 8 pairs of cancer tissue (figure 1B). To further demonstrate TIMP3 level in oral cell line, human oral squamous cell carcinoma cell line (Ca9-22, Cal-27, HSC3, SAS, SCC9, TW2.6) and normal oral cell line (SG, HOK) were used to analyze their TIMP3 protein and mRNA level . Result show that TIMP3 is downregulated in cancer cell line compare to the normal cell line (figure 1C). TIMP3 mRNA level of oral cell lines were also analyzed by real-time PCR and show similar result (figure 1D).

Down-regulation of TIMP3 is regulated by DNA methylation in oral cancer cell line

Next, to investigate whether TIMP3 inactivation may due to promoter hypermethylation, we treated these cancer cell lines with 5-aza 2'-deoxycytidine (5-aza) which can inhibit DNA methylation by blocking DNA methyltransferases (DNMTs) activity. After treatment with 5-aza, TIMP3 mRNA level were increased in all of cancer cell lines (figure 2A). It mean that loss of TIMP3 in oral cancer may due to DNA hypermethylation. Thus, we chose two cell lines SCC9 and TW2.6 to analyze cell motility, migration and invasion ability which TIMP3 expression is more significantly up-regulated after demethylation. Data reveal that motility, migration and invasion were all decrease after

treatment with 5-aza (figure 2B, C, D).

Suppression of TIMP3 by DNA methylation involves inhibition of binding of Sp1 to the TIMP3 promoter.

The human TIMP3 promoter contains a number of binding sites for transcription factor Sp1 [33]. To figure out the relationship between Sp1 and TIMP3, Sp1 overexpression vector was transfected into SCC9 and TW2.6. Western blot results showed that protein expression of TIMP3 was increased when Sp1 restoration (figure 3A). To further examine if TIMP3 promoter activity was regulated by Sp1, we construct a luciferase reporter vector which contain TIMP3 promoter sequence (-940 to +376). Sp1 overexpression in SCC9 and TW2.6 increased the activity of the human TIMP3 promoter in luciferase reporter assays (figure 3B). Moreover, siRNA knockdown of Sp1 inhibited upregulation of TIMP3 by 5-aza (figure 3C, D). Loss of gene may due to transcriptional silencing by interfering binding of transcription factors to DNA promoter. To figure out that SCC9 and TW2.6 were treated with 5-aza and analyze the Sp1 binding ability to TIMP3 promoter. In support, 5-aza markedly enhanced binding of endogenous Sp1 to the TIMP3 promoter in both cell lines (figure 3E)

Restoration of TIMP3 inhibits migration and invasion ability in oral cancer

To confirm the expression of the transgene in infected cells, we first performed Western blot analysis on whole cell lysates prepared from stable clone to analyse the expression of TIMP-3. TIMP3 is overexpression in SCC9-TIMP3 stable clones (T4 and T9) and TW2.6-TIMP3 stable clones (T18

and T21) compare to the control clones (figure 4A). And there were no significant differences between TIMP3 stable cells and control cells on proliferation by MTT assay (figure 4B). Next we investigate the motility of TIMP3-stable clones by wound healing. Overexpression of TIMP3 reduced the cell motility compare to control cells (figure 4C). In migration and invasion assay, overexpression of TIMP3 also inhibited migration and invasion ability in SCC9-TIMP3 and TW2.6-TIMP3 stable clones (figure 4D). We next investigate both the migratory and invasive capabilities of SCC9 and TW2.6 using their own stable conditioned medium (CM+ from TIMP3-stable clones and CM- from control clones) as chemoattractant. CM+ in bottom chamber reduced migration ability (63% in SCC9 and 60% in TW2.6) and invasion ability (55% in SCC9 and 67% in TW2.6) (figure 4E). Furthermore, TIMP3 recombinant protein reduced migration ability (50% in SCC9 and 40% in TW2.6) and invasion ability (39% in SCC9 and 47% in TW2.6) (figure 4F). Moreover, knockdown of TIMP3 recovered migration and invasion ability in SCC9-T9 and TW2.6-T18 clones (figure 4G).

Overexpression of TIMP3 increased cell size, adhesion ability and regulated EMT

It is known that EMT as the mechanism by which epithelial cancers progress toward more aggressive phenotype with increased cell motility and invasive capabilities [20, 30]. Therefore, we hypothesized that the up-regulation of TIMP3 may promote EMT in oral cancer cells. Interestingly, restoration of TIMP3 substantially changed the visible cell morphology, causing increased clustering of cells into epithelial-like islands with higher degree of adherence between neighboring cells and reduced fibroblastic-like morphology. Moreover, TIMP3 overexpression resulted in an increase in

cell area compared with control (figure 5A), and increased cell size correlates with a transition to an epithelial cell morphology and a less motile phenotype [19]. In previous study, TIMP3 has reported to modulate adhesion ability in thyroid tumor cells [2]. We next check the adhesion ability after TIMP3 overexpression in SCC9 and TW2.6 stable clone. Results demonstrate that TIMP3 increased cell adhesion ability in TIMP3 stable clones (figure 5B). To elucidate the possible molecular pathways underlying the connection between TIMP3 expression and EMT process, mRNA array was used to analyze it. We found that overexpression of TIMP3 in oral cancer cell increased E-cadherin expression and decreased expression of fibronectin (figure 5C), real-time PCR and Western blot results were also be used to confirm the array data (figure 5D, E). Moreover, TIMP3 knockdown reversed the expression of epithelial markers (E-cadherin and ZO-1) and decreasing expression levels of the mesenchymal markers (vimentin, fibronectin snail and twist). (Figure 5F).

Ras-ERK pathway is required for TIMP3 regulating EMT, migration and invasion in oral cancer

Activation of Ras-ERK signaling is known to trigger expression of EMT-promoting factors [22], so we hypothesized that TIMP3 regulated EMT was ERK-dependent. First, we analyzed the Ras-ERK pathway in our stable clones. Result showed that restoration of TIMP3 suppresses activation of p-Raf, p-MEK and p-ERK in TIMP3 stable clones (figure 6A), and knockdown of TIMP3 in TIMP3 stable clones activated expression of p-Raf, p-MEK and p-ERK (figure 6B). Next, we used ERK inhibitor PD98059 to suppressed ERK pathway in control stable. Result suggested that blocking of

ERK pathway by PD98059 up-regulated epithelial markers and down-regulated mesenchymal marker (figure 6C). Moreover, migration and invasion ability were also decreased after blocking of ERK pathway (figure 6D). Last, SCC9 T9 and TW2.6 T18 were transfected with TIMP3 siRNA before treating with PD98059. Results revealed that PD98059 reversed TIMP3 suppression-mediated EMT by increased epithelial markers and decreased mesenchymal marker (figure 6E), and further inhibited cell migration and invasion ability (figure 6F). Taken together, these results demonstrate that TIMP3 regulation of EMT, migration and invasion is at least partially ERK-dependent.

TIMP3 suppresses lymph node metastasis in a TW2.6 orthotopic graft model.

As shown in the above experiments, TIMP3 inhibits migration, invasion and induces EMT of oral cancer cells. To further evaluate if TIMP3 overexpression affects metastasis in vivo, luciferase-expressing TW2.6-Luc cells were established to analyze tumor growth and metastasis. There were no significant differences between Control TW2.6 cells (TW2.6/pcDNA3-Luc) and TW2.6/TIMP3-Luc in tumor growth after cell injected into mice at 35 days (Figure 7A, B). Mice were killed at the end of the experiment, and in vivo or ex vivo images of their neck lymph nodes revealed a lower intensity in TW2.6/TIMP3-Luc injected mice than in TW2.6/pcDNA3-Luc injected mice (Figure 7C, D). Most mice developed neck lymph node metastasis within 35 days after cancer cell injection; we further determined the frequency of neck lymph node metastasis and volume of lymph node excised from the TW2.6/pcDNA3 and TW2.6/TIMP3 groups. The mean numbers and volume of neck metastatic lymph nodes in TW2.6/TIMP3 mice significantly decreased compared with those in TW2.6/pcDNA3

mice (Figure 7E, F).

