LOSS OF EPITHELIAL MEMBRANE PROTEIN-2 PROMOTES EPITHELIAL TO MESENCHYMAL TRANSITION IN BREAST CANCER PROGRESSION

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ABSTRACT

Loss of epithelial membrane protein-2 promotes epithelial to mesenchymal transition in breast cancer progression

Metastasis is the main causing death in cancer patients. The epithelial mesenchymal transition (EMT) is the major mechanism involved in cancer metastasis. During EMT, epithelial cells lose cell-cell adhesions to gain mesenchymal-like properties. Epithelial membrane protein 2 (EMP2), a member of the peripheral myelin protein 22 (PMP-22) family of tetraspan proteins, is related with a variety of cellular processes, such as invasion, proliferation and their trafficking to glycolipid-enriched lipid raft domains is important in receptor signaling. However, the function of EMP2 in breast cancers has not yet been studied. Here I showed that the EMP2 controls EMT in breast cancer metastasis. I demonstrated that the role of EMP2 using the mouse mammary tumor virus-polyoma middle T antigen (MMTV-PyMT) model, MMTV-PyMT /EMP2$^{+/+}$ and MMTV-PyMT/EMP2$^{-/-}$. I found that loss of EMP2 leads to increase the expressions levels of mesenchymal markers such as N-cadherin, vimentin, ZEB1 and decrease the expression of the epithelial marker E-cadherin in mouse mammary tumor cells and human breast cancer cells. Also, I found that loss of EMP2 causes an increase metastasis to lung in MMTV-PyMT mouse model. EMP2 silenced cells including SKBR3 cancer cells exhibited enhanced

migration and invasion, which are the typical characteristics of EMT. These results suggest that EMP2 functions as a metastasis suppressor in breast cancer.

Key words: breast cancer, metaetasis, EMT, EMP2, MMTV-PyMT mouse

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List of Abbreviation

EMT:	epithelial–mesenchymal transition
EMT-TFs:	EMT transcription factors
GEMMs:	genetically engineered mouse models
EMP2:	the epithelial membrane protein-2 (human)
Emp2:	the epithelial membrane protein-2 (mouse)
FAK:	focal adhesion kinase
GAS3/PMP22:	the growth arrest specific-3/peripheral myelin protein-22
ECM:	extracellular matrix
MMTV-PyMT:	mouse mammary tumor virus-polyoma middle T antigen
FBS:	fetal bovine serum
DMEM:	Dulbecco's modified Eagle's medium
PBS:	phosphate-buffered saline
ABC:	the avidin-biotin complex
DAB:	3,3'-diaminobenzidine
SDS:	sodium dodecyl sulfate
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
cDNA:	complementary DNA
RT-PCR:	reverse transcriptase polymerase chain reaction
qRT-PCR:	quantitative real-time PCR
HR:	hazard ratio

1. Introduction

1.1. Breast cancer metastasis

Being the most common cancer in women both in the developed and less developed countries, breast cancer has more than 250,000 new cases and over 70,000 women died in 2017 due to breast cancer[1]. It is the second-leading cause of cancer death in women. Metastasis is the major cause of death in patients with breast cancer. The main reason causing death in breast cancer is metastasis.

Metastasis is the phenomenon while a primary tumor locally invades to other organs. In metastasizing process, cancer cells may invade to adjacent tissue before migrating to another adjacent site through blood circulation[2].

Previous studies showed that there are various model systems to investigate the complex and multi-step progress of metastasis [3]. *In vitro* provided a prominent insights to understand and become the starting point for testing hypotheses. Lung metastasis is also one of common sites to be spread from primary breast cancer cells or tissues[4]. Pulmonary metastasis is one of frequent sites of breast cancer by mediating of some genes including MMP1, CXCL1, PTGS2, ID1, VCAM1 and EREG[5].

In my study, I used SKBR3 cells as an *in vitro* models because of their high expression in breast cancer. SKBR3 cells is breast cancer cells from mammary gland tissues which derived from metastatic site. It was established in 1970 from the pleural effusion of malignant breast adenocarcinoma from 43-year-old Caucasian women. So it is suitable for cancer metastasis research.

