PTEN Insufficiency Increases Breast Cancer Cell Metastasis *In Vitro* and *In Vivo* in a Xenograft Zebrafish Model

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Abstract. *Background/Aim: Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) insufficiency is commonly found in breast cancer patients with metastasis. We investigated the mechanisms by which PTEN affects breast cancer metastatic behavior. Materials and Methods: Migration and invasion assay, western blot, immunofluorescent staining and zebrafish animal model were applied. Results: We showed that PTEN insufficiency induced an increase in MCF-7 cell migration and invasion through induction of epithelial-mesenchymal transition (EMT), which was triggered by up-regulation of the EMT-inducing transcriptional factors Zeb1, Zeb2, Snail, Slug and Twist. Simultaneously, E-cadherin expression was inhibited and P-cadherin was up-regulated. Further, WNT1 inducible signaling pathway protein 1 (WISP1) and lipocalin-2 (LCN2) expressions were increased after PTEN knockdown in MCF-7 cells, which also exhibited increased filamentous actin (F-actin) synthesis and extracellular matrix metalloproteinase-2 (MMP-2) and MMP-9 expression. We further showed that PTEN knockdown in MCF-7 cells could increase cell migration in the xenograft zebrafish model. Conclusion: Our findings reveal new therapeutic targets for breast cancer patients with PTEN insufficiency.*

Breast cancer, the second most diagnosed malignancy in women, is found in approximately one of every eight women (1, 2). Although cytotoxic chemotherapies and target therapies for breast cancer have seen great improvements in the past decades, breast metastasis still eventually develops in 25-50% of breast cancer patients (3), which results in a 5-year survival rate of less than 25% (4, 5). Thus, a deeper understanding of the mechanisms that trigger breast cancer metastasis is urgently needed to improve the survival of breast cancer patients.

Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), located on chromosome 10q23, was first identified in 1997 and has been widely deemed as a tumor suppressor gene (6, 7). The PI3K/Akt/mTOR signal pathway is crucial for breast cancer cell proliferation (8) and PTEN expression is negatively associated with PI3K/Akt/mTOR pathway activity. Thus, it is not surprising that PTEN insufficiency is associated with poor prognosis in breast cancer patients (9, 10). About 25% of breast cancer cases have been shown to have reduced PTEN expression (11-13). Wikman *et al*. further showed that PTEN insufficiency is associated with brain metastasis in breast cancer patients (14). Taken together, these data indicate that reduced PTEN

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expression is a common phenomenon in breast cancer and appears to be associated with metastasis and poor prognosis.

Although the role of *PTEN* as a tumor suppressor gene is well known, the details of the mechanism by which *PTEN* affects breast cancer metastasis is not well-understood so far. In this study, we aimed to investigate how PTEN insufficiency influences breast cancer cell metastasis by knocking down *PTEN* in MCF-7 cells. The underlying mechanisms by which PTEN modulates MCF-7 invasiveness, in addition to cell migration and invasion, were also studied. The *in vivo* metastatic potential of *PTEN* knockdown MCF-7 cells was evaluated using a xenograft zebrafish model. Through our current work, we aimed to identify new therapeutic targets to overcome PTEN insufficiency in breast cancer patients to prevent cancer cell metastasis.

Materials and Methods

Cell culture. The human breast carcinoma cell line, MCF-7, was purchased from BCRC (Hsinchu, Taiwan). MCF-7-COLsi cells (*PTEN* mock knockdown MCF-7 cells) and MCF-7-PTENsi (*PTEN* knockdown MCF-7 cells) cells were grown in DMEM (Gibco, Waltham, MA, USA) supplemented with 5% fetal bovine serum (FBS). The culture medium was changed 3 times per week.

PTEN knockdown. MCF-7 cells were transducted using shRNA control transduction particles (H1(shRNA-Ctr)-GP; GenTarget Inc., San Diego, CA, USA) or PTEN shRNA lentiviral transduction particles (GenTarget Inc.) according to the manufacturer's protocol. The detailed procedure was as previously described (15). MCF-7-PTENsi cells and MCF-7-COLsi cells were generated as described previously (15).

Matrigel invasion assay. The matrigel invasion assay was conducted as previously described (16). Cells invading to the opposite side of the matrigel-coated membrane were fixed with 4% paraformaldehyde in 1× PBS (pH 7.5). The invading cells were stained, digitally photographed and counted under a microscope (IX71; Olympus, Tokyo, Japan). Experiments were performed in triplicates and repeated at least three times.

Trans-well filter migration assay. The MCF-7-COLsi and MCF-7-PTENsi cells were seeded on each trans-well filter with 8.0 μm pores (Costar, Cambridge, MA, USA). The procedure was conducted as previously described (17). The migrating cells on the lower surface of the filter were stained and counted under four random high-power microscopic fields (HPF; 100X) per filter. The experiments were performed in triplicates.