Discussion

Loss of TIMP3 has reported in many human cancers [2, 10]. In this study, we observed a significant down-regulation of TIMP3 expression of oral cancer tissues compared with adjacent normal tissues, suggesting that the loss of TIMP3 might be an important event in pathogenesis of oral cancer. Moreover, in vitro data also demonstrated that TIMP3 mRNA and protein were down-regulated in oral cancer cell lines compare to normal cell lines (HOK and S-G).

Loss or down-regulation of TIMP-3 expression has been linked to TIMP-3 gene methylation in esophageal adenocarcinom, gastric cancer and non–small cell lung cancer in previous reports [8, 16, 36]. Although our results did not find the definite sequence which DNA methylation is responsible for suppression of TIMP3, it is known that loss of TIMP3 protein expression in human gastric cancer cell lines is closely correlated with hypermethylation of TIMP3 in the region near the transcription start site (-116 to 64) [16]. Nevertheless, transcriptional repression by DNA methylation involves inhibition of binding of transcription factors to the active region of gene promoter, and Sp1 is one of the major transcription factors which enhanced the promoter activity of TIMP3 has been reported in chondrocytes and glioblastoma [24, 41]. Consistently, we found that DNA methylation inhibited binding of the transcription factor Sp1 to a region of the TIMP3 promoter that was active when DNA methylation was absent. In conclusion, these findings identify Sp1 as an important transcription factor that activates TIMP3 transcription, should TIMP3 DNA methylation be removed.

To determine the contribution of TIMP3 to oral carcinogenesis, we restored TIMP3 in SCC9 and TW2.6 oral cancer cell lines. Different results of TIMP3 expression on tumor cell growth have been

reported. No effect on in vitro proliferation of human leukemia and thyroid tumor cells was observed [2, 40]; on the contrary, in human melanoma and prostate cancer, TIMP3 was reported to induce cell apoptosis [1, 10, 42]. In our data, TIMP3 expression did not influence the cell growth compare to the control cell. Nevertheless, we demonstrated that TIMP3 plays an important role in regulating migration and invasive processes in oral cancer. TIMP3 restoration in oral cancer cell lines decrease wound healing, migration and invasion ability. Moreover, recombinant protein of TIMP3 or condition medium from TIMP3-stable cell also decreased cancer cell migration and invasion ability, and knockdown of TIMP3 expression in TIMP3-stable cell recovered the cell migration and invasion ability was used to confirm the above results. TIMP3 has broad metalloproteinase inhibitory activity against MMP, ADAMT and ADAMTS family [4, 34]. In animal model, TIMP3 restoration has reported to inhibit leukemia cell growth and angiogenesis in nude mice [40], and $TIMP3^{-/-}$ mice enhanced melanoma and lymphoma cells metastatic dissemination to multiple organs [6]. To gain further insight on how TIMP3 regulate oral cancer invasion and metastasis in vivo, metastatic orthotopic tongue carcinoma nude mouse model was used to examine it. Interestingly, TIMP3 overexpression in oral cancer dramatically decreased metastasis to the lymph nodes and to the lung in an orthotopic implant model, demonstrating spontaneous metastasis from the tongue.

EMT is a key step during tumor invasion and metastasis, and several molecular pathways that mediate EMT in cancer cells have been identified [15, 21, 30]. Previous study has indicated that TIMP3 increased cell adhesion ability in thyroid tumor [2]. We observed that TIMP3 changed cell morphology from fibroblastic-like to epithelial-like island and increased cell adhesion ability in oral cancer. Our

microarray analyses suggested that TIMP3 may function as an activator of EMT directly regulate the expression of a number of genes involved in EMT. TIMP3 restoration increased cell adhesion ability by up-regulation of epithelial markers E-cadherin and down-regulation of mesenchymal markers (vimentin and fibronectin). Moreover, knockdown of TIMP3 reversed EMT process by decreasing E-cadherin expression in SCC9-T3 stable cell and increasing vimentin expression in TW2.6-T3 stable cell.

Snail, Slug, Twist and ZEB1/2 are involved in transcriptional factor contribute to the regulatory network during EMT [9, 22]. In this study, suppression of TIMP3 enhanced the expression of Snail and Twist in oral cancer. Furthermore, Snail and Twist expression was negatively correlated with E-cadherin. As EMT is one of the pathways mediated by mitogen-activated protein kinase (MAPK) signaling or PI3K/AKT signaling pathway [11, 37], EMT might be regulated by TIMP3 through these signal pathways. TIMP3 expression decreased expression of p-ERK and p-AKT, and two pathways were restored by knockdown of TIMP3. Combination of these findings, we suggested that TIMP3 may active EMT by regulating Snail and Twist through ERK and signaling pathway.

In summary, we have identified TIMP3 as a clinical marker for prediction of oral cancer. In addition, the expression of TIMP3 was regulated by promoter hypermethylation, and loss of TIMP3 may due to blocking Sp1 binding to the TIMP3 promoter. Moreover, TIMP3 regulated EMT by increasing expression levels of the epithelial markers E-cadherin and decreasing expression levels of the mesenchymal markers (snail, twist, vimentin and fibronectin). In conclusion, these results suggest that suppression of TIMP-3 by DNA methylation contributes to oral cancer metastasis.

Materials and Methods

Patient specimens

Oral cancer tissue and their corresponding normal tissue were obtained at the time of initial surgery for NSCLC at a Changhua Christian Hospital. Samples were immediately shock frozen in liquid nitrogen and stored at −80°C. The tumor samples were checked for the percentage of tumor cells by histology, and only tumor biopsies with at least 70% cancer cells were used for subsequent analyses. Similarly, cancer-free control samples were also confirmed by histologic examination.

Cell and cell culture

Human oral squamous cell carcinoma cell line Cal-27, Ca9-22, HSC3, SAS and SCC9 were cultured in appropriate medium. Human oral gingival cells (SG) and human oral keratinocyte (HOK) was cultured in DMEM medium and Keratinocyte-SFM. All cell cultures were maintained at 37℃ in a humidified atmosphere of 5% CO_2 .

Western Blot Analysis

The cell lysates was separated in a 10% polyacrylamide gel and transferred onto a nitrocellulose membrane. The blot was subsequently incubated with 5% nonfat milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) for 1 h to block non- specific binding and then overnight with antibodies against TIMP3, E-cadherin, vimentin, fibronectin, ZEB1, Snail, Slug, Twist and β-actin. Afterwards, signal was detected by using enhanced chemiluminescence (ECL) commercial kit

(Amersham Biosciences) and relative photographic density was quantitated by scanning the photographic negatives on a gel documentation and analysis system (AlphaImager 2000, Alpha Innotech Corporation,San Leandro, CA, USA).

RT–PCR and real-time PCR

Total RNA was extracted from oral tissue and cell line using Total RNA Mini Kit (Geneaid). Total RNA (2 μg) was reverse transcribed into cDNA by SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA). The PCR was performed in a reaction mixture containing 2 μL cDNA, 0.2 mM dNTP mixture, 2 mM of each primers, 1 U Taq DNA polymerase, and 1-fold concentration of Thermal Pol Buffer (New England BioLabs, MA, USA) by denaturation at 95℃ for 5 min, followed by amplification of indicated cycles of 95℃ for 30 sec, 60℃ for 30 sec, and 72 ℃ for 60 sec. The specific primer sequences for these genes are as following: TIMP3: 5'-CTGACAGGTCGCGTCTATGA-3'(forward), 5'-GGCGTAGTGTTTGGACTGGT -3' (reverse), and GAPDH: 5'- CGGAGTCAACGGAT TTGGTCGTAT -3'(forward), 5'-AGCCTTCTCCATGGTTGGTGAAGAC -3'(reverse). In quantitative real-time PCR, TIMP3, E-cadherin, vimentin, fibronectin, ZEB1, SNAIL, SLUG, TWIST, Actin and GAPDH expressions were determined using Power SYBR Green PCR Master Mix (Applied Biosystems). Cycling conditions were: 10 min at 95 ℃ followed by 50 repeats of the following cycle: 95 ℃ for 15 s, annealing at the appropriate temperature for 60 s. GAPDH expression was used for normalisation of target gene expression.

Construction of TIMP3 expression vector

The TIMP3 cDNA was isolated by RT–PCR from a human blood cDNA using the following primers: 5’-GAATTCCAGCGGCAATGACCCCTTG-3’ and 5’-GGATC CGCGCTCAGGGGTCTGTGG-3’, containing the EcoRI and the BamHI restriction sites, respectively. The 636 nucleotides PCR product was digested with EcoRI and BamHI endonucleases and inserted into the pcDNA3.

Treatment with DNMT inhibitor and TIMP3 recombinant protein

Twenty-four hours prior to treatment, cells were plated at 5 x 10^5 cells/6 cm plate. Treatments consisted of 5-aza-2’-deoxycytidine (5-aza; Sigma) for 96 h. Drug levels were maintained by replacing media containing the relevant concentration of drug every 24 h. The recombinant TIMP3 protein (R&D Systems, Minneapolis, MN, USA) was used at 50 nM.

Wound healing assay

9 x 10^5 cells were plated in 6 cm plates for 24 h, wounded by scratching with a pipette tip, and then incubated with DMEM containing 0.5% FBS for 0, 24 and 24 h. Cells were photographed using a phase-contrast microscope.

Migration and invasion assay

In the migration assay, cells were seeded in the upper chamber (3 x 10^5 cells per well) of 24-well

Transwell plates equipped with polycarbonate filters (Costar; Corning Incorporated, NY, USA) in serum-free medium. Condition medium were added to the lower chamber as chemoattractant. After 24 h of incubation at 37 ℃, filters were fixed with methanol and stained with Giemsa stain (Sigma). Migrated cells were counted under an inverted microscope in six randomly chosen fields. In the invasion assay, cells were seeded at 1.5 x 10^4 cells per well in modified Boyden chamber invasion assay with serum-free medium. Condition medium were added to the lower chamber as chemoattractant. After 48 h of incubation at 37 ℃, filters were fixed with methanol and stained with Giemsa stain (Sigma). Invasive cells were counted under an inverted microscope in six randomly chosen fields.

Adhesion assay

TIMP3-stable cell and control cell were seeded in 24-well plate (1x10^5 cells per well) pretreated with type IV collagen. After 30 min of incubation, non -attached cells were removed by gently washing twice with 1X PBS. Attached cells were fixed with methanol for 20min at room temperature, followed by staining in crystal violet for 30min. Stained cells were lysed by destain buffer and the intensity of stain was quantified by a spectrometer at the absorbance of 595 nm.

RNA interference experiments

The human small interfering ribonucleic acids (siRNA) for TIMP-3, E-cadherin and scrambled siRNA were from Ambion Inc. Cells were transfected with siRNA with Lipofectamine RNAiMAX

reagent (Invitrogen).

Luciferase assay

Adensity of 8 x 10^4 cells per well was plated in 24-well plates for 24 h. The pGL3-control vector, pGL3-basic vector, and pGL3-TIMP3 vector (promoter -940 to +376) were co-transfected with a b-galactosidase expression vector (pCH110) into cells using Lipofectamine 2000 (Invitrogen). After 24 h of transfection, cell lysates were harvested, and luciferase activity was determined using a luciferase assay kit. The value of the luciferase activity was normalized to transfection efficiency and monitored by b-galactosidase expression.

Chromatin immunoprecipitation analysis (ChIP)

Chromatin immunoprecipitation analysis was performed as described previously [38]. DNA immunoprecipitated with anti-SP-1 was purified and extracted using phenol–chloroform. Immunoprecipitated DNA was analyzed by RT-PCR using specific primers according to previous study [41]. The primers used for PCR to amplify the *TIMP3* promoter encompassing the Sp1 binding sites were *TIMP3* Fw, 5′-CCACGGCGGCATTATTCCCTATAA-3′, *TIMP3* Rev,′-AGGAGCAAGAGGAG GAGGAGAA-3′. The expected size of the PCR product is 266 bps.

Orthotopic implantation.

All animal studies were performed according to protocols approved by the Institutional Animal Care

and Use Committee of Taipei Medical University. Age-matched male severe combined immunodeficient (SCID) mice (6–8 weeks old) were used in assays for tumor growth and metastasis in an orthotopic graft model. TIMP3-expressing luciferase-tagged TW2.6 cells (5×10^5) or the control vector were suspended in phosphate-buffered saline:Matrigel (1:1) and directly injected into the lips of SCID mice (n = 6 per group). After detecting tumour growth, lymph node metastasis was monitored once per week by using a non-invasive bioluminescent imaging system (Xenogen IVIS-200 system). Metastatic neck lymph nodes were enumerated, and the volume was quantified 35 days after cell injection.

Statistical analysis

Statistical significances of difference throughout this study were calculated by Student's t-test (Sigma-Stat 2.0, Jandel Scientific, San Rafael, CA, USA). A difference at $P < 0.05$ was considered to be statistically significant and the experiments were repeated three times.

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Figure 1

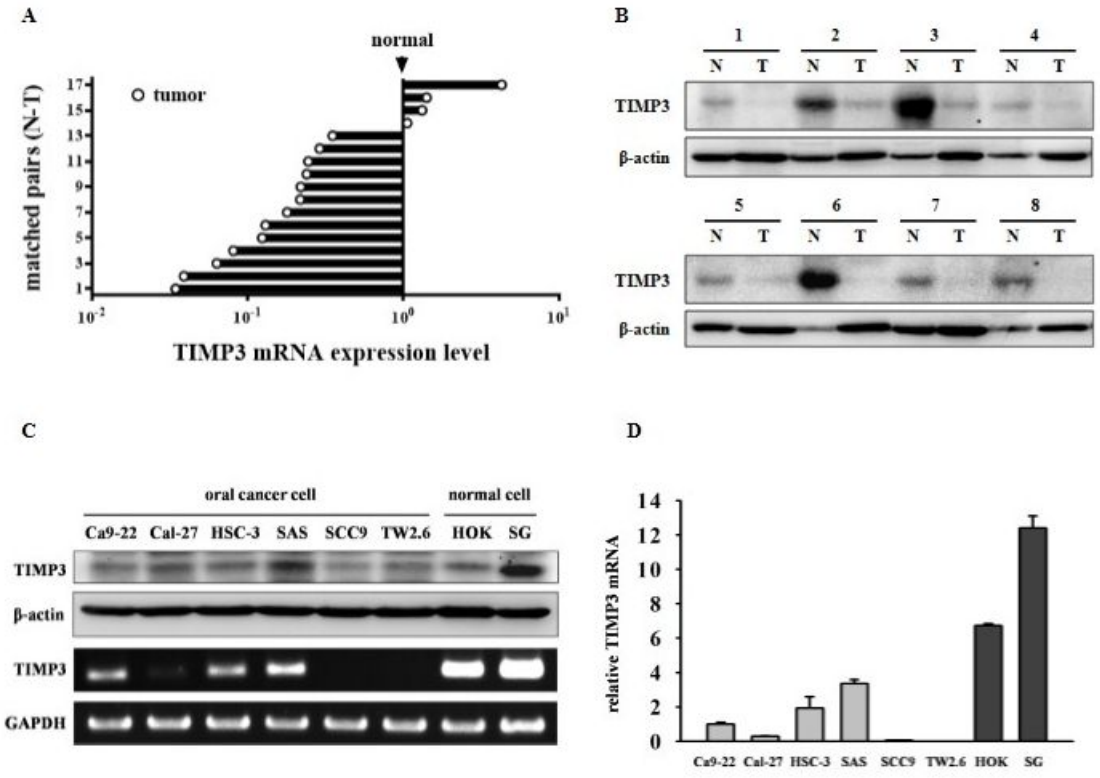

Figure 1. Loss of TIMP3 was frequent in oral cancer (A) TIMP3 mRNA level of oral tissue, GAPDH was used as internal control. (B) TIMP3 protein level of oral tissue, α-tubulin was used as internal control. (C) Protein and mRNA expression of TIMP3 in oral cell lines. Human oral squamous cell carcinoma cell line: Cal-27, Ca9-22, SCC9, HSC3 and SAS. Human oral keratinocyte: HOK. Human oral gingival cells: SG. (D) Real-time PCR results of oral cell lines. The relative TIMP3 expression was normalised to GAPDH.

Figure 2

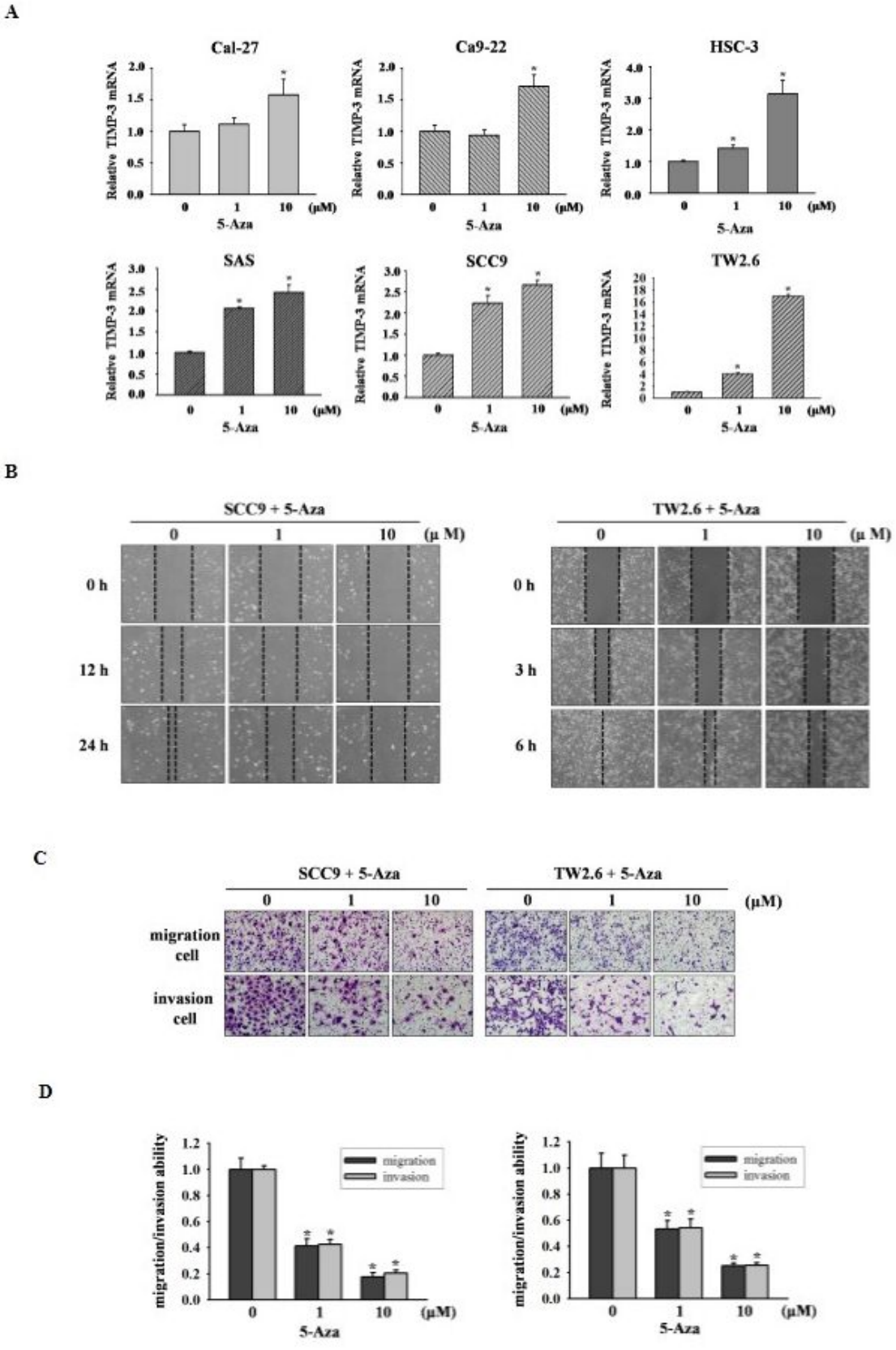
A
Cal-27
Ca9-22
HSC-3
SAS
SCC9
TW2.6
Relative TIMP-3 mRNA
5-Aza
0
1
10
(μM)
B
SCC9 + 5-Aza
TW2.6 + 5-Aza
(μ M)
0 h
12 h
24 h
3 h
6 h
C
SCC9 + 5-Aza
TW2.6 + 5-Aza
(μM)
migration cell
invasion cell
D
migration/invasion ability
migration
invasion

Figure 2. Loss of TIMP3 is regulated by DNA methylation in oral cancer cell line. (A) TIMP3 mRNA is up-regulated in oral cell lines after treatment with 5-Aza. (B) Wound healing assay for SCC9 and TW2.6 after treatment with 5-Aza. (C)(D) Migration and invasion assay for SCC9 and TW2.6 after treatment with 5-Aza. *p<0.05 compared with 0 μM group.

Figure 3

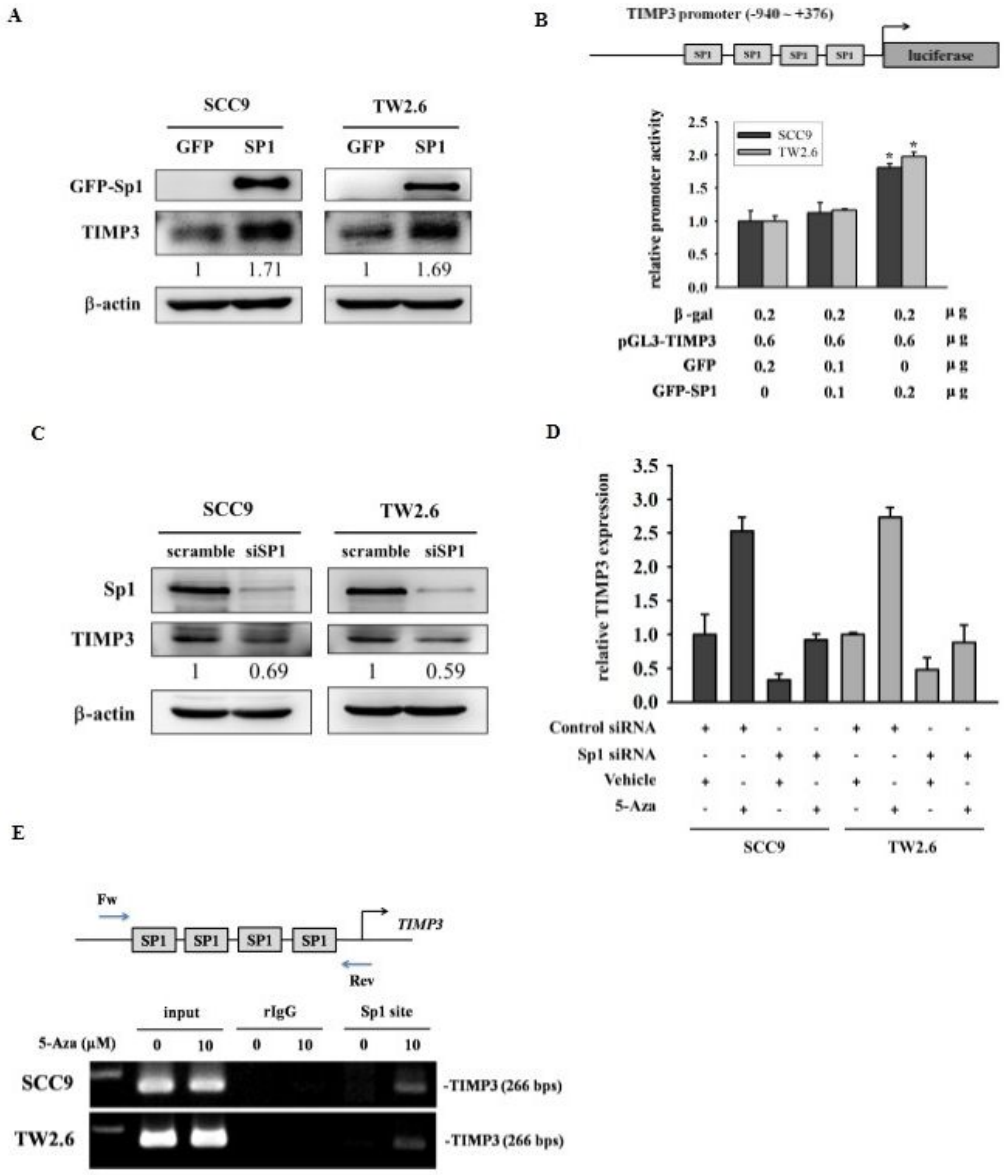

Figure 3. Suppression of TIMP3 by DNA methylation involves inhibition of binding of Sp1 to the TIMP3 promoter. (A) Wesern blot results of Sp1 and TIMP3, β-actin was used as internal control. (B) TIMP3 promoter activity after transfection of Sp1 overexpression vector, β-gal was used to normalize transfection efficiency. (C) SCC9 and TW2.6 cells were transfected with the control siRNA and Sp1 siRNA1. After 24 h, whole-cell lysates were subjected to western blot analysis. (D) SCC9 and TW2.6 cells were transfected with the control siRNA, Sp1 siRNA1. After 24 h, cells were treated with the vehicle control (DMSO) or decitabine (10 μM) for 96 h before total RNA was subjected to qPCR analysis. (E) Formaldehyde-cross-linked chromatin of SCC9 and TW2.6 cell treated with the vehicle control (DMSO) or 5-aza (10 μM) for 96 h was subjected to immunoprecipitation with an antibody against Sp1. The precipitates were subjected to PCR amplification using primers directed to Sp1 binding site of the TIMP3 promoter.

Figure 4

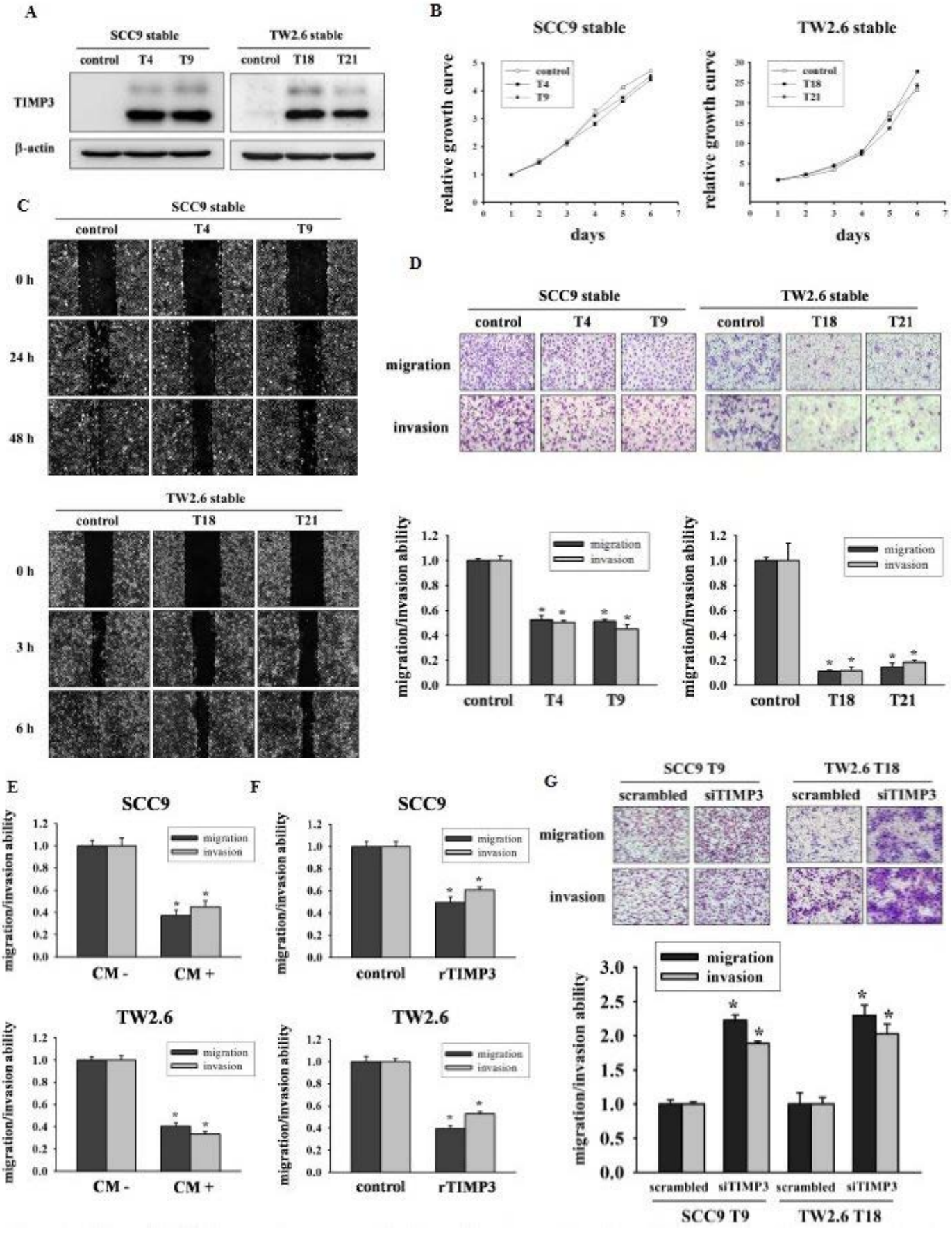
A
SCC9 stable
TW2.6 stable
control
T4
T9
T18
T21
TIMP3
β-actin
B
SCC9 stable
TW2.6 stable
relative growth curve
days
C
SCC9 stable
TW2.6 stable
0 h
24 h
48 h
3 h
6 h
D
migration
invasion
migration/invasion ability
E
SCC9
TW2.6
CM -
CM +
F
control
rTIMP3
G
SCC9 T9
TW2.6 T18
scrambled
siTIMP3

Figure 4. Effect of TIMP3 expression on wound healing, migration and invasion. (A) Western blot of stable clones, β-actin was used as internal control. (B) Cell proliferation assay by MTT assay. (C) Clones were wounded for 0 h, 24 h and 48 h (SCC9) or 0 h, 3h and 6h (TW2.6). Phase-contrast pictures of the wounds at three different locations were taken. (D) Migration and invasion assay were measured after 24 h and 48 h. *p<0.05 compared with control group. (E) Migration and invasion of SCC9 and TW2.6 cell exposed to their own stable conditioned medium (CM-: control cells; CM +: SCC9-T9 and TW2.6-T18) as chemoattractant, or to (F) recombinant TIMP3 protein (rTIMP3). (G) Migration and invasion of SCC9-T9 and TW2.6-T18 were measured at 24 h and 48 h after transfection of scrambled siRNA and TIMP3 siRNA. *p<0.05 compared with scrambled siRNA.

Figure 5

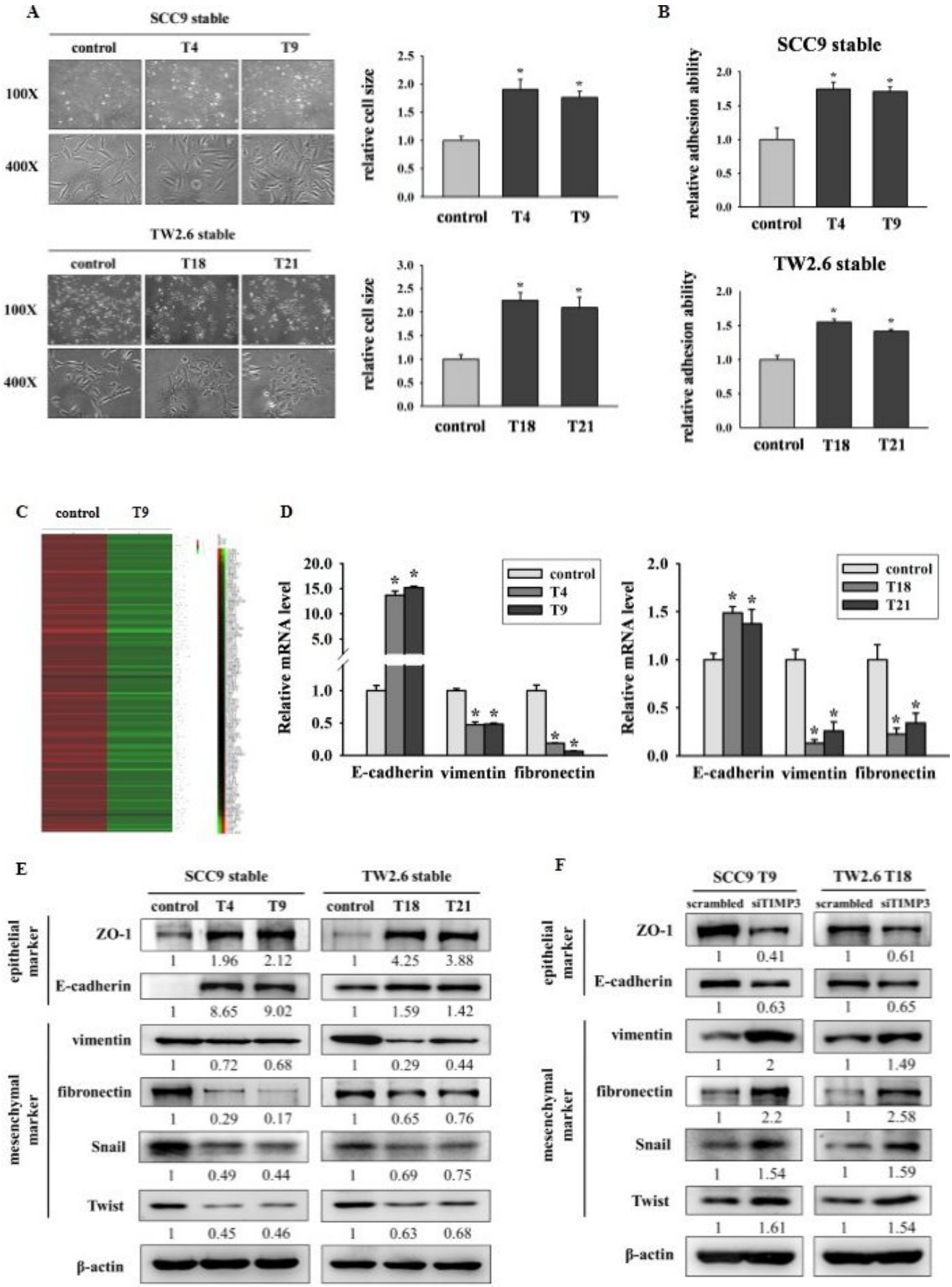
A
SCC9 stable
control
T4
T9
100X
400X
relative cell size
control
T4
T9
TW2.6 stable
control
T18
T21
100X
400X
relative cell size
control
T18
T21
B
SCC9 stable
relative adhesion ability
control
T4
T9
TW2.6 stable
relative adhesion ability
control
T18
T21
C
control
T9
D
Relative mRNA level
control
T4
T9
E-cadherin
vimentin
fibronectin
Relative mRNA level
control
T18
T21
E-cadherin
vimentin
fibronectin
E
SCC9 stable
TW2.6 stable
control
T4
T9
control
T18
T21
epithelial marker
ZO-1
1
1.96
2.12
1
4.25
3.88
E-cadherin
1
8.65
9.02
1
1.59
1.42
mesenchymal marker
vimentin
1
0.72
0.68
1
0.29
0.44
fibronectin
1
0.29
0.17
1
0.65
0.76
Snail
1
0.49
0.44
1
0.69
0.75
Twist
1
0.45
0.46
1
0.63
0.68
β-actin
F
SCC9 T9
TW2.6 T18
scrambled
siTIMP3
scrambled
siTIMP3
epithelial marker
ZO-1
1
0.41
1
0.61
E-cadherin
1
0.63
1
0.65
mesenchymal marker
vimentin
1
2
1
1.49
fibronectin
1
2.2
1
2.58
Snail
1
1.54
1
1.59
Twist
1
1.61
1
1.54
β-actin

Figure 5. **Overexpression of TIMP3 increased cell size, adhesion ability and regulated EMT**. (A) Morphology and cell size of SCC9 and TW2.6 stable clones. *p<0.05 compared with control cells. (B) Adhesion assay of stable clones after 30 min. *p<0.05 compared with control cells (C) mRNA array data of SCC9-control and SCC9-TIMP3 (D) Real-time PCR of stable clones. The relative mRNA expression was normalised to GAPDH. *p<0.05 compared with the control cells. (E) EMT-related protein expression. β-actin was used as loading control. (F) EMT-related protein expression after transfection of scrambled siRNA and TIMP3 siRNA. β-actin was used as loading control.

Figure 6

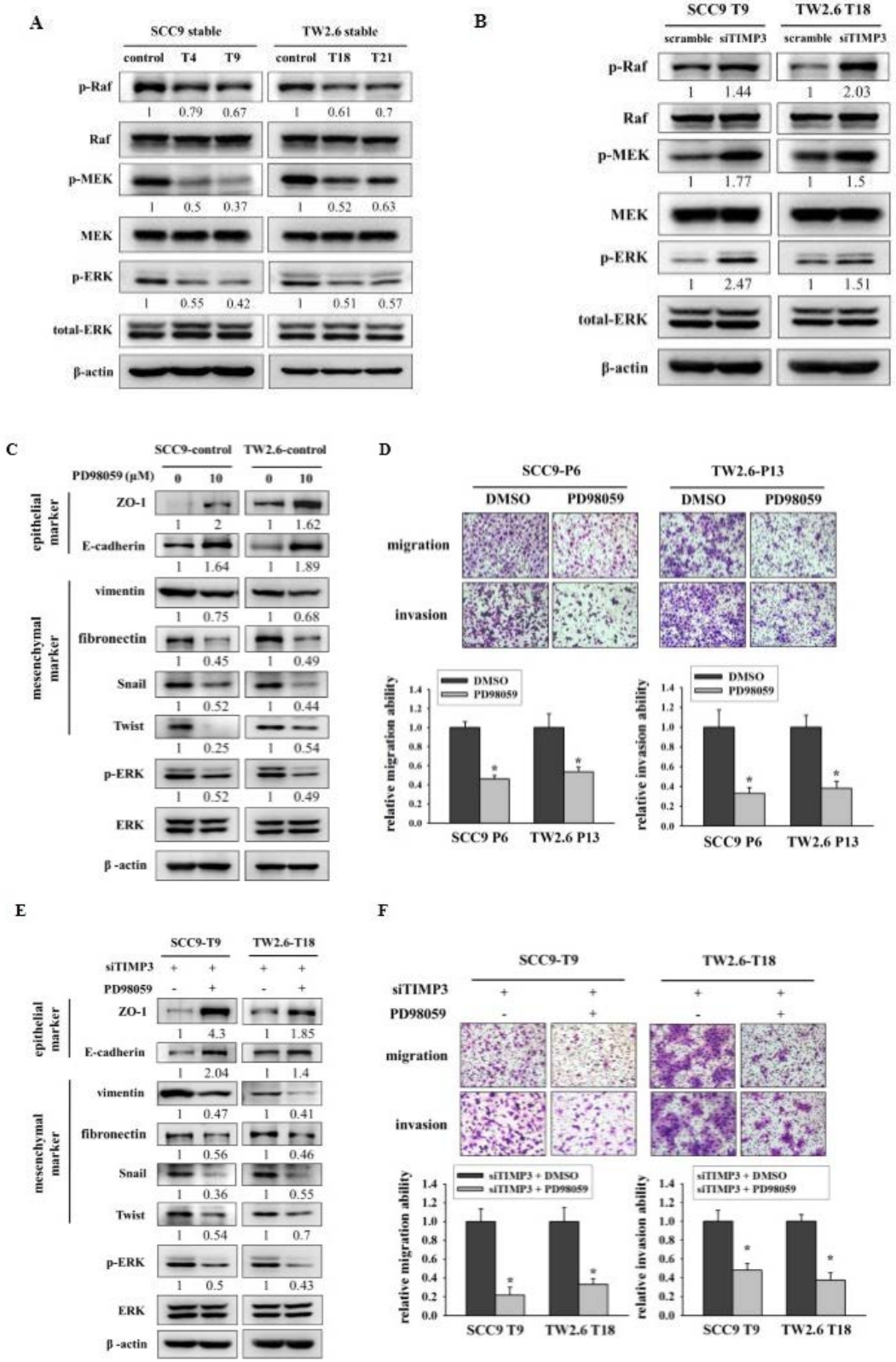
A
SCC9 stable
TW2.6 stable
control T4 T9
control T18 T21
p-Raf
Raf
p-MEK
MEK
p-ERK
total-ERK
β-actin
B
SCC9 T9
TW2.6 T18
scramble siTIMP3
scramble siTIMP3
p-Raf
Raf
p-MEK
MEK
p-ERK
total-ERK
β-actin
C
SCC9-control
TW2.6-control
PD98059 (μM)
epithelial marker
mesenchymal marker
ZO-1
E-cadherin
vimentin
fibronectin
Snail
Twist
p-ERK
ERK
β -actin
D
SCC9-P6
TW2.6-P13
DMSO
PD98059
migration
invasion
relative migration ability
relative invasion ability
SCC9 P6
TW2.6 P13
E
SCC9-T9
TW2.6-T18
siTIMP3
PD98059
F
SCC9-T9
TW2.6-T18
siTIMP3 + DMSO
siTIMP3 + PD98059
SCC9 T9
TW2.6 T18

Figure 6. **Ras-ERK pathway is required for TIMP3 regulating EMT, migration and invasion in oral cancer.** (A) EMT-related pathway protein expression. β-actin was used as loading control. (B EMT-related pathway protein expression after transfection of scrambled siRNA and TIMP3 siRNA. β-actin was used as loading control. (C) EMT-related protein expression after treatment of PD98059 for 48hrs. β-actin was used as loading control. (D) Migration and invasion assay after treatment of PD98059 for 48hrs. *p<0.05 compared with DMSO. (E) EMT-related protein expression after transfection of siRNA for 24hr and treatment of PD98059 for another 24hrs. β-actin was used as loading control. (F) Migration and invasion assay after transfection of siRNA for 24hr and treatment of PD98059 for another 24hrs. *p<0.05 compared to treatment with siTIMP3 and DMSO.

Figure 7

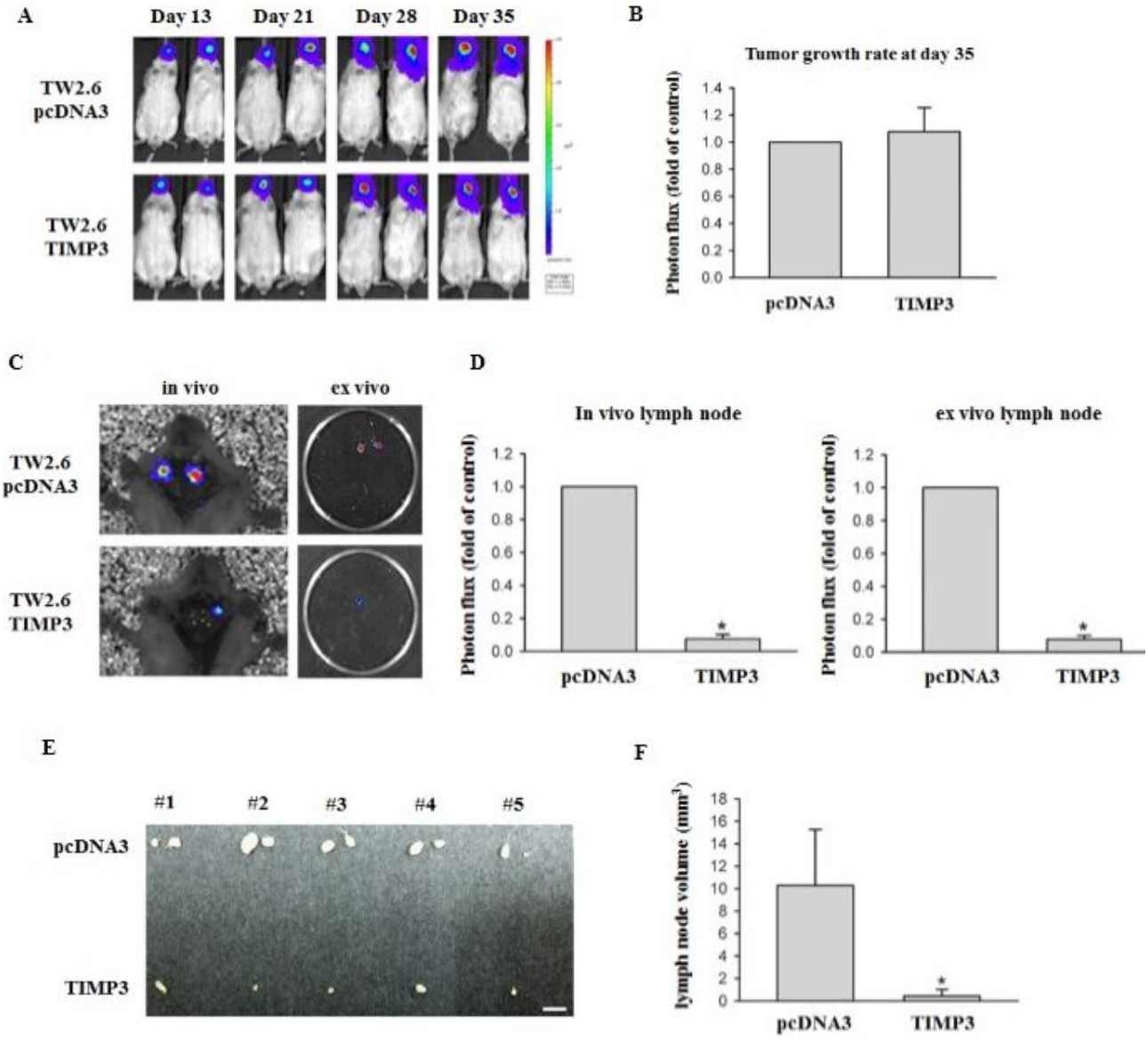

Figure 7. TIMP3 suppresses lymph node metastasis in a TW2.6 orthotopic graft model. (A) Luciferase activity image of mice after injecting with luciferase-tagged TW2.6/pcDNA3 or TW2.6/TIMP3 cells. (B) After 35 days of tumor cell injection, tumors from six mice injected with TW2.6/pcDNA3 or TW2.6/TIMP3 were quantified by measuring the photon influx. (C, D) Lymph node metastasis was imaged at the end of the study with the mean signal for each group indicated (n = 6). *P < 0.05 compared with the vehicle groups. (E, F) Macroscopic analysis of neck lymph nodes. The appearance, number and volume of neck lymph nodes were photographed, enumerated and measured after removal. The metastatic lymph node number and volume were significantly lower in TW2.6/TIMP3 mice than in TW2.6/pcDNA3 mice. *P < 0.05 compared with the vehicle groups.

科技部補助專題研究計畫出席國際學術會議心得報告

日期：105 年 10 月 19 日

計畫編號	MOST 104－2314－B－040－009－		
計畫名稱	第三型基質金屬蛋白酶組織抑制因子其甲基化程度與蛋白表現在口腔癌致癌過程的研究及調控轉錄因子 SP-1 的相關性探討		
出國人員姓名	林巧雯	服務機構及職稱	中山醫學大學口腔科學研究所 副教授
會議時間	2016/07/09- 2016/07/12	會議地點	英國-曼徹斯特
會議名稱	(中文)第 24 屆歐洲癌症學會 (英文)24th Biennial Congress of the European Association for Cancer Research		
發表題目	(中文) Neutrophil gelatinase-associated lipocalin 抑制口腔癌轉移透過 miRNA4505 調控 CAIX 的機制 (英文) Neutrophil gelatinase-associated lipocalin suppress metastases of oral cancer through miRNA4505 regulated carbonic anhydrase IX		

一、參加會議經過

7/8 從倫敦前往曼徹斯特。7/9 前往位在市中心的會議地點(Manchester Central Convention Complex)報到及領取大會議程及摘要手冊，並聆聽演講。7/10 將準備好的論文海報張貼於指定位置。並於會議尚未開始的時間參觀會場內其他相關發表，下午於論文海報張貼處介紹自己的研究成果。

二、與會心得

此次的會議舉辦地點在 Manchester Central Convention Complex，在市中心且交通方便，因此參與會議者眾多。在會場也遇到許多台灣的教授。而除了在會場中觀摩展示海報發表的研究內容外，也參與多場口頭報告發表，聆聽一些關於 Invasion and Metastasis 與 Inflammation and Cancer 的相關研究。藉由參與國際大型會議，可認識國際上頂尖的研究學者，了解大師級的思維，對平日教學與研究頗有助益。

三、發表論文全文或摘要

Background: Neutrophil gelatinase-associated lipocalin (NGAL) is a secreted glycoprotein and dysregulated in different types of human cancer. However, the role of NGAL in the metastasis of human oral cancer is largely unknown.

Methods: We modified NGAL levels in two human oral cancer cell lines and analyzed the effects on carbonic anhydrase IX (CAIX) expression and oral cancer migratory ability by Transwell assays. Prediction of microRNA (miRNA) binding to 3'-UTR of CAIX mRNA was performed by bioinformatics analyses and confirmed by a luciferase reporter assay using miRNA-4505-modifed oral cancer cells.

Results: Overexpression of NGAL in oral cancer cell lines reduced in vitro migration/invasion whereas its silencing induced the increase of cell motility. Mechanistically, NGAL inhibited the cell motility of oral cancer cells through transcriptional inhibition of the carbonic anhydrase IX (CAIX). Moreover, in oral cancer cells, NGAL significantly increased the levels of miRNA-4505, which suppressed the translation of CAIX mRNA via 3'-UTR binding. Overexpression of miRNA-4505 significantly suppressed migration and

invasion in oral cancer cells ($p<0.001$).

Conclusion: MiRNA-4505 inhibits translation of CAIX through binding to the 3'-UTR of CAIX mRNA in oral cancer cell lines. NGAL increases miRNA-4505, which suppress CAIX-mediated oral cancer invasion.

四、建議

國內也應增加舉辦如此大型會議、增加補助出國額度、或盡量補助教師與學生出國開會或短期研究之經費，使學生可以訓練其外語能力與加強國際觀。

五、攜回資料名稱及內容

會議議程手冊

六、其他

科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/24

科技部補助計畫	計畫名稱: 第三型基質金屬蛋白酶組織抑制因子其甲基化程度與蛋白表現在口腔癌致癌過程的研究及調控轉錄因子SP-1的相關性探討
	計畫主持人: 林巧雯
	計畫編號: 104-2314-B-040-009- 學門領域: 牙醫

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：林巧雯	**計畫編號：**104-2314-B-040-009-
計畫名稱：第三型基質金屬蛋白酶組織抑制因子其甲基化程度與蛋白表現在口腔癌致癌過程的研究及調控轉錄因子SP-1的相關性探討	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		

		其他	0		
	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	1		
		博士後研究員	0		
		專任助理	1		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   癌症的形成中包含許多基因層次上的改變。由過去的文獻指出，許多抑制癌症的基因在癌症中都有被高度甲基化的現象。在口腔癌中許多抑癌基因的甲基化都已被證實參與在癌症的進程中，但有關第三型基質金屬蛋白酶組織抑制因子甲基化對口腔癌的研究並不多。在本篇研究中，我們發現在口腔癌病人的組織中，TIMP3的表現相對於周邊正常組織有減少的趨勢，此外，在口腔癌細胞株中，TIMP3 的表現相較於正常口腔細胞株 HOK 及 S-G 有下降的趨勢。而 TIMP3 的表現會受到啟動子 CpG island 上甲基化的影響並調控轉錄因子 Sp1 與 TIMP3 啟動子的結合能力。此外，TIMP3 能透過調控 EMT相關的蛋白表現，影響細胞的貼附、爬行及侵襲的能力。綜合結果，我們認為 TIMP3 甲基化能抑制 TIMP3 的表現並調控下游 EMT 的表現來導致口腔癌的轉移。期望透過本研究在口腔癌藥物的開發與治療上得到運用。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是
   說明：（以150字為限）