1.1.1. Epithelial–mesenchymal transition

Epithelial–mesenchymal transitions (EMTs) are beginning of multi-step metastasis process. Elizabeth Ha *et al*. found that EMTs have first defined as an important role in vertebrate embryonic development [6]. This transition from an epithelial to a mesenchymal phenotype of cells is a process which changed distinct morphology, when epithelial cells reduce their cell-cell adhesion, apical-basolateral polarity to gain migratory behaviors, invasive capacities[7]. They are proposed by numerous publications to be a potential mechanism that enhances the cancer metastasis from primary tumor (Fig. 1.).

EMT is mainly described by loss of epithelial markers including E-cadherin, cytokeratins, syndecan-1, zonaoccludens 1. The transition of epithelial cells also increases their nuclear expression of several transcription factors including Snail, Slug, ZEB1, Twist[8]. de Herreros AG *et al*. suggested that several transcription factors are EMT inducers by direct transcriptional repression of E-cadherin recognize the E-Box DNA sequence placed near E-cadherin transcriptional initiation site, forming transcriptional co-factors and histone deacetylases[9]. Hence, I study epithelial markers and mesenchymal markers to determine characteristic of cells and metastasis.

EMT transcription factors (EMT-TFs) are key drivers of mesenchymal programs not only in almost states of cancer progression and metastasis to colonization but also in resistance to therapy [10]. Activation of EMT-TFs contributes the stem-cell-like properties of epithelial cells to increase tumor expressions.

Moreover, EMT-TFs can be directly activated the transcription of inflammatory and immunosuppressive cytokines genes in cancer cells and during fibrosis, therefore triggering tumor-promoting effects which involve the infiltrating immune system on the composition of the tumor microenvironment [11]. Regarding to Lee *et al.* studies, they have demonstrated that cells bearing this hybrid phenotype have been recommended to as “metastable” [11], reflecting the flexibility of these cells to decrease or regress the EMT process [12].

In recently study, they use genetically engineered mouse models (GEMMs) to directly examine the EMT-TFs function, aims to directly confirm whether EMT-related TFs are responsible for chemoresistance [13-16]. From above results, it is clearly that EMT-TFs have pleiotropic roles from initiation stage to metastasis in cancer progression.

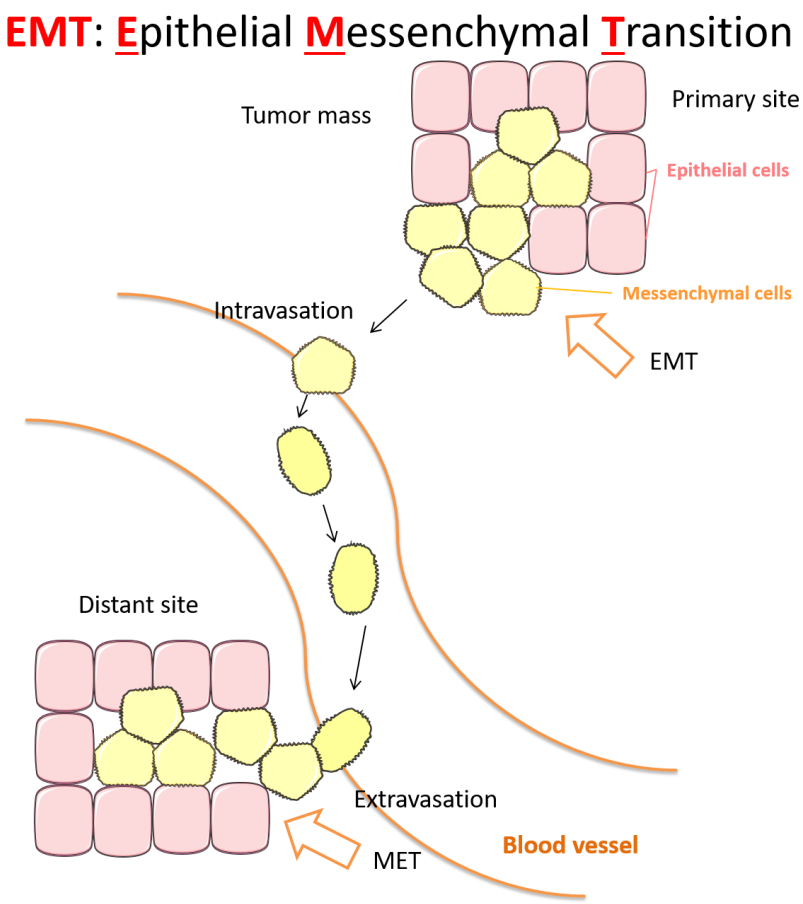

Figure 1. Pulmonary metastasis mechanism from breast cancer primary tumor under EMT

The first step in metastasis, EMT is a process which changed distinct morphology, when epithelial cells reduce their cell-cell adhesion, apical-basolateral polarity to gain migratory behaviors, invasive capacities to become mesenchymal cells. After coming to the secondary organ, the MET process, which is changed morphology from mesenchymal cells to epithelial cells, to be adapted with new

organs.

1.1.2. The epithelial membrane protein-2 (EMP2)

EMP2 is a tetraspan (4-transmembrane) protein which belongs to the growth arrest specific-3/peripheral myelin protein-22 (GAS3/PMP22) family that facilitates plasma membrane delivery of certain integrins [17]. Structure of EMP2 (Fig. 2.) show their characteristics that GAS33/PMP22 facilitates plasma membrane delivery of certain integrins [18]. EMP2 decreased level of caveolin-1 protein, a scaffolding protein which is the primary component of the caveolae [19]. Caveolin-1 promotes recruitment of β-catenin to the plasma membrane which associates with E-cadherin. In addition, EMP2 assists the formation and surface trafficking of lipid rafts carrying glycosylphosphatidyl inositol-anchored proteins, and decreases caveolin expression, resulting in impaired formation of caveolae. Therefore, EMP2 plays an important role in recycling apical membrane [17]. Studies reported that EMP2 associates with β1 integrins, it adjusts the repertoire of α6β1 to α5β1 on the cell surface reciprocally. Then, EMP2 consequently controls the cell-stromal adhesion [20].

EMP2 is also involved in cell proliferation and cell-cell interactions [21]. EMP2 associates to the activation of focal adhesion kinase (FAK) and its activation through phosphorylation play a crucial role for cell cycle progression, migration, invasion, survival, proliferation and contraction [22]. Integrin receptor ligation can initiate FAK phosphorylation by extracellular matrix (ECM) components.

EMP2 enhanced tumor formation *in vivo* by activating αvβ3 integrin surface expression, FAK and Src kinases to promote focal adhesion, cell migration, invasion and tumor metastasis [23] .

Other studies are confirmed that the effects of EMP2 induce G2/M cell cycle arrest, suppress cell proliferation, viability and independent cell growth by regulating G2/M checkpoints in bladder cancer[24]. In urothelial cancer, lack of EMP2 expression is positively associated with muscle invasion of upper urinary tract, disease progression, and cancer-related patient death [25]. Additional evidence is nasopharyngeal carcinoma, these analyses demonstrated that loss of EMP2 gene is an unrelated prognosticator for decreasing survival [26]. EMP2 is induced the death of B-cell lymphoma cells on serum deprivation *in vitro*, this is prevented by caspase inhibitors Z-VAD and Z-DEVD treatments. EMP2 is also inhibited B-lymphoma tumorigenicity through a functional tumor suppressor phenotype *in vivo* [27]. So, EMP2 function as an oncogene or a tumor suppressor gene is based on types of cancer. The function of EMP2 in breast cancer progression still has not been studied yet.

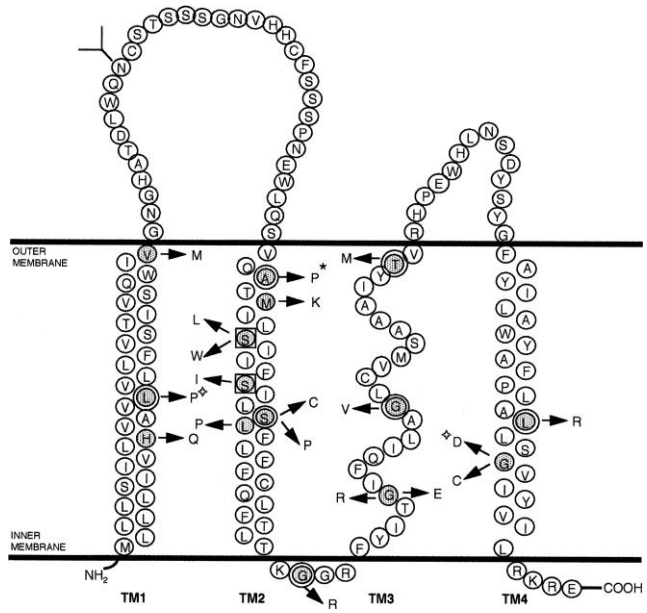

Figure 2. Structure of EMP2

EMP2 is a tetraspan (4-transmembrane) protein which belongs to the growth arrest specific-3/peripheral myelin protein-22 (GAS3/PMP22) family that facilitates plasma membrane delivery of certain integrins.

1.1.3. Mouse mammary tumor virus-polyoma middle T antigen

Mouse mammary tumor virus-polyoma middle T antigen (MMTV-PyMT) is the well-characterized transgenic animal model of breast cancer metastasis [28]. The characteristics of MMTV-PyMT breast cancer mouse model is described by short latency, high penetrance. They develop palpable mammary tumors as 5 week-old mice, eventually show 100% tumor penetrance and 94% tumor-bearing mice are observed pulmonary metastasis[29]. In addition, high percentage of pulmonary metastasis which have been observed in female mice at the age of 14 weeks, unrelated with pregnancy and with a reproducible kinetics of progression [30].

Recently studies display that changes in cell-cell adhesion plays a crucial role in tumorigenesis [31]. Because of the adherent junction, E-cadherin is referred as a tumor suppressor [32]. E-cadherin expression prevents tumor cell migration and invasion, in addition, inhibition of E-cadherin increases invasion and metastatic dissemination of tumor cell. In addition, loss of E-cadherin is related with tumor metastasis, also with invasive carcinoma. Therefore, in my study, I used transgenic mouse models to study the change of markers. I also found that loss of EMP2 induces EMT in breast cancer progression.

In my laboratory, we replaced exon 3 with a puromycin cassete to remove *EMP2* gene. After that, we verified the absent of EMP2 by RT-PCR analysis using RNA isolated from the *EMP2*$^{-/-}$ mouse and a primer specific for the knockout. Furthermore, we also used western blot to confirm these results.

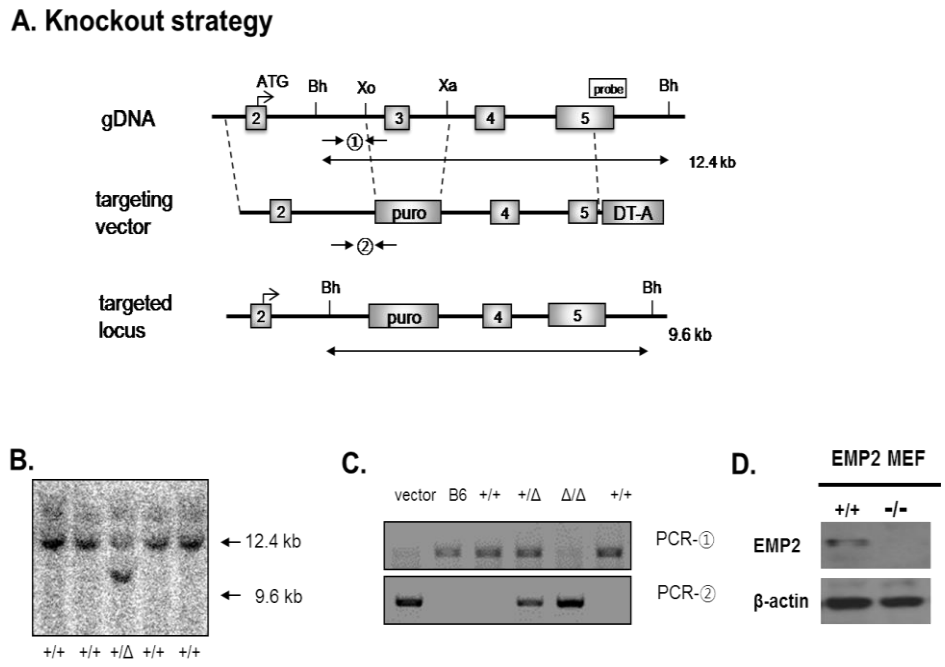

Figure 3. Establishment of *Emp2*$^{-/-}$ mice

(A)Knockout strategy for establishing Emp2$^{-/-}$ mice (B) Southern blot analysis for verifying the absent of Emp2$^{-/-}$ gene (C) RT-PCR analysis using RNA isolated from the Emp2$^{-/-}$ mouse and a primer specific for the knockout (D) Western blot analysis to confirm Emp2$^{-/-}$ mice.

1.2. Purpose and objectives

The present study aims to establish the functional role of EMP2 in breast cancer progression and metastasis remains largely unknown. Therefore, the purpose of this study is;

1. To investigate the function of EMP2 in breast cancer progression and metastasis.

2. To identify the role of Emp2 in breast cancer mouse model.

3. To investigate the EMT and EMT related genes expression in breast cancer cell.

2. Materials and methods

2.1. Animal studies

For all experiments, MMTV-PyMT/EMP2$^{-/-}$ (n=20) and control littermate animals (n=20) were used. They were born and bred in laboratory animal research center of national cancer center, Republic of Korea which ancestors were from The Jackson Laboratory. These mice (n≤5 for each cage) were under specific pathogen free conditions as constant temperature of 22±1^{0}C, relative humidity of 55±10%, cycle light/dark every 12 hours, fed rodent standard chow (Samyang, Korea) and purified water. Animals were checked once to twice per week.

2.2. Cell culture

Human breast cancer cell lines SKBR3 (the American Type Culture Collection, Rockville, USA) were cultured in fetal bovine serum, FBS (WelGENE Inc., Korea). These cells were cultured from Dulbecco's modified Eagle's medium (DMEM) adding penicillin-streptomycin (10,000 IU/ml and 10,000 μg/ml, respectively) and sodium pyruvate (1 mM). All cells were incubated at 37℃ in a humidified atmosphere containing 5% CO_2. Before the respective experiments, these cells were washed twice in serum-free DMEM and they were incubated in serum-free DMEM 18 hours.

2.3. Hematoxylin and Eosin (H&E) staining

Lung tissues were fixed with 10% formalin on overnight, dehydrated in ethanol, embedded in paraffin, sectioned at 4μm by Thermo Scientific Rotary Microtome Microm HM355S (Thermo Scientific, Germany). MMTV-PyMT/EMP2$^{+/+}$ (n=10) and MMTV-PyMT/EMP2$^{-/-}$ (n=10) lung tumor sections in 12 and 16 weeks were seeded onto glass coverslips and processed as deparaffinized (perform the following washes: twice Xylene to Xylene: Ethanol 1:1) and rehydrated (Ethanol 100% - 95% - 90% - 70% - 50% in water). Finally rinse to tap water. The tissue sections were stained by hematoxylin (nuclear) (Vector Laboratories, Burlingame, CA, USA), rinsed by running tap water, after that counterstained by eosin (cytoplasm) (Vector Laboratories), rinsed by running tap water, dehydrate and mount with resinous mounting medium (Vector Laboratories). Lung tumor nodules and areas of lung tumors are counted by using microscope (Zeiss Axiophot, Carl Zeiss, Oberkochen, Germany) (1.25X magnification).

2.4. Immunohistochemistry

Mammary gland tissues were dissected and fixed with 10% formalin on overnight, dehydrated in ethanol, embedded in paraffin, sectioned at 4 μm by Thermo Scientific Rotary Microtome Microm HM355S (Thermo Scientific, Walldorf, Germany). Tissues were treated in 0.3% H_2O_2 in phosphate-buffered saline (PBS) to block endogenous peroxidase for 30 minutes at room temperature.

Then, each slide was blocked by normal serum to prevent nonspecific binding of the antibodies. After that, these slides were incubated by primary antibodies such as E-cadherin (BD Transduction Laboratories, San Jose, CA, USA), N-cadherin (abcam, Cambridge, UK), vimentin (Santa Cruz, Beverly, MA, USA), ZEB1 (Santa Cruz) and EMP2 (abcam) diluted from 1:40 to 1:200 overnight in 4^{o}C or 30 minutes at room temperature. Next is incubating the recommended conjugated secondary antibodies (Vector Laboratories) diluted 1:200 according to standard protocols. Detection of antibodies was performed by the avidin-biotin complex (ABC) reagent (Vector Laboratories) for 30 minutes at room temperature. These sections were stained with DAB (3,3'-diaminobenzidine) peroxidase substrate kit (Vector Laboratories). Then, the tissue sections were counterstained by hematoxylin. Dehydrate (washes again by ratio ethanol 50% to 100%, xylene: ethanol 1:1 to Xylene) to clear DAB or any other organic chromogen developed sections. Stabilizing with mounting medium (Vector Laboratories). Images were obtained by microscope (Zeiss Axiophot) (40X magnification).

2.5. Western blot analysis

The cells were harvested and lysed in 50mM Tris-Cl (pH 7.5), 150 mM sodium chloride [NaCl], 1% sodium deoxycholate, 1% triton X-100, 2 mM ethylenediaminetetraacetic acid [EDTA], 0.1% SDS and protease inhibitors (Gendepot, USA). As recommended by the manufacturer, the protein concentrations were examined using Coomassie Plus (Thermo Scientific Inc.,

Waltham, MA, USA). Protein extracts were denatured by boiling for 5 min in sodium dodecyl sulfate (SDS) sample buffer. 30 µl of proteins were separated by 8-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After that, these proteins were transferred to polyvinylidene difluoride membrane (Pall, USA). After blocking by 5% skim milk in mixture of tris-buffered saline and tween 20 (TBST), I incubated membranes by primary antibodies such as E-cadherin (BD Transduction Laboratories), N-cadherin (abcam), vimentin (Santa Cruz), ZEB1 (Santa Cruz) Snail (Santa Cruz), EMP2 (Santa Cruz) and β-actin (Santa Cruz) diluted 1:1000 to 1:2500 overnight in 4^{o}C. Next, membranes were incubated by the recommended dilution of conjugated secondary antibodies (GenDEPOT, Katy, TX, USA) diluted 1:5000 in blocking buffer at room temperature for 1 hour. Developing and acquire image development techniques for chemiluminescence (Thermo Scientific, Madison, WI, USA) and exposure to X-ray film (Kodak, Rochester, NY, USA).

2.6. Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR is used to detect gene expression qualitatively through creation of 1 µl complementary DNA (cDNA) transcripts from RNA produce multiple copies of particular DNA isolates, primers, water and 10 µl premix in 30-35 cycles through amplification by PCR machine (Eppendorf AG, Germany). Sequences of each primer are: human E-cadherin; 5’- GTC ATC CAA CGG GAA TGC A - 3’ and

3’- TGA TCG GTT ACC GTG ATC AAA A - 5’, human N-cadherin; 5’ - TGG AGA ACC CCA TTG ACA TT - 3’ and 3’ - TGA TCC CTC AGG AAC TGT CC - 5’, human vimentin; 5’ - TCG TTT CGA GGT TTT CGC GTT AGA GAC - 3’ and3’ - CGA CTA AAA CTC GAC CGA CTC GCG A - 5’, human Snail; 5’ - TTC AAC TGC AAA TAC TGC AAC AAG - 3’ and 3’ - CGT GTG GCT TCG GAT GTG - 5’, human ZEB1; 5’ - GCA CCT GAA GAG GAC CAG AG - 3’ and 3’ - GTG TAA CTG CAC AGG GAG CA - 5’, human EMP2; 5’ - AGA ATA GAC TAT GAA GGC TGG TA - 3’ and 3’ - CTC CTC TCC TCC CAT CAC - 5’, human GAPDH; 5’ - CTC CAA AAT CAA GTG GGG CG - 3’ and 3’ - GGG CAG AGA TGA TGA CCC TT - 5’, mouse E-cadherin; 5’ - TCT CCT CAT GGC TTT GCC AG - 3’ and 3’ - TCT CCA GGT GCC TCT CCG - 5’, mouse N-cadherin; 5’ - CGC CCA GGG AAG GGA AAA G - 3’ and 3’ - GAG GAT ACG GAG GTG GCG - 5’, mouse vimentin; 5’ - TGT CTA CCA GGT CTG TGT CCT - 3’ and 3’ - GAC GTG GTC ACA TAG CTC CG - 5’, mouse Snail; 5’ - GGA CGC GTG TGT GGA GTT - 3’ and 3’ - GGG GTT GAG GAC CTC GGG - 5’, mouse ZEB1; 5’ - CCA CTG TGG AGG ACC AGA AT - 3’ and 3’ - CTC GTG AGG CCT CTT ACC TG - 5’, mouse EMP2; 5’ - CTC TGG AGA GTG TGC ACC AA - 3’ and 3’ - CGT CAG GAC GAA CCT CTC TC - 5’, mouse GAPDH; 5’ - TGT CGT GGA GTC TAC TGG TGT C - 3’ and 3’ - GCT AAG CAG TTG GTG GTG CAG G - 5’. Detect by gel electrophoresis.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR analysis of EMT related transcription factors from short hairpin Control and EMP2 RNA. Total RNA was isolated from SKBR3 cells or mammary gland, RT-PCR was performed on a qRT-PCR machine (LightCycler 480, Roche Diagnostics, Mannheim, Germany), using suitable primers, 2 μl template cDNA, 5 μl SYBR Green Supermix (Takara, Shiga, Japan). Sequences of each primer are shown in 2.6. Data presented as the mean ± SD. A p value <0.05 was considered significantly by Student's t test.

2.8. Cell migration assay

For transwell migration assays, I used transwell (Neuro Probe, Inc., Gaithersburg, MD, USA) inserts covered with 25 μg/ml fibronectin in the back side of the upper chamber overnight. Then these cells were suspended 10 minutes in 3% FBS media and 1.5 x 10^4 cells were put to the upper chamber of the transwell inserts. These cells are incubated 5 hours after adding 25 μl chemical-treated cells. I removed all nonmigrated cells on the upper surface of the membrane. Then, the transwell membranes were fixed and stained with Diff Quik staining kit (Sysmex Corporation, Kobe, Japan). Images were obtained by microscope (Zeiss Axiophot) (40X magnifications). Three random fields were chosen and cell numbers were averaged.

2.9. Cell invasion assay

For matrigel invasion assays, inserts were coated on the inside in the transwell insert, incubated, stained according to standard protocols. Three random fields were chosen and cell numbers were averaged. In matrigel invasion assays, I used transwell (BD BioCoat^{TM}Matrigel^{TM} invasion chamber) inserts coated with matrigel 500 μg/ml (10 μl/ well) on 4 hours in room temperature. Then, these inserts were warm in serum free media for 30 minutes. 2 x 10^5 cells were added onto the matrigel in the 10% FBS media to incubate for 20 hours at 37^{o}C. After fixing with methanol 10 minutes, the slides were incubated by hematoxin and eosin. Images were obtained by microscope (Zeiss Axiophot) (40X magnification). Three random fields were chosen and cell numbers were averaged.

2.10. Statistical analysis

The data were expressed as the mean ± S.E.M. of at least three independent experiments performed in triplicate. A p value <0.05 was considered significantly.

3. Results

3.1. EMP2 expression correlates with progression of human breast cancer

The relationship between EMP2 expression and clinical outcome in breast cancer patient was assessed by overall survival (n = 56, hazard ratio [HR]: