Cellular Signalling 59 (2019) 1–12

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

SLFN5 suppresses cancer cell migration and invasion by inhibiting MT1-MMP expression *via* AKT/GSK-3β/β-catenin pathway

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ARTICLE INFO

Keywords:
Schlafen family member-5 (SLFN5)
Matrix metalloproteinase 14
Invasion
β-catenin
Cancer

ABSTRACT

Human SLFN5 inhibits invasions of IFNα-sensitive renal clear-cell carcinoma and melanoma cells. However, whether this inhibition is confined to these IFNα-sensitive cancers is unclear. Here we show that SLFN5 expressions on both mRNA and protein levels are significantly higher in non/low-invasive cancer cell lines (breast cancer cell line MCF7, colorectal cancer cell line HCT116 and lung cancer cell line A549) than in highly-invasive cancer cell lines (fibrosarcoma cell line HT1080 and renal clear cell cancer cell line 786-0). SLFN5 knockdown in non/low-invasive cancer cell lines enhanced MT1-MMP expression and increased migration and invasion *in vitro*, and *in vivo*. Furthermore, SLFN5 overexpression in HT1080 and 786-0 inhibited MT1-MMP expression and repressed migration and invasion. MT1-MMP is instrumental in SLFN5-controlled inhibition of cancer cell migration and invasion, as shown by MT1-MMP-knockdown and -overexpression analyses. SLFN5 knockdown activated AKT/GSK-3β/β-catenin pathway by promotion AKT phosphorylation and subsequent GSK-3β phosphorylation, further β-catenin translocation into nucleus as un-phosphorylated protein at Ser33, 37 and 45 and Thr41 sites. This is the first study to report that SLFN5 inhibits cancer migration and invasiveness in several common cancer cell lines by repressing MT1-MMP expression *via* the AKT/GSK-3β/β-catenin signalling pathway, suggesting that SLFN5 plays wide inhibitory roles in various cancers.

1. Introduction

Schlafen (SLFN) proteins were firstly reported to be abundant in immune cells, and to regulates immune cell growth and differentiation [1,2]. Currently, 49 organisms have been found to have orthologous *SLFN genes*, of which ten of the murine SLFNs (SLFN1, 1L, 2, 3, 4, 5, 8, 9, 10 and 14), are the most widely studied [3,4]. Only 5 human SLFN proteins have been discovered (SLFN5, 11, 12, 13 and 14), and differ greatly from murine SLFNs in structure and function [3,4]. Some human SLFNs reportedly regulate monocyte differentiation, inhibit virus replication and mediate cancer cell functions [5–10].

The cytokine interferon (IFN) has many functions, including inhibiting cell division, regulating immunity, and suppressing viruses and tumours [11–13]. Cells that display IFN receptors are divided into type I and type II [14]. Type I alpha (IFNα) is mainly produced by white blood cells and is used to treat tumours; melanoma and renal clear-cell carcinoma (RCC) are especially sensitive to IFNα therapy [14]. When melanoma and RCC cell lines are exposed to IFNα, SLFN5 is significantly upregulated, and has an inhibitory effect on motility and invasiveness, which implies a possible mechanism for the effect of IFNα on these cancers [8,9]. However, SLFN5 was recently shown to affect intestinal metaplasia progression to gastric cancer [15], which implies that SLFN5 may also affect IFNα non-sensitive cancers.

Cancer cells invade extracellular matrix and metastasize from primary sites to remote organs [16–19]. Matrix metalloproteinase (MMP) proteins are responsible for the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodelling, as well as in disease processes, such

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https://doi.org/10.1016/j.cellsig.2019.03.004
Received 13 January 2019; Received in revised form 17 February 2019; Accepted 2 March 2019
Available online 04 March 2019
0898-6568/

as arthritis progression and cancer metastasis [20–24]. Of the 27 known members of MMP family, most are secreted as inactive proproteins, which are activated after cleavage by extracellular proteinases [18,19,21]; six members are called membrane-type MMP (MTs-MMP), as they contain a transmembrane domain with which they tether to cell surface [18,19,21]. Differing localizations (extracellular soluble or transmembrane), may influence their functions in tumour progression [25].

MMP14 (MT1-MMP) is associated with invasive and metastatic behaviours in many cancers and cancer cell lines [26–29]. Introduction of MT1-MMP into human breast cancer MCF7 cells confers invasive activity, *in vitro* and *in vivo* (with chick embryos) [27]. Interestingly, inhibiting MT1-MMP by specific siRNA in highly malignant cells, such as breast cancer MDA-MB-231 or fibrosarcoma HT1080 cells, represses invasiveness [27–29]. Other MMPs, such as MMP-1, -2, -9, and -13, are also reported to be associated with cell invasiveness [30,31]. Sassano et al. reported that MMP-1 and MMP-13 were upregulated in SLFN5 knockdown (KD) RCC cells [9]. However, we downloaded and analysed their published RNA sequencing data and found that MT1-MMP expression was also upregulated in *SLNF5*-KD RCC cells. This raised our curiosity as to whether MT1-MMP interacts with SLFN5 in mediating invasiveness. So far, no study has investigated this relationship.

MT1-MMP is regulated by some factors or processes, such as growth factor PDGF-BB [32], cytokine IL-6 [33], the Wnt/β-catenin signalling pathway [34], and the p53-regulated pathway [35], as well as inhibitory transcriptional factors, such as Snail [36,37], to drive cell invasion, as previously reported by us and other researchers. However, whether and how it is regulated by SLFN5 remains unclear. Herein, we show that *SLFN5* KD activated the β-catenin signalling pathway and enhanced MT1-MMP expression in non- or low-invasive cancer cell lines MCF7, HCT116 and A549, and thereafter increased in migration and invasion activities *in vitro* and chick embryo *in vivo*. Moreover, SLFN5 overexpression in invasive cell lines HT1080 and 786-0 inhibited MT1-MMP expression at mRNA and protein levels, and repressed migration and invasion activities in both *in vitro* and *in vivo*. Loss of MT1-MMP by siRNA treatment reversed SLFN5 KD-induced upregulation of migration and invasion. Similarly, gain of MT1-MMP by plasmid transfection reversed SLFN5 overexpression-induced downregulation of migration and invasion. These findings indicate that MT1-MMP is responsible for SLFN5 regulation of migration and invasion in cancer cells. Moreover, β-catenin was translocated from cytosol into nucleus and regulated MT1-MMP expression in SLFN5-KD cells, suggesting that SLFN5 regulates MT1-MMP expression through β-catenin pathway. To our knowledge, this is the first report of unstimulated human SLFN5 having an inhibitory function in cancer invasiveness, *in vitro* and *in vivo*, and in several different cell lines, by repressing MT1-MMP expression *via* the β-catenin pathway.

2. Results

2.1. SLFN5 expression varies in morphologically different cancer cells

Although SLFN5 reportedly affects IFNα-induced invasiveness of melanoma cells and RCC cells, we investigated whether it has an effect on activity in other cancers. We assessed 5 typical cancer cell lines—MCF7 for breast cancer, HCT116 for colorectal cancer, A549 for lung cancer, 786-0 for renal cell cancer, and HT1080 for fibrosarcoma—for SLFN5 expression at mRNA and protein levels, using real-time PCR and western blot (WB), respectively. We found that SLFN5 mRNA and protein levels (891 amino acid residues, 101kD) in MCF7, HCT116, and A549 cells were markedly higher than those in 786-0 and HT1080 cells (Fig. 1A, B). We therefore divided MCF7, HCT116, and A549 cells into the high SLFN5-expressing group, and 786-0 and HT1080 cells into the low SLFN5-expressing group.

Interestingly, the high SLFN5-expressing group exhibited epithelial morphology, and the low SLFN5-expressing group showed mesenchymal morphology (Fig. 1C). As we and others have reported, MCF7, HCT116 and A549 display a spherical non-invasive phenotype, whereas mesenchymal cells such as HT1080 display a long-stretched fibroblast phenotype in 3-dimensional (3D) collagen matrix (Fig. 1C, middle panels). Further, when these cells were buried in a 3D collagen island, the MCF7, HCT116, and A549 cells stayed near their original locations, but the 786-0 and HT1080 cells migrated from their original locations and invaded the surrounding collagen. Therefore, SLFN5 level seems inversely correlated with invasiveness. However, whether SLFN5 functions on these cell invasion and related mechanisms are unclear.

2.2. SLFN5-KD lung cancer A549 cells show upregulated MT1-MMP expression and associated migration and invasion

Real-time PCR results showed that the A549 cell line expressed more SLFN5 mRNA than did the HT1080 cell line (Fig. 2A). Therefore, we transfected A549 with three *SLFN5* siRNAs that had different targeting sequences (Fig. 2B), and found siRNA1 (si-1) had the best KD efficiency; it was therefore used in our subsequent experiments. In SLFN5-KD A549 cells, MT1-MMP mRNA expression was strongly upregulated (Fig. 2B), which suggested that MT1-MMP is regulated at the transcriptional level by SLFN5. In fact, the online GEO database (https://www.ncbi.nlm.nih.gov/geo, GSE64399) reported MT1-MMP in SLFN5-KD 786-0 cells to be upregulated 160% relative to cells with control siRNA (Ctro-Si; Fig. 2D). Consistently, MT1-MMP protein levels in SLFN5-Si cells approximately doubled, as assayed by WB, using a specific catalytic domain antibody (band at 55 kD; Fig. 2E). Immunofluorescence cytochemistry to detect SLFN5 and MT1-MMP expression and localization showed that SLFN5 was localized in nucleus in Ctro-Si cells and diminished in SLFN5-KD cells (as expected), whereas MT1-MMP expression was greatly increased in SLFN5-KD cells, but showed only background staining in Ctro-Si cells (Fig. 2F).

To assess the function of SLFN5 in cell migration and invasion, we used Transwell migration and invasion assays. The SLFN5-KD group had more than twice as many migrated or invading cells than the Ctro-Si group (Fig. 2I, J, upper panels), indicating that SLFN5 inhibits cancer cell motility.

However, whether MT1-MMP affects SLFN5 regulation of migration and invasion was unclear. After assessing knockdown efficiency of MT1-MMP-specific siRNA (Fig. 2G, H), we evaluated MT1-MMP-Si combined with SLFN5-Si cells, which showed significant decreases in both migration and invasion, relative to cells treated with Ctro-Si combined with SLFN5-Si (Fig. 2I, J, lower rows). Taken together, these data suggest that SLFN5 suppresses A549 cell invasiveness by downregulating MT1-MMP expression.

2.3. SLFN5 KD in colorectal cancer HCT116 cells and breast cancer MCF7 cells promotes MT1-MMP expression and associated migration and invasion

Although the above data demonstrated the effects of SLFN5 KD on MT1-MMP expression and migration and invasion in A549 lung cancer cells, whether SLFN5 has the same function in colorectal cancer HCT116 cells and breast cancer MCF7 cells, which express relatively high levels of SLFN5 (Fig. 1A, B) was unclear. After checking SLFN5 KD efficiency in these cells (using real-time PCR and RT-PCR for mRNA and WB for protein; Fig. 3A–C), we found that MT1-MMP protein and mRNA expressions were significantly elevated in SLFN5-Si HCT116 cells, relative to those in Ctro-Si cells (as assessed by real-time PCR, RT-PCR, and WB; Fig. 3D–F). We similarly evaluated MT1-MMP expressions in SLFN5-Si treated MCF7 breast cancer cells and found that MT1-MMP was strongly induced by SLFN5-Si treatment (Fig. 3G–I). Taken together, these data from HCT116 and MCF7 cells, as well as from A549 cells (Fig. 2), indicate that SLFN5 suppresses MT1-MMP transcription and expression in these cancer cell lines.

As predicted, SLFN5-Si transfection led to greater numbers of migrating or invading HCT116 cells per field, compared with Ctro-Si cells

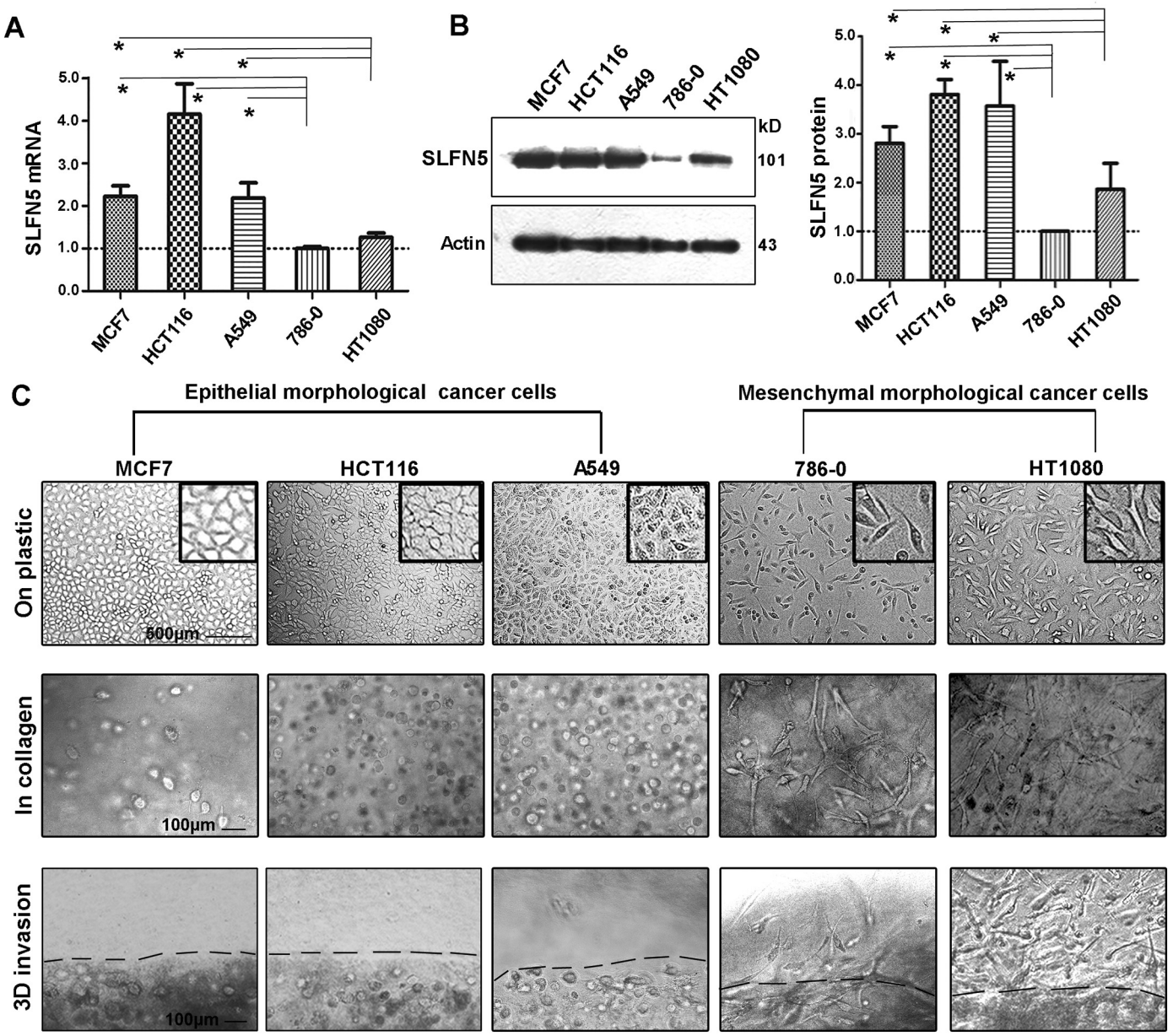
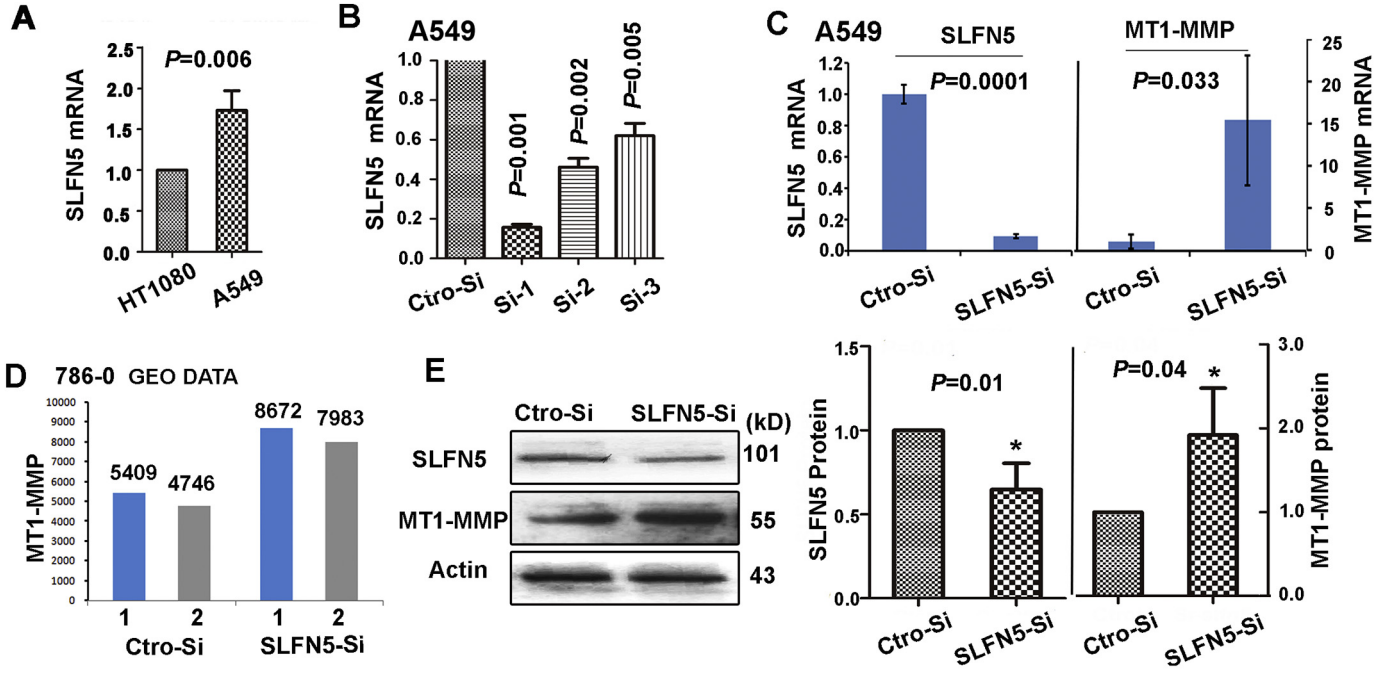


Fig. 1. SLFN5 expression levels in 3 epithelial and 2 mesenchymal morphological cancer cell lines.
(A) Real-time PCR analysis of human *SLFN5* mRNA expression in MCF7, HCT116, A549, 786-0 and HT1080 cell lines. (B) Western blot analyses of human SLFN5 protein expression in these 5 cell lines (loading control: actin; band intensity shown as mean ± SE of 3 experiments). (C) Morphologies of these five cell lines, cultured on plastic plates or in collagen, observed with inverted microscopy (upper right corner: enlarged versions; Scale bars, top row: 500 μm, middle and bottom rows: 100 μm; black dashed lines: initial collagen-cell mixture boundary). *$P < .05$.

(Fig. 3J, K, upper panels). As SLFN5 KD increases MT1-MMP, whether greater migration and invasion by SLFN5-KD cells is resulted from MT1-MMP upregulation need to investigate. To study this possibility, we used MT1-MMP siRNA (MT1-Si) along with SLFN5-Si to transfect HCT116 cells. Both migration and invasion activities were significantly repressed in MT1-KD combined with SLFN5-KD cells, compared to those co-transfected with Ctro-Si and SLFN5-Si (Fig. 3J, K, lower panels). Together these data indicate that MT1-MMP is downstream of SLFN5 and responsible for SLFN5 inhibitory functions on non- or low-invasive cancer cell migration and invasion *in vitro*.

2.4. SLFN5 KD promotes AKT and GSK-3β phosphorylation and β-catenin translocation into nucleus, and regulates MT1-MMP expression

To find how SLFN5 inhibits MT1-MMP expression, we investigated MT1-MMP upstream pathways, such as the WNT signalling pathway in A549 cells. AKT phosphorylation was found to be enhanced in SLFN5-Si A549 cells compared with Ctro-Si A549 cells (Fig. 4A), indicating that AKT signalling pathway was activated. As a direct substrate of phosphorylated AKT, GSK-3β was increased in phosphorylation level significantly in SLFN5-Si cells compared with Ctro-Si cells (Fig. 4A). Further, SLFN5 KD increased β-catenin protein expression levels, compared with Ctro-Si cells (Fig. 4B). However, β-catenin that was phosphorylated at amino acid sites Ser33, 37 and 45 and Thr41 was down-regulated significantly more in SLFN5-Si cells than in Ctro-Si cells (Fig. 4B). The decrease in phosphorylated β-catenin, together with increased total β-catenin, in SLFN5-Si cells, suggests that SLFN5-KD keeps β-catenin in an un-phosphorylated state, thus allowing it to enter the nucleus and subsequently regulate transcriptions of target genes. To further investigate this possibility, nucleus and cytosol protein fractions



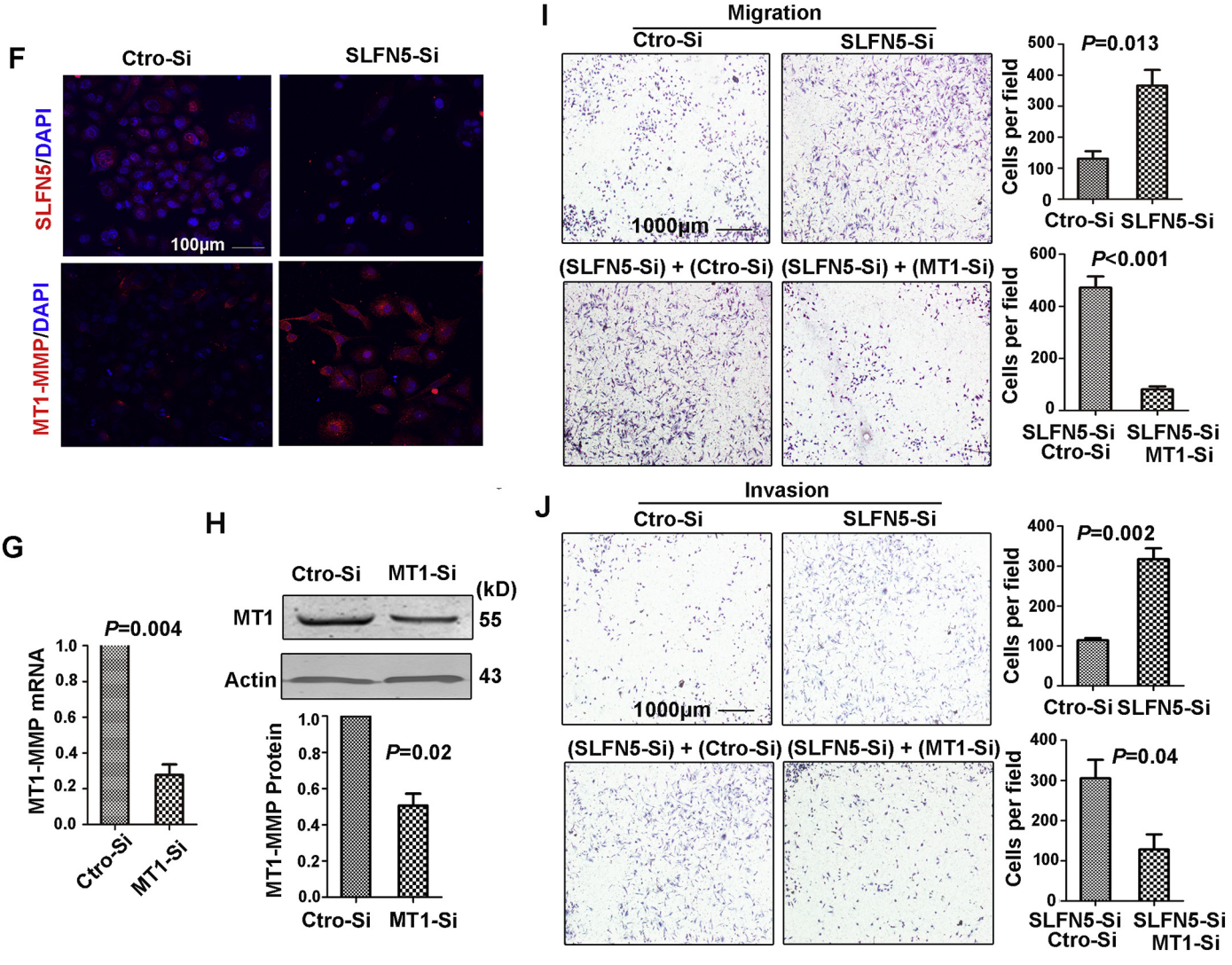


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Fig. 2. Effects of *SLFN5* knockdown (KD) on MT1-MMP expression in A549 and cell migration and invasion.
(A) Real-time PCR analysis confirmed different expression levels of human *SLFN5* mRNA levels in HT1080 and A549 cancer cell lines. (B) Real-time PCR analysis of KD efficiency of 3 siRNAs specific for *SLFN5*, using scrambled siRNA as control (Ctro-Si). We used Si-1 which had the highest efficiency, in our KD experiments. (C) Real-time PCR analysis of *SLFN5* and *MT1-MMP* mRNA expressions in *SLFN5-KD* cells. (D) Data downloaded from online GEO profile (https://www.ncbi.nlm.nih.gov/geo, GSE64399) about *SLFN5* KD effect on *MT1-MMP* mRNA expression in 786-0 renal cancer cells. (E) Western blot analysis of SLFN5 and MT1-MMP protein expressions in *SLFN5*-KD A549 cells. (F) Immunofluorescence of SLFN5 and MT1-MMP protein localization in A549 cells transfected with Ctro-Si or SLFN5-Si. DAPI staining (blue): nucleus. (G and H) Real-time PCR and western blot analyses of *MT1-MMP*-KD transfection efficiencies. (I and J) Migration and invasion of A549 cells transfected with Ctro-Si, or SLFN5-Si, or SLFN5-Si combined with Ctro-Si, or with SLFN5-Si combined with MT1-Si (scale bars: 1000 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were isolated from SLFN5-Si to Ctro-Si cells, and assessed by WB (using histone-3 as nucleus protein loading control, and β-actin as cytosol control). Firstly, SLFN5 protein was clearly detectable in nucleus fractions (Fig. 4C) in Ctro-Si cells, but not in SLFN5-Si cells, consistent with the results shown in Fig. 2F, and shows SLFN5 to be a nuclear protein (the presence of SLFN5 in cytosol due to protein synthesis occurring in cytosol). Phosphorylated β-catenin (at Ser33, 37 and 45 and Thr41) decreased significantly in SLFN5-Si cells compared with Ctro-Si cells, as known phosphorylation of β-catenin at these amino acid residues leads to ubiquitination and degradation. Therefore, decreased phosphorylation of β-catenin in the cytosol fraction of SLFN5-Si cells resulted in presence of active β-catenin and its translocation into the nucleus. No phosphorylated β-catenin was found in the nucleus fractions of either SLFN5-Si or Ctro-Si cells. In both nucleus and cytosol fractions, total β-catenin levels were higher in SLFN5-Si than those in Ctro-Si cells, indicating that β-catenin proteins translocate from cytosol into the nucleus, where it regulates gene transcriptions. To further detect whether β-catenin accumulated in nucleus regulates MT1-MMP expression, β-catenin siRNA transfection was performed with scrambled siRNA transfection as control. Real-time PCR and WB results showed that MT1-MMP expressions at both mRNA and protein levels were blocked in β-catenin siRNA treated cells, compared with scrambled siRNA cells (Fig. 4D, E), which suggested that MT1-MMP is regulated by SLFN5 *via* β-catenin pathway.

2.5. SLFN5 overexpression inhibits MT1-MMP expression in RCC 786-0 cells and fibrosarcoma HT1080 cells, and represses cell migration and invasion

The above data on the function of SLFN5 in cancer migration and invasion came from non- or low-invasive cancer cell lines. We therefore wanted to conduct similar evaluations of high-invasive cancer cell lines. We found that 786-0 (a typical high-invasive renal cancer cell line) and HT1080 (a typical high-invasive fibrosarcoma cell line) both had relatively low expressions of SLFN5 at mRNA and protein levels compared with the non- or low-invasive MCF7, A549 and HCT116 cells (Fig. 1A and B), therefore we selected them for transfection by a SLFN5 plasmid to study SLFN5 function. After transfecting a SLFN5 plasmid (SLFN5-pl) into 786-0 and HT1080 cells, their SLFN5 mRNA expression greatly increased (as shown by RT-PCR and real-time PCR; Fig. 5A, B, E, F), as did SLFN5 protein expression in both cell lines (Fig. 5C, G). Migration and invasion activities were both suppressed by SLFN5 overexpression (Fig. 5I, J). As MT1-MMP was found to act as a downstream player in migration and invasion, controlled by endogenous SLFN5 in non-invasive cancer cell lines (Figs. 2 and 3), we wanted to ascertain whether this pathway existed similarly in the high-invasive 786-0 and HT1080 cell lines. MT1-MMP expression were downregulated at both mRNA and protein levels in SLFN5-pl-transfected 786-0 and HT1080 cell lines (Fig. 5A, B, D for 786-0; Fig. 5E, F, H for HT1080). Co-transfection with MT1-MMP plasmid (MT1-pl) and SLFN5-pl reversed cell migration and invasion abilities, compared with SLFN5-pl transfection alone (Fig. 5I, J). Taken together, these data indicate that overexpression of SLFN5 also inhibits MT1-MMP expression in invasive cancer cell lines. Moreover, low SLFN5 levels and high MT1-MMP levels could lead to greater high migration and invasiveness in highly invasive cancer cells.

2.6. SLFN5 inhibits cancer cell invasiveness in chick embryos in vivo

The above data indicated that SLFN5 play inhibitory regulation of cancer cell invasion *in vitro*, to assess the role of SLFN5 in regulating cancer cell trafficking *in vivo*, we directly monitored the migratory patterns of human cancer cells in 11-day old chick embryos. In this model, cancer cells are fluorescently labelled (green) and cultured atop the chick chorioallantoic membrane (CAM; Fig. 6). After 2 days' culture, HT1080 transfected with vector control plasmid (Ctro-pl) rapidly crossed the CAM surface and penetrated the underlying tissues (Fig. 6A). By contrast, HT1080 transfected with SLFN5 plasmid (SLFN5-pl) were almost static and remained on the surface of CAM, with minimal invasive cell numbers or invasion depth (Fig. 6A); 786-0 cancer cells transfected with Ctro-pl or SLFN5-pl demonstrated similar invasion patterns (Fig. 6B). However, SLFN5-KD A549 and HCT116 cells both demonstrated clearly invasive activity, by crossing the chick CAM surface and invading into the underlying stromal tissues, with invasive cells accounting for 90% of total cells and deeper invasive activities, compared with the no or extremely weak invasiveness of Ctro-Si A549 or HCT116 cells (Fig. 6C, D). Together, these data indicate that SLFN5 inhibits cancer cell invasiveness *in vivo*.

3. Discussion

When triggered by IFNα stimulation, SLFN5 inhibits invasiveness in melanoma and RCC cells. To examine whether SLFN5 can inhibit cancer in a wider variety of cancers under no-stimulation conditions, we undertook the present study, using five cell lines, MCF7, A549, HCT116, HT1080, and 786-0, which are commonly used to study breast cancer, lung cancer, colorectal cancer, fibrosarcoma and RCC, respectively.

Interestingly, of these five cell lines, the three considered to be non- or low-invasive (MCF7, A549 and HCT116) express high levels of SLFN5, whereas the other two considered to be high-invasive cell lines (HT1080 and 786-0) have relatively low SLFN5 expression. This expression patterns imply that SLFN5 expression is inversely associated with invasiveness.

Furthermore, targeting SLFN5 expression led to greatly enhanced migration and invasion in non- or low-invasive MCF7, A549 and HCT116 cell lines; whereas introduction of SLFN5 plasmids into HT1080 and 786-0 cell lines inhibited their migration and invasion capability. Collectively, these findings demonstrate that SLFN5 has a critical function in maintaining cells in a non-invasive state. Moreover, its introduction can reverse invasiveness, which suggests it could provide the basis of a potential drug development program. In the present study, we used 3 non- or low-invasive cell lines, and 2 high-invasive cell lines; future studies would require more cell lines and clinical data to further illuminate its effect on cancer invasion and metastasis. Our findings are consistent with the inhibitory roles of SLFN5 in the invasiveness of malignant melanoma cells and RCC Cells [8,9]. However, there is an opposing report that it appears that human SLFN5 may have promoting roles in invasiveness of glioblastoma, resulted from the different transcription factors and genes regulated by SLFN5. In the case of glioblastoma, SLFN5 expression positively correlates with the malignant phenotype as a negative regulator of STAT1-dependent transcriptional activation of interferon-stimulated genes as reported by Arslan et al. [38].

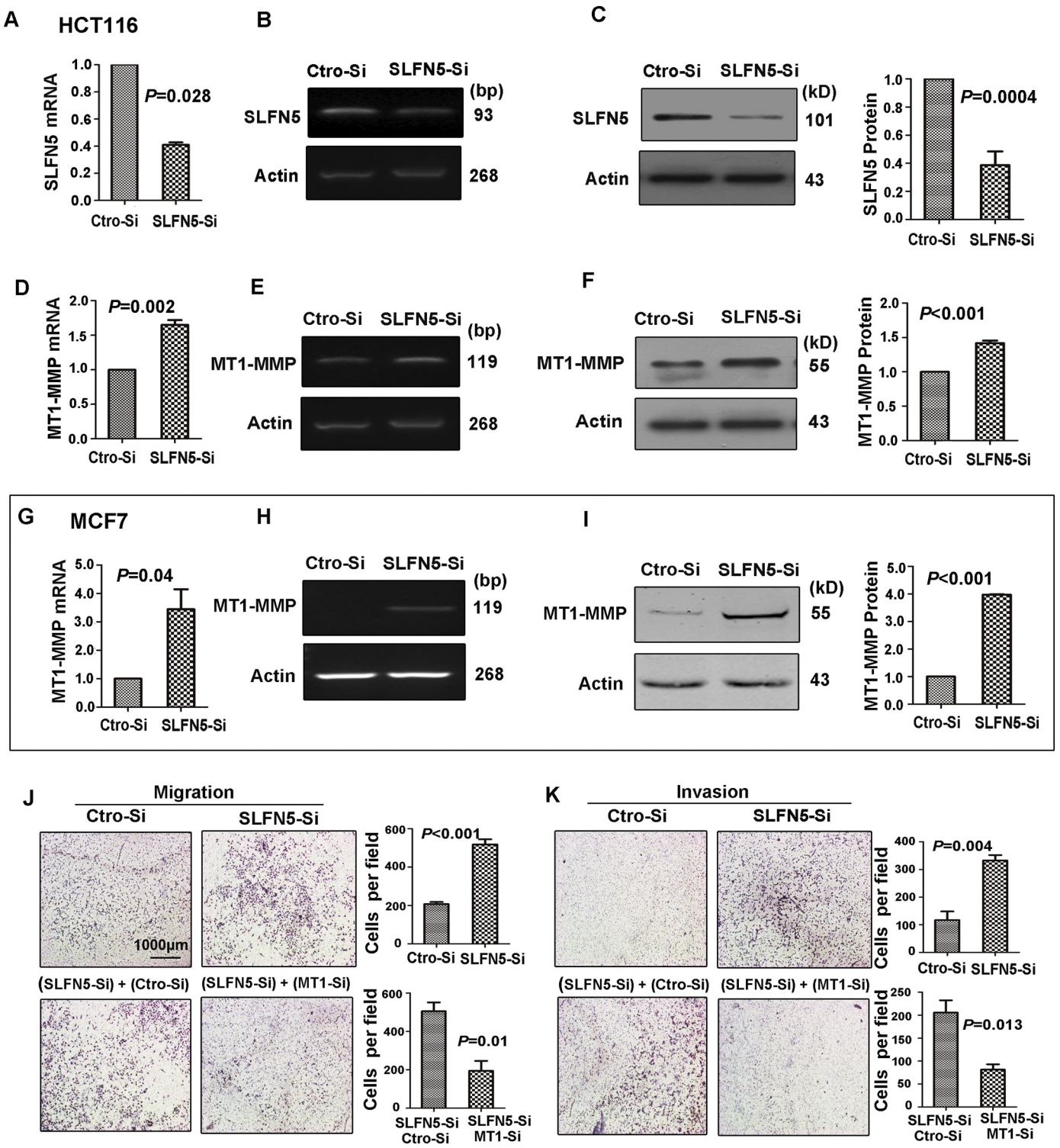

Fig. 3. Influence of *SLFN5* knockdown (KD) on MT1-MMP and migration and invasion of HCT116 and MCF7 cells.
(A–C) Real-time PCR, RT-PCR and western blotting analyses of SLFN5 siRNA transfection efficiency. (D–F) Real-time PCR, RT-PCR and western blotting analyses of MT1-MMP expression in *SLFN5*-KD HCT116 cells. (G–I) mRNA and Protein of MT1-MMP expression in *SLFN5*-KD MCF7 cells. (J and K) Migration and invasion analyses of HCT116 cells transfected with Ctro-Si, or SLFN5-Si, or SLFN5-Si combined with Ctro-Si, or SLFN5-Si combined with MT1-Si (scale bars, 1000 μm).

During metastasis, cancer cells must drive proteolytic enzymes to degrade extracellular matrix, basement membrane and surrounding matrix, to traverse these barriers and escape to remote organs [24]. Both exogenous stimulatory factors (such as growth factor PDGF-BB, cytokine IL-6 and Wnt) and endogenous factors (such as mutations in invasiveness-related genes) can influence invasiveness [32–34,39–42]. Here, we show that SLFN5, as an endogenous nuclear transcriptional factor, inhibits invasiveness.

We have also determined that the terminal enzyme for migration and invasion in executing SLFN5 inhibitory function is MT1-MMP, based on our findings: (a) MT1-MMP expression, both mRNA and protein, were affected in opposing directions, by SLFN5 KD and

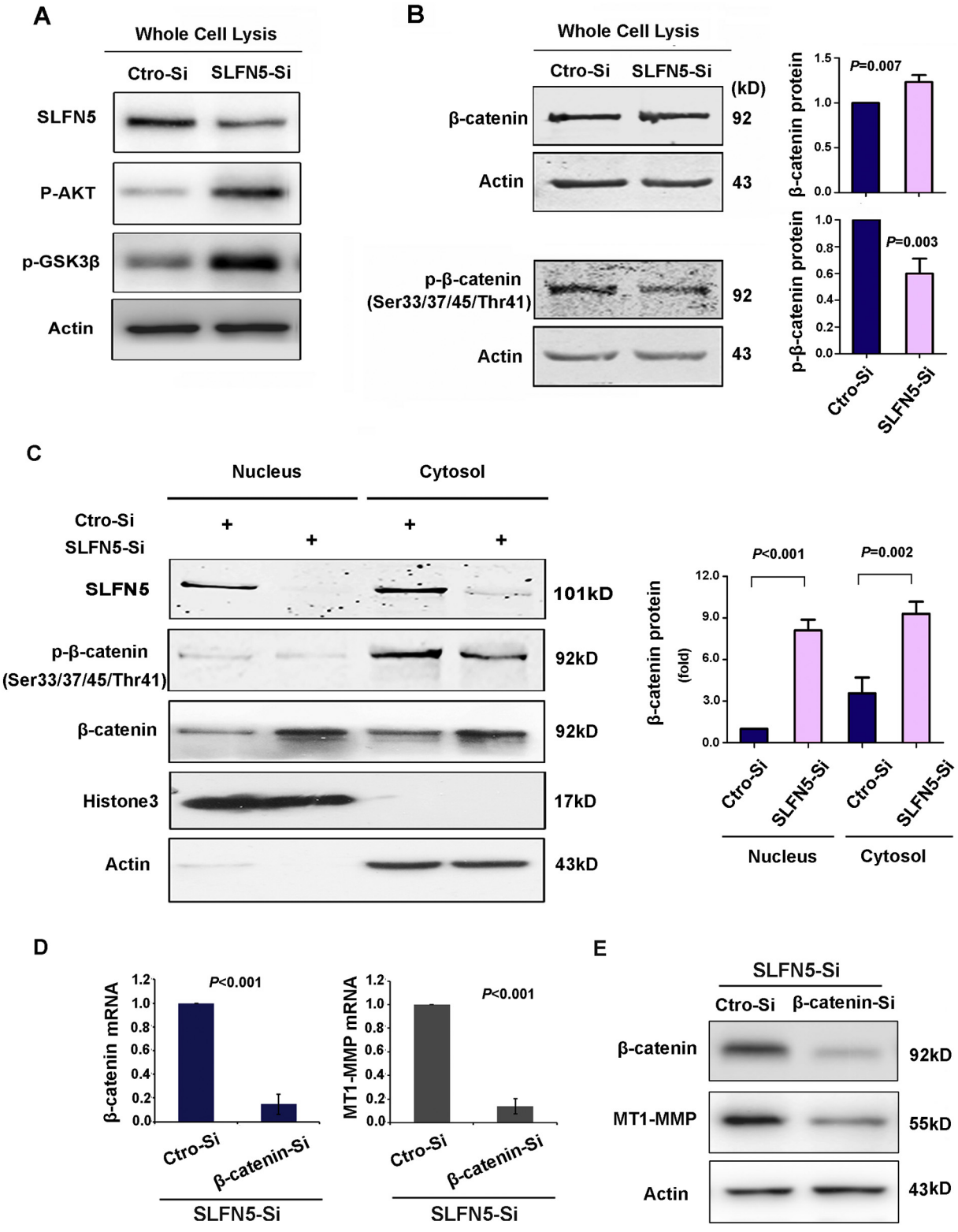

Fig. 4. Knockdown *SLFN5* activates β-catenin to translocate into nucleus and regulates MT1-MMP expression in A549 cells.
(A) Western blotting analysis of total β-catenin expression in whole-cell lysates of SLFN5-Si- or Ctro-Si-transfected cells. (B) Phosphorylation antibodies were used to detect β-catenin phosphorylation at amino acid sites Ser33, 37 and 45 and Thr41 by western blot. (C) Nucleus or cytosol fractions of cells transfected with SLFN5-Si or Ctro-Si were used to detect total and phosphorylated β-catenin at Ser33, 37 and 45 and Thr41 (nucleus loading control: histone 3; cytosol loading control: actin). (D and E) Real-time PCR and western blotting analyses of β-catenin knockdown effect on MT1-MMP expression in SLFN5-Si cells.

overexpression; (b) whereas SLFN5 KD increased migration and invasiveness in non- or low-invasive cancer cell lines, MT1-MMP siRNA transfected into SLFN5-KD cells cancelled this effect; (c) and *vice versa*, MT1-MMP plasmid transfection reversed SLFN5 overexpression resultant inhibition on migration and invasion in high-invasive cancer cells. Together these evidences, we conclude that SLFN5 plays

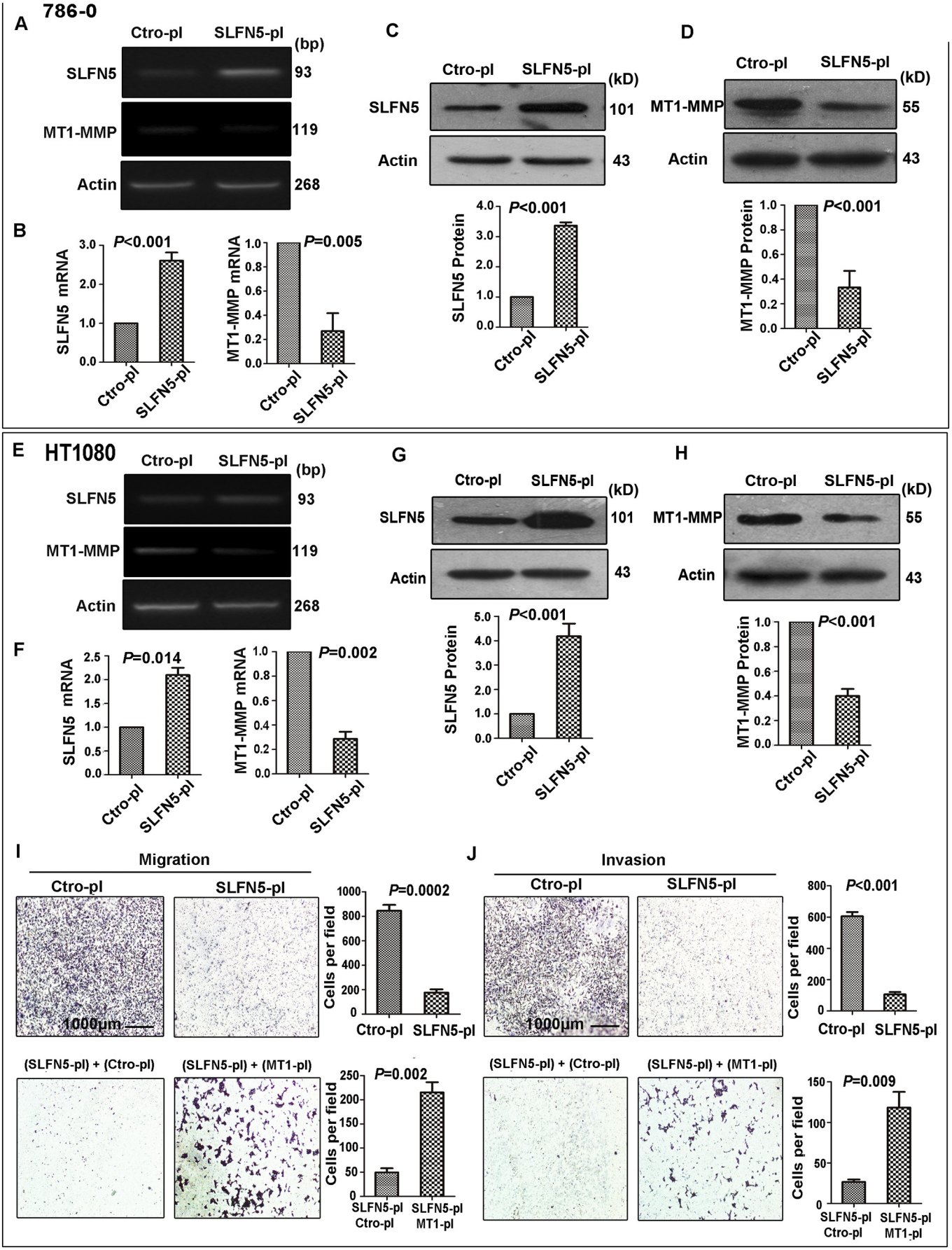

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Fig. 5. Overexpression of SLFN5 affects MT1-MMP expression, and migration and invasion of renal cancer 786-0 cells and fibrosarcoma HT1080 cells.
(A–D). RT-PCR, real-time PCR and western blot analyses of SLFN5 and MT1-MMP expression in 786-0 cells transfected with SLFN5 plasmid (SLFN5-pl) or control plasmid (Ctro-pl). (E–H). Detection of SLFN5 and MT1-MMP expression in HT1080 cells transfected with SLFN5-pl or control plasmid Ctro-pl. (I and J) Migration and invasion analyses of HT1080 cells transfected with Ctro-pl or SLFN5-pl, or SLFN5-pl combined with Ctro-pl, or SLFN5-pl combined with MT1-pl (scale bars: 1000 μm).

inhibitory functions on cancer cell invasion through controlling MT1-MMP expression.

As to how MT1-MMP expression is regulated by SLFN5, we found that the β-catenin pathway mediates SLFN5 regulation of MT1-MMP expression. The β-catenin pathway is a canonical Wnt pathway that affects cancer proliferation and metastasis [40,42–44]. We examined several molecules expression related to the Wnt/β-catenin pathway, including DKK-1, APC and Axin, but none of them showed detectably changes in SLFN5-KD or -overexpressing cells (data not shown). However, we found AKT phosphorylation was enhanced significantly in SLFN5-KD cells. Further, as a direct substrate of phosphorylated AKT, GSK-3β was phosphorylated in SLFN5-KD cells. WB analysis of cellular fractional lysates clearly showed β-catenin to translocate into the cellular nucleus from its original cytosol site. β-catenin is phosphorylated at serine residues 33, 37 and 45 by GSK-3β in a GSK-3β/Axin/APC complex, and at a threonine residue 41 by CK1, in the absence of Wnt stimulation (off-state). Phosphorylation of β-catenin at these sites can lead to ubiquitination and degradation [45,46]. While stimulated by Wnt, GSK-3β is inactive, and β-catenin is therefore un-phosphorylated and stabilized; as a result, it is translocated into the nucleus *via* Rac1 and other factors, whereby it cooperates with transcription factors to bind the promoter sequences of genes related to the Wnt/β-catenin pathway, thus regulating their expressions (“on” state). Hence, β-catenin nuclear translocation is a key event in WNT/β-catenin signalling activation. In the present study, we found β-catenin was un-phosphorylated and translocated into the nucleus in SLFN5-KD cells, which indicates that the β-catenin signalling pathway is activated under the SLFN5-KD condition. MT1-MMP expressions were inhibited at both mRNA and protein levels after β-catenin was targeted with specific siRNAs in SLFN5-KD cells, suggesting that β-catenin is the pathway for SLFN5 to regulate MT1-MMP in cancer cells. Therefore, SLFN5 controls the β-catenin pathway in an “off” state wherein MT1-MMP expression is low or undetectable, in high SLFN5-expressing cancer cells, ultimately leading to little or no invasive capability.

Taken together, we found SLFN5 inhibits cancer cell invasiveness by repressing MT1-MMP expression through the β-catenin pathway, in several cancer cell lines. This suggests a target for drug development, with the potential to treat a wide range of malignancies.

4. Materials and methods

4.1. Cell culture

The human breast cancer cell line MCF7, lung cancer cell line A549, colorectal cancer cell line HCT116, fibrosarcoma cell line HT1080, and RCC cell line 786-0 were all purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The 786-0 cells were cultured in RPMI-1640 medium supplemented with 10% foetal bovine serum (Gibco, Paisley, Scotland), 100 U/mL penicillin, 100 mg/mL streptomycin (HyClone) and 2 mM L-glutamine (HyClone) cultured in an incubator containing 5% CO_2. The other cell lines were maintained in high-glucose Dulbecco's modified Eagle's medium (HyClone) supplemented with 10% foetal bovine serum and antibiotics.

4.2. RT-PCR and quantitative real-time PCR

Total RNA was isolated and reverse-transcribed to cDNA as previously reported [47]. We performed qPCR using SYBR Green qPCR master mix (Applied Biosystems). We quantified mRNA levels of *SLFN5* and *MT1-MMP* as the fold change compared with control samples, with β-actin as internal standard. Primers used for both RT-PCR and real-time PCR were: *SLFN5* (NM_144975.3), forward 5′-CATCCGACGCATCACCGATCTG-3′ and reverse 5′-CATCCGACGCATCACCGATCTG-3′ (amplified product length: 93 bp); *MT1-MMP* (NM_004995.3), forward 5′-GTGGATGGACACGGAGAAT-3′ and reverse 5′-GGGAGGTAGTCCTGGGTTG-3′ (product: 119 bp); *β-catenin* (NM_001904), forward 5′-CCAAGTGGGTGGTATAGAGG-3′ and reverse 5′-AGTCCATAGTGAAGGCGAAC-3′ (product: 156 bp); *β-actin*, forward 5′-TACCTCATGAAGATCCTCACC and reverse 5′-TTTCGTGGATGCCACAGGAC-3′ (product: 268 bp). Primers used in this study were synthesized by Sangon Biotechnology (Shanghai, P.R.C). RT-PCR was used to verify the sizes of amplified fragments.

4.3. Construction of SLFN5 plasmid and transient transfection

Homo SLFN5 (NM_144975.3) open reading frame full length (2676 nt) was amplified by PCR and ligated into the GV219 vector *via* the *Xho*I/*Kpn*I restriction enzyme recognition site. Recombinant plasmids were screened by PCR and sequencing. We transfected 1 μg each of recombinant plasmids into 786-0 or HT1080 cells using Lipofectamine 2000 Transfection Reagent according to the manufacturer's instructions (Cat#11668-027, Invitrogen, California, USA), with the GV219 empty vector used as transfection control. SLFN5 recombinant expression efficiency was assayed using RT-PCR and real-time PCR at 24 h after transfection, and WB at 48 h after transfection.

4.4. RNA interference KD of SLFN5, MT1-MMP and β-catenin

Transient knockdown (KD) of homo *SLFN5* (NM_144975.3), matrix metallopeptidase 14 (*MT1-MMP*, NM_004995.3), and *β-catenin* (NM_001904) was performed using specific small interfering RNAs (siRNAs) and negative control siRNA purchased from GenePharma (Shanghai, P.R.C), using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. The siRNAs were used as follows:

Homo *SLFN5* siRNA 1 target sequence: sense, 5′-GACUCAGACUCCAACGAAUTT-3′,

antisense, 5′-AUUCGUUGGAGUCUGAGUCTT-3′;

SLFN5 siRNA 2: sense, 5′-GUGGUAUAUACUCCAGAAATT-3′,

antisense, 5′-UUUCUGGAGUAUAUACCACTT-3′;

SLFN5 siRNA: sense, 5′-CCGGCUAACAUUCUCUACATT-3′,

antisense, 5′-UGUAGAGAAUGUUAGCCGGTT-3′.

Homo *MT1-MMP* siRNA: sense, 5′-AACAGGCAAAGCUGAUGCAGATT-3′,

antisense, 5′-UCUGCAUCAGCUUUGCCUGUUTT-3′.

homo *β-catenin* siRNA: sense, 5′-AGGCUAGCUGUUGGUUGAUUTT-3′,

antisense, 5′-AAAUCAAUCCAACAGCUAGCCUTT-3′.

Scrambled siRNA was used as negative control. Knockdown efficiency was monitored by real-time PCR, RT-PCR, and WB; cells thus assessed were used thereafter.

4.5. Western blotting

Western blotting was performed as described previously. Briefly, cells were lysed in RIPA lysis buffer with 50 mM Tris (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, supplemented with sodium fluoride, EDTA, leupeptin, sodium

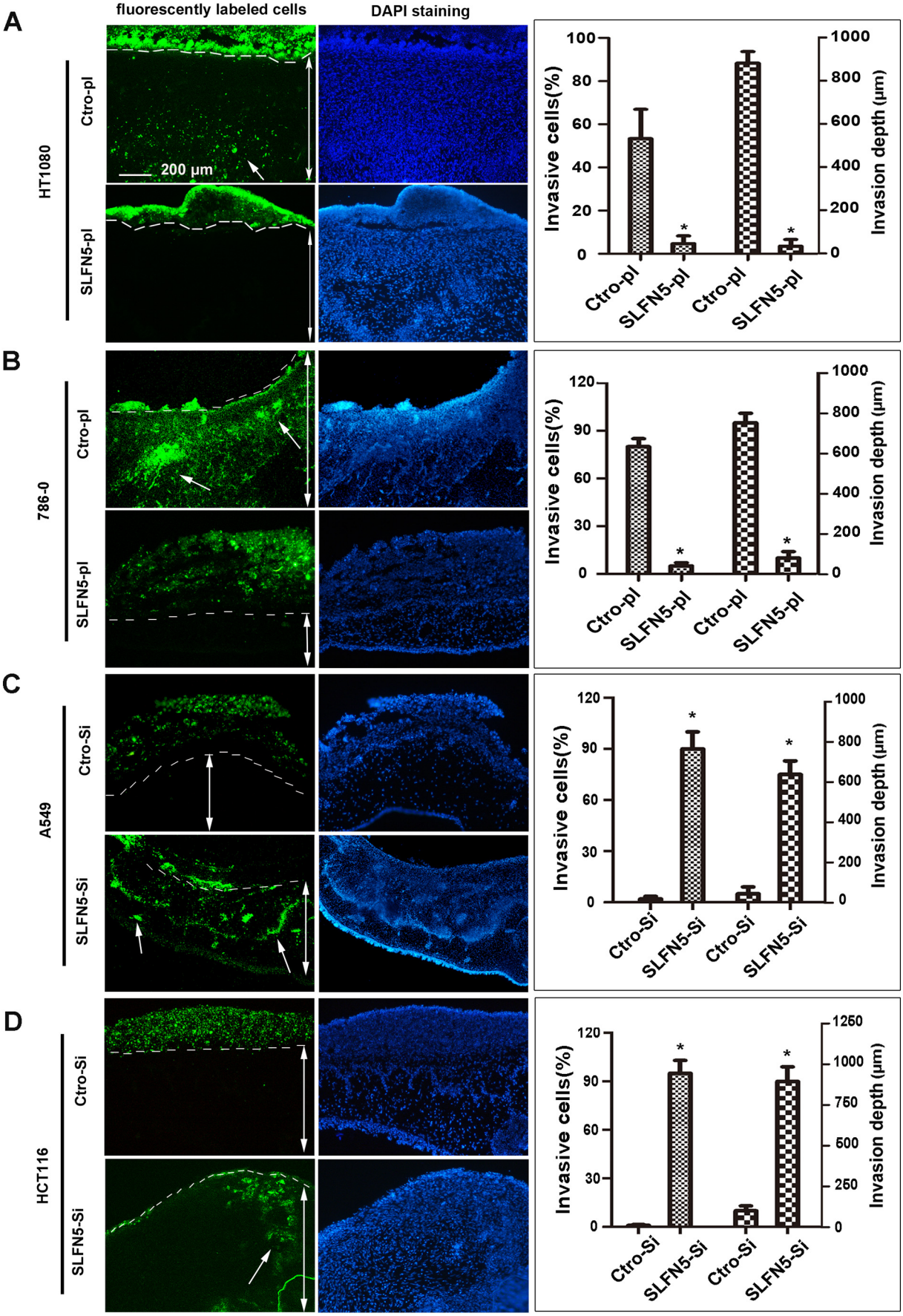

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Fig. 6. SLFN5 inhibits cancer cell invasion in chick embryos *in vivo*.
HT1080 (A) and 786-0 (B) cells transfected with Ctro-pl or SLFN5-pl, and A549 (C) and HCT116 (D) transfected with Ctro-Si or SLFN5-Si, were labelled with green fluorescent nanobeads and seeded on the chorioallantoic membrane (CAM) of 11-day-old chick embryos for 2 days. Frozen sections were stained with DAPI for nuclei. Right: quantification of invasive cells (%) and invasion depth (μm). White dashed lines: location of CAM surface. White arrow: cells invading CAM stromal tissues. Double-headed arrow: depth underneath CAM (scale bar: 200 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

orthovanadate and PMSF from Beyotime Technology (Shanghai, P.R.C.) on ice. Where indicated, nucleus and cytosol proteins were separated using specific isolation kits (Cat #P2007, Beyotime Biotechnology). Proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes (Millipore) followed by primary and secondary antibody incubations. Antibodies used were anti-SLFN5 rabbit antibody (Cat #HAP017760, Sigma-Aldrich, 1:1000), anti-MMP14 rabbit polyclonal Antibody (Cat # ab53712, Abcam, 1:1000), histone H3 mouse monoclonal antibody (Cat #AF0009, Beyotime Biotechnology, 1:1000), β-Catenin (D10A8) XP® rabbit monoclonal antibody (Cat #8480, Cell Signalling Technology, 1:1000), phospho-β-catenin (Ser33/37/Thr41 and Thr41/Ser45, Cat #9561 and #9565, respectively, Cell Signalling Technology, both at 1:1000), total and phospho-AKT (Cat # 4691 and 4060, Cell Signalling Technology, 1:1000), total and phospho-GSK-3β (Cat #12456 and 9331, Cell Signalling Technology, 1:1000), rabbit polyclonal antibody (Cat #9561, Cell Signalling Technology, 1:1000), flag mouse monoclonal antibody (Cat #AF519, Beyotime Biotechnology, 1:2000), and HRP-conjugated secondary antibodies. Probed proteins on membranes were visualized by the chemiluminescence method, using enhanced ECL reagent (Amersham Pharmacia, NJ, USA) followed by X-ray film exposure in the dark. Bio-Rad software was used to quantify protein band intensity (mean ± SE). Experiments were performed at least 3 times from cell treatment.

4.6. Immunofluorescence cytochemistry

Cells were seeded on Lab-Tek II Chambered Coverglasses (Thermo Fisher Scientific), cultured in medium overnight and then fixed in 1% polyformaldehyde. After permeabilization with 0.1% Triton X-100 in PBS and blocking with 3% goat serum for 30 min at room temperature, cells were incubated with antibodies against SLFN5 (Cat #HAP017760, Sigma-Aldrich, dilution 1:200) or MMP-14 (Cat # ab53712, Abcam, dilution 1:100) overnight at 4 °C. As secondary antibody, we used Alexa Fluor 594 donkey anti-rabbit lgG (H + L) at dilution 1:200 for 1 h at room temperature. Photographs were taken with a Leica confocal microscope TCS SP5 using 20× or 40× objectives. Laser intensity, magnification, and microscope settings per each channel were maintained equal throughout the different experimental conditions.

4.7. Transwell migration and invasion assay

Migration and invasion experiments were performed using Boyden chambers consisting of Transwell membrane filter inserts (Cat # 3422, Corning Costar). In brief, 5×10^4 cells were seeded into each 24-well Transwell chamber (8 μm pore size) for migration assay, or into chambers coated with Matrigel for the invasion assay, in complete medium with 10% FBS. Migration and invasion cultural periods were 24 h and 48 h, respectively. Cells that did not penetrate the filter were wiped off, and cells on the lower surface of the filter were stained with 0.4% crystal violet. The numbers of migrating or invading cells were counted under a light microscope from five fields in a single chamber of three samples (mean ± SE).

4.8. Chick CAM invasion in vivo

We used 11-day-old chick embryos to assess cancer cell invasion *in vivo*, as described previously [48,49]. Briefly, cancer cells were incubated with Fluoresbrite carboxylate microspheres (Polysciences, Inc., Niles, IL) for 10 min, seeded on CAM, and incubated for 2 days. The CAM was then frozen-sectioned, stained with DAPI for cell nuclei, or stained with H&E. Fluorescent images were captured using a fluorescence microscope. Invading cells were quantified in at least five randomly selected fields of three samples (mean ± SE). The invasion depth from the top of each CAM was defined as the leading front in randomly selected fields, measured in at least five randomly selected fields of three samples (mean ± SE).

Acknowledgment

This work was supported by the National Natural Science Foundation of China (81272376 and 81772829), the Special Program for Collaborative Innovation, and Shanghai Municipal Education Commission (Class II Plateau Disciplinary Construction Program for Medical Technology of SUMHS, 2018–2020).

Conflicts of interest

The authors declare no conflicts of interest.

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