Western blot. Western blot was conducted as described previously (18). The primary antibodies used in this study were monoclonal antibodies against E-cadherin (1:1,000, #3195; Cell Signaling Technology, Irvine, CA, USA), P-cadherin (1:1,000, #2189; Cell Signaling Technology), Zeb1 (1:500, TA802313; OriGene Technologies, Inc, Rockville, MD, USA), Zeb 2 (1:500, TA802113; OriGene Technologies), Snail (1:100, PA5-23472; Thermo Fisher Scientific, Waltham, MA, USA), Slug (1:1,000, #9585; Cell Signaling Technology), Twist (1:100, sc-15393; Santa Cruz Biotechnology, Dallas, TX, USA), fibronectin (1:1,000, MAB1918; R&D Systems, Minneapolis, MN, USA), WNT1 inducible signaling pathway protein 1 (WISP1) (1:300, PA5-29150; Thermo Fisher Scientific) and lipocalin-2 (LCN2) (1:500, PAB9543; Abnova, Taipei, Taiwan). The secondary antibodies (1:5,000) were anti-rabbit (111-035-003; Jackson Immunoresearch, West Grove, PA, USA) or anti-mouse secondary antibodies (Zymed 81-6520; Zymed, Waltham, MA, USA). The blots were visualized using enhanced chemiluminescence (ECL) reagents (WBKLS0500; Millipore, Billerica, MA, USA). Membranes were visualized using the VersaDoc™ Imaging System (Bio-Rad, Hercules, CA, USA) for analysis.

Filamentous actin (F-actin) staining. The MCF-7-COLsi and MCF-7-PTENsi cells were seeded on glass coverslips in a culture dish and allowed to attach overnight. The cells were then fixed with 4% paraformaldehyde in 1× PBS (pH 7.5) at room temperature. The F-actin protein was fluorescently stained by incubation with fluorescein isothiocyanate (FITC)-conjugated phalloidin and then examined using a confocal microscope (LSM510 Meta; Zeiss, Oberkochen, Germany).

Zebrafish xenotransplantation assay. All the experiments were performed in strict accordance with the standard guidelines for zebrafish work and approved by the Institutional Animal Care and Use Committee of Chang Gung University (IACUC approval number: 20150931). For zebrafish xenotransplantation, a wild-type AB strain of zebrafish embryos was maintained in 0.3% phenylthiourea ((PTU), Sigma-Aldrich) 24 h post-fertilization (hpf). The chorions were removed at 48 h and anesthetized in 0.04 mg/ml tricaine (Sigma-Aldrich) before tumor cell injection. Subconfluent cultured breast cancer cells were harvested, washed in Hank's balanced salt solution (HBSS) and labeled with a green fluorescent tracer, 5(6)-Carboxyfluorescein diacetate N-hydroxysuccinimidyl ester (CFSE), according to the manufacturer's instructions (Abcam, Cambridge, MA, USA). After CFSE labeling, the cells were washed, centrifuged and re-suspended in HBSS. Thereafter, approximately 50 CFSE-labeled cells were injected into the hindbrain ventricle of each embryo. After confirmation of a proper visible tumor cell mass at the injection site, the zebrafish embryos were transferred individually into a 24-well dish and maintained at 35˚C. Later, individual recipient embryos were imaged at the different time points as indicated in the Figures' legends for tumor cell metastasis under a fluorescence microscope (Leica).

Statistics. The data from each group were compared using the Student's *t*-test. *p*-Values <0.05 were considered to indicate a significant difference. Excel 2010 was used to conduct the statistical analyses.

Results

Effect of PTEN insufficiency on the metastatic potential of the MCF-7 cells. To investigate the effect of *PTEN* knockdown on the metastatic potential of the MCF-7 cells, we compared the migration and invasion of the MCF-7-COLsi cells to those of the MCF-7-PTENsi cells. As shown in Figure 1A, the MCF-7-PTENsi cells showed 165±4% migration and 195±10% invasion (Figure 1B). Based on our

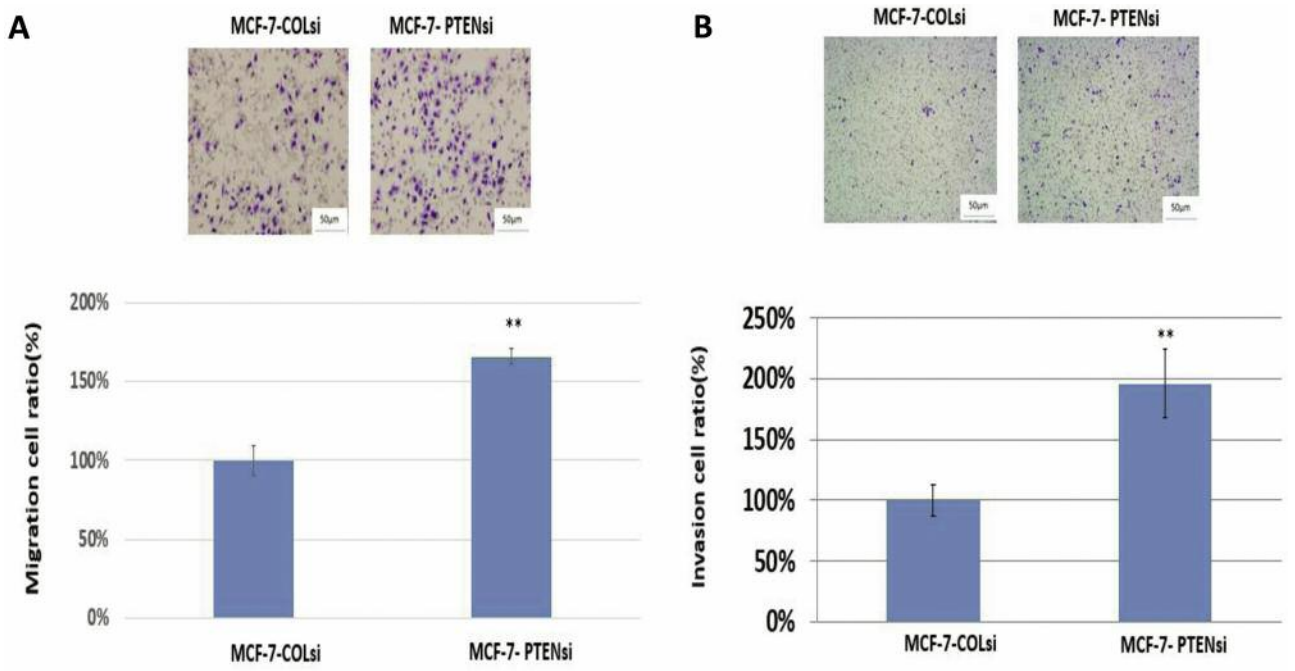

Figure 1. *The effect of PTEN insufficiency on MCF-7 cell migration and invasion. (A) The photograph shows the migrating MCF-7-COLsi and MCF-7-PTENsi cells after 16 hours of migration time (upper panel) and the quantitative result of the migration study (lower panel). MCF-7-PTENsi cells have an obvious more migration ability as compared to MCF-7-COLsi cells. Migrating cells through filters were counted under a IX71 microscope (Olympus, Tokyo, Japan). Experiments were performed in triplicate and repeated at least three times. (**p<0.01). (B) The photograph shows the invading MCF-7-COLsi and MCF-7-PTENsi cells after 48 hours of invasion time (upper panel) and the quantitative result of the invasion study (lower panel). PTEN knockdown in MCF-7 cells increased the invasion ability. Invading cells through filters were counted under a IX71 microscope (Olympus, Tokyo, Japan). Experiments were performed in triplicate and repeated at least three times. (**p<0.01).*

data, we concluded that PTEN insufficiency in MCF-7 cells could increase cell migration and invasion, thus increasing metastasis.

Effect of PTEN insufficiency on E-cadherin and P-cadherin expression in the MCF-7 cells. Two cadherins, E- and P-cadherin, are closely associated with breast cancer cell metastasis. Therefore, we evaluated the expression of E-cadherin and P-cadherin in the MCF-7-PTENsi and MCF-7-COLsi cells using western blot. As shown in Figure 2, PTEN insufficiency decreased E-cadherin expression 0.76±0.08-fold in the MCF-7 cells but increased P-cadherin expression to 1.3±0.09-fold, compared to the control.

Effect of PTEN insufficiency on the expression of EMT-related transcriptional factors, Zeb-1, Zeb-2, Snail, Slug and Twist, in the MCF-7 cells. Since EMT is a vital process for tumor metastasis, we evaluated the transcriptional factors responsible for EMT in MCF-7 cells after *PTEN* knockdown. Figure 3A shows that the expression of Zeb-1 and Zeb-2 was up-regulated to 1.32±0.1- and 1.3±0.11-fold in the MCF-7-PTENsi cells, as compared to the control. The expression of Snail, Slug and Twist in the MCF-7 cells after *PTEN* knockdown increased 1.32±0.09-, 1.5±0.1- and 1.7±0.09-fold, respectively, compared to the control (Figure 3B). Our results suggest that PTEN insufficiency stimulated the EMT process in the MCF-7 cells through up-regulation of Zeb-1, Zeb-2, Snail, Slug and Twist.

Effect of PTEN insufficiency on the expression of the mesenchymal cell marker fibronectin in the MCF-7 cells. Since PTEN insufficiency could induce an increase in the expression of EMT-promoting transcriptional factors, we then investigated the expression of the mesenchymal cell marker fibronectin. Figure 3C shows that the MCF-7-PTENsi cells had 2.45±0.12-fold higher fibronectin expression as compared to expression in the MCF-7-COLsi cells, indicating that PTEN insufficiency did induce EMT in the MCF-7 cells.

Effect of PTEN insufficiency on the extra-cellular expression of MMP-2 and MMP-9 in the MCF-7 cells. Since MMP-2 and MMP-9 are two major collagenases to digest extracellular matrix, we then measured extra-cellular expression of MMP-2

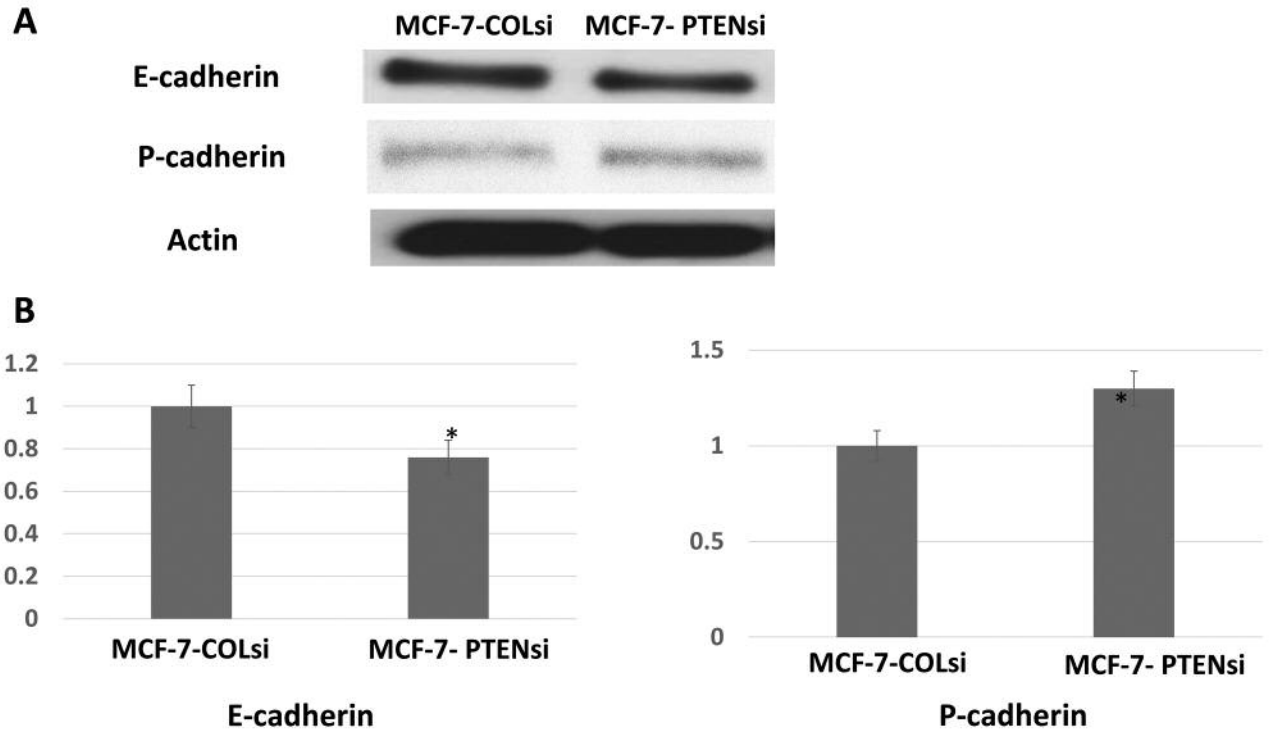

Figure 2. *The effect of PTEN insufficiency on E-cadherin and P-cadherin expression in MCF-7 cells. (A) Western blot showing E-cadherin and P-cadherin expression in MCF-7-COLsi and MCF-7-PTENsi cells. Actin was used as the loading control. (B) Quantitative result of the Western blot. PTEN insufficiency decreased E-cadherin but increase P-cadherin in MCF-7 cells. Each value shows the mean±SD of three independent determinations (*p<0.05).*

and MMP-9 in MCF-7-COLsi and MCF-7-PTENsi cells. As shown in Figures 4A, the extracellular expression of MMP-2 and MMP-9 was markedly increased in the MCF-7-PTENsi cells.

Effect of PTEN insufficiency on the expression of WISP1 and NGAL in the MCF-7 cells. We then evaluated the effect of PTEN insufficiency on the expression of *WSIP1* and *NGAL*, two oncogenes for breast cancer (19-21). Figure 4B shows that WSIP1 and NGAL were up-regulated 1.4±0.1- and 1.45±0.11-fold in MCF-7-PTENsi cells, compared to their expression in the MCF-7-COLsi cells.

Effect PTEN insufficiency on actin synthesis in the MCF-7 cells. The MCF-7-COLsi and MCF-7-PTENsi cells were double stained with FITC-conjugated phalloidin (green) and PI (blue) for F-actin and nuclear fluorescence detection, respectively (Figure 5A). The intensity of F-actin staining was markedly increased in the cytoplasm of the MCF-7-PTENsi cells.

Effect of PTEN insufficiency on the metastatic ability of the MCF-7 cells in vivo. We used the zebrafish model to evaluate the effect of *PTEN* on breast cancer cell migration *in vivo*. The MCF-7 cells with or without transfected *PTEN* siRNA were labeled with a green fluorescent dye, CFSE, and then injected into the hindbrain ventricle of zebrafish embryos at 48 hpf at which time the immune system of the fish is not yet fully developed. The zebrafish were first imaged 8 h after injection (Figure 5B). The *PTEN*-knockdown cells had significantly disseminated away from the primary injection site, whereas most of the control cells remained at the primary site. This result indicates that PTEN insufficiency could increase metastasis *in vivo* in the MCF-7 cells.

Discussion

PTEN, a well-known tumor suppressor gene, is associated with initiation and progression in a variety of cancers. Although the role of *PTEN* in the poor prognosis of breast cancer patients is obvious (10), the detailed mechanisms responsible for the effect of PTEN on breast cancer cell metastasis has not been well studied. Our current work showed that PTEN insufficiency increased MCF-7 cell metastatic potential *in vitro* and *in vivo* through induction of EMT, up-regulation of P-cadherin, WISP1, LCN2 and F-actin synthesis, down-regulation of E-cadherin, as well as through increase in MMP-2 and MMP-9 secretion.

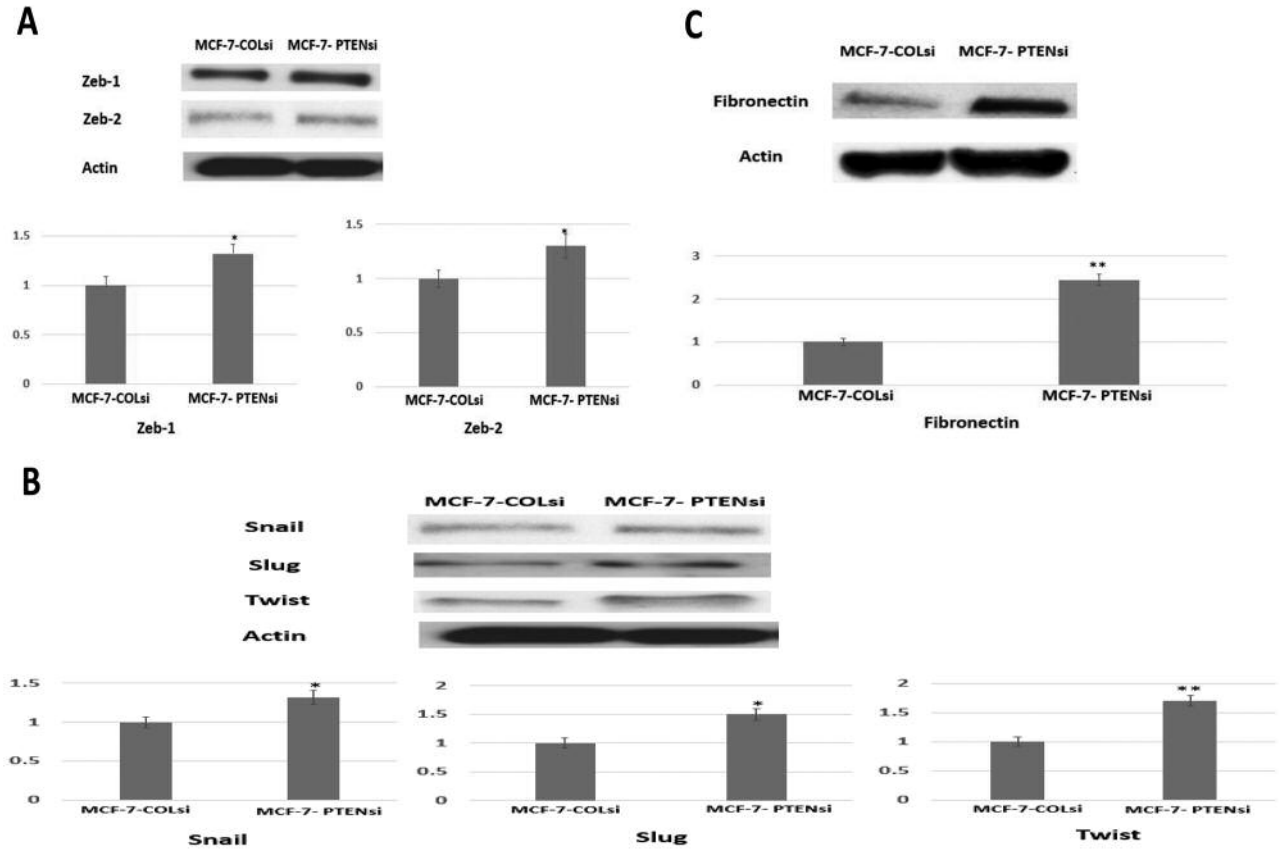

Figure 3. *The effect of PTEN insufficiency on Zeb-1, Zeb-2, Snail, Slug, Twist and fibronectin expression in MCF-7 cells. (A) Western blot showing Zeb-1 and Zeb-2 expression in MCF-7-COLsi and MCF-7-PTENsi cells (upper panel; actin was used as the loading control) and its quantitative result (lower panel). Zeb-1 and Zeb-2 expressions were increased in MCF-7 cells after PTEN knockdown. Each value shows the mean±SD of three independent determinations (*p<0.05). (B) Western blot showing Snail, Slug and Twist expression in MCF-7-COLsi and MCF-7-PTENsi cells (upper panel; actin was used as the loading control) and its quantitative result. PTEN knockdown in MCF-7 cells increased Snail, Slug and Twist expression. Each value shows the mean±SD of three independent determinations (*p<0.05, **p<0.01). (C) Western blot showing fibronectin expression in MCF-7-COLsi and MCF-7-PTENsi cells (upper panel; actin was used as the loading control) and its quantitative result (lower panel). The data indicate that PTEN insufficiency could induce fibronectin expression in MCF-7 cells. Each value shows the mean±SD of three independent determinations (**p<0.01).*

At the start of cancer metastasis, the cancer cells need to detach from the primary tumor to move to other sites. To achieve this, the cell-cell interaction has to be broken. E-cadherin, a member of type-1 classical cadherins, has been found to mediate cell-cell interactions (22). Decreased E-cadherin expression has been linked to a higher incidence of cancer metastasis or recurrence (23, 24). We showed that PTEN insufficiency attenuated E-cadherin expression in MCF-7 cells (Figure 2), thus inducing an increase in the metastatic ability of the MCF-7-PTENsi cells. The function of P-cadherin, another classical cadherin, unlike that of E-cadherin, is still controversial. For malignant melanomas, low P-cadherin expression has been associated with advanced disease (25, 26). With regard to breast cancer, however, P-cadherin is deemed as an oncogene since P-cadherin was able to increase breast cancer cell aggressiveness and was negatively associated with poor prognosis in breast cancer patients (27). Figure 2 shows that P-cadherin expression in MCF-7-PTENsi cells was higher than that in the MCF-7-COLsi cells. Collectively, our results suggest that PTEN insufficiency in breast cancer cells reduced E-cadherin expression and increased P-cadherin expression, thus leading to the higher metastatic potential.

After a cancer cell detaches from the primary site, it needs to achieve motility to move to another site and to penetrate into vessels for dissemination. EMT plays a vital role in this process. The transition of epithelial cells to mesenchymal cells renders the cell motility and induces invasiveness (28, 29). Clinically, the expression of EMT markers has been associated with more advanced disease stages or poor prognosis in colon, breast and prostate cancers (30-33). In normal cells, EMT is well orchestrated and controlled by a

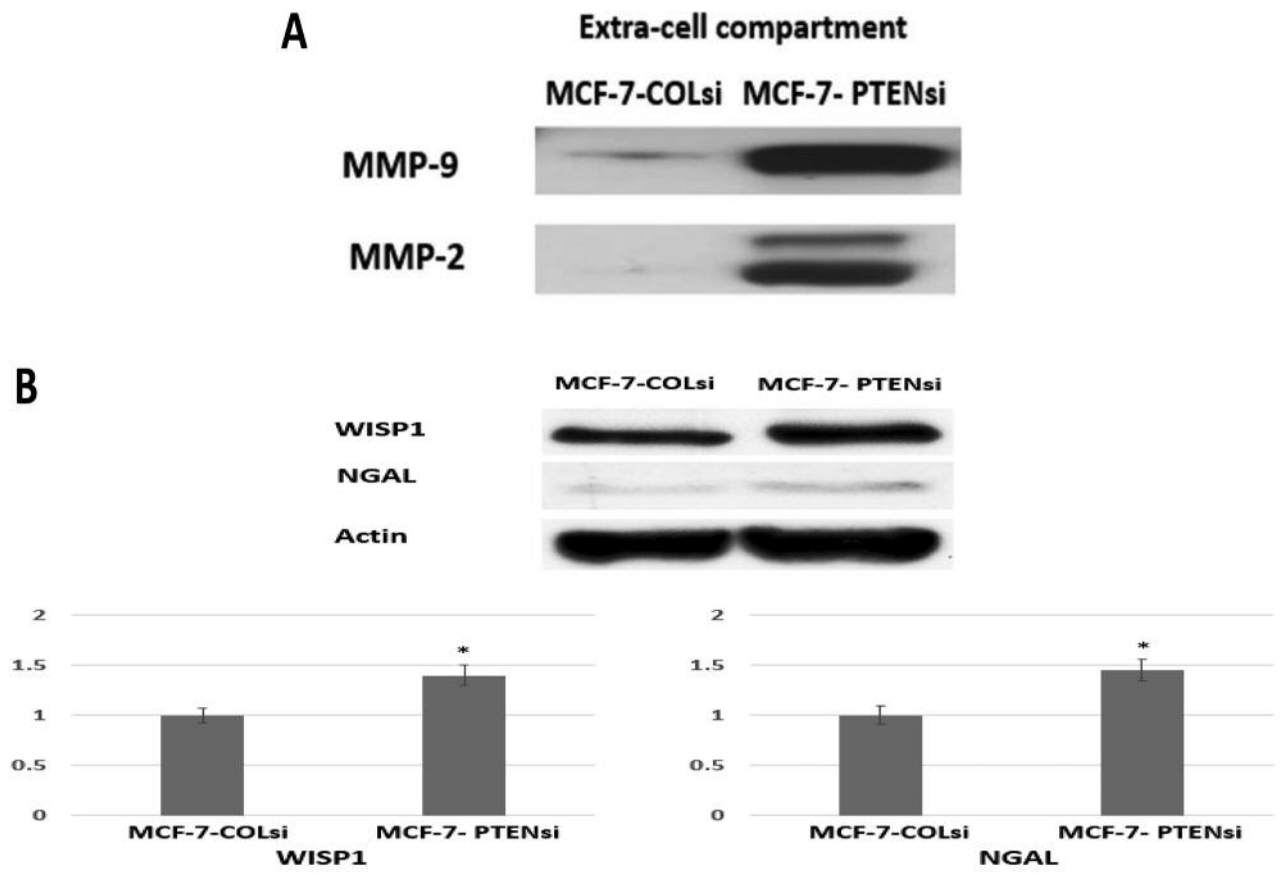

Figure 4. *The effect of PTEN insufficiency on extracellular MMP-2, MMP-9, WISP1 and LCN2 expression in MCF-7 cells. (A) Western blot showing extracellular MMP-2 and MMP-9 expression in MCF-7-COLsi and MCF-7-PTENsi cells. MCF-7-PTENsi cells demonstrate an obvious MMP-2 and MMP-9 expression in the extracellular compartment as comparted to MCF-7 cells. (B) Western blot showing WSIP1 and LCN2 expression in MCF-7-COLsi and MCF-7-PTENsi cells (upper panel; actin was used as the loading control) and its quantitative result. WISP1 and LCN2 expressions were increased in MCF-7 cells after PTEN knockdown. Each value shows the mean±SD of three independent determinations (*p<0.05).*

complex crosstalk between signaling pathways. The regulation of EMT could be translational, translational or post-translational (34). Zeb-1, Zeb-2, Snail, Slug and Twist, among other factors, have been shown to be EMT-inducing transcriptional factors (35). As shown in Figures 3A and 3B, all the aforementioned transcriptional factors were up-regulated by the knockdown of *PTEN* in the MCF-7 cells, indicating that PTEN insufficiency could induce EMT in breast cancer cells. For further verification, we measured the expression of fibronectin, a mesenchymal cell marker (34), in the *PTEN*-knockdown MCF-7 cells. Figure 3C shows that MCF-7-PTENsi cells exhibited higher fibronectin expression as compared to MCF-7-COLsi cells, suggesting that PTEN insufficiency did induce EMT in the MCF-7 cells, which leads to the increased invasiveness *in vitro* and *in vivo*.

Cancer cells need to digest the extracellular matrix and basement membrane before penetrating the vessels after detachment from the primary tumor. Thus, the ability to digest the barriers is proportional to the metastatic potential of cancer cells. MMPs are proteases used to digest the extracellular matrix and basement membrane. MMP-2 and MMP-9 are two of the main proteases. Figure 4A shows that the extracellular expression of MMP-2 and MMP-9 were increased as *PTEN* knockdown in MCF-7 cells, which increased the ability of the cells to digest the extracellular barriers and, thus, increased their invasiveness.

WISP1, a type of secreted protein, belongs to a subset of the extracellular matrix (ECM) and can influence a variety of important cellular processes (36). Previously, we showed that WISP1 plays the role of an oncogene in human breast cancer; overexpression of WISP1 in breast cancer cells induces cell proliferation and EMT, which further increase metastasis (19). In this study, we showed that WISP1 was up-regulated after *PTEN* knockdown in the MCF-7 cells, resulting in an increase in the aggressive behavior of the MCF-7-PTENsi cells (Figure 4B).

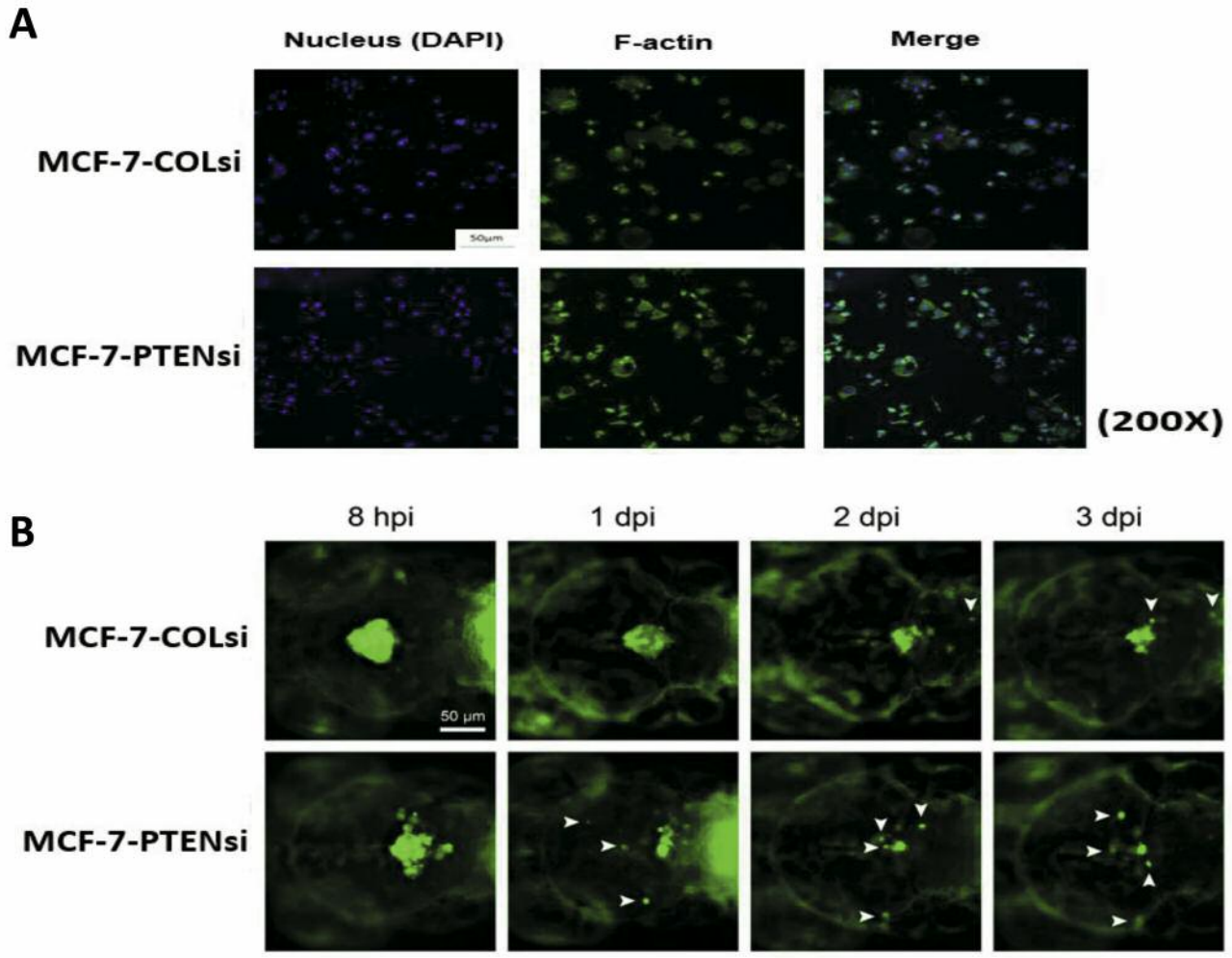

Figure 5. *The effect of PTEN insufficiency on MCF-7 cells' F-actin synthesis and metastasis in the brain of zebrafish embryo. (A) Expression and cellular distribution of F-actin in MCF-7-COLsi and MCF-7-PTENsi cells that were stained with FITC-conjugated phalloidin (green). PI (blue) was applied to counter-stain the nucleus. PTEN knockdown in MCF-7 cells demonstrated an obvious increase of F-actin synthesis. (B) MCF-7-COLsi and MCF-7-PTENsi cells were labeled with the green fluorescent dye CFSE and injected in the hindbrain ventricle of zebrafish embryos (approximately 50 cells per embryo) at 48 h post-fertilization. Arrowheads indicate metastatic cells. MCF-7-PTENsi cells showed higher metastatic ability as compared to MCF-7-COLsi cells.*

LCN2 is a type of secreted glycoprotein whose expression is elevated in human breast cancer. It has been shown that inhibition of LCN 2 expression can reduce breast cancer tumorigenesis and metastasis and high LCN2 expression is associated with poor prognosis in breast cancer patients (20, 21). Taken together, these data indicate that LCN2 plays the role of an oncogene in human breast cancer. Figure 4B shows that higher LCN2 expression was observed in the MCF-7 cells with PTEN knockdown, indicating that *LCN2* is also one of the downstream genes of *PTEN* in breast cancer cells and contributes to the *PTEN* insufficiency-induced increase in the metastasis of the MCF-7 cells.

The cytoskeletal networks of F-actin play a vital role in the regulation of cell shape and force generation during migration (37). The immunofluorescent staining of F-actin and the nucleus in the MCF-7-COLsi and MCF-7-PTENsi cells indicates that F-actin expression was increased in the MCF-7-PTENsi cells. Thus, *PTEN* knockdown in the MCF-7 cells strengthened F-actin synthesis (Figure 5A), leading to an increase in the migration of the cells.

Our *in vivo* xenograft study using the zebrafish model further demonstrates that, in addition to the increased migration and invasion noted *in vitro*, PTEN insufficiency could also increase MCF-7 cell metastasis *in vivo* (Figure 5B), as indicated by the fact that the xenografted MCF-7-

PTENsi cells could migrate over longer distances than the MCF-7-COLsi cells in the zebrafish embryos.

Conclusion

Although *PTEN* is a well-known tumor suppressor gene, the mechanisms underlying the effect of PTEN on breast cancer metastasis has not been investigated yet. The high incidence of PTEN insufficiency in breast cancer and association between increased metastasis of breast cancer and PTEN insufficiency warrants a further understanding of the role of PTEN in breast cancer metastasis. Our current work indicates that PTEN insufficiency in]MCF-7 cells promoted EMT by increasing the expression of EMT-related transcriptional factors, along with the up-regulation of P-cadherin, WISP1, LCN2, extracellular MMP-2 and MMP-9, as well as F-actin, and the down-regulation of E-cadherin, thus resulting in the increased invasiveness of these cells *in vitro* and *in vivo*. Our work suggests molecular targets for therapies in breast cancer patients with PTEN insufficiency that need to be studied in further detail with the aim to reduce cancer metastasis in the clinical setting.

Conflicts of Interest

All Authors declare that there are no competing financial interests.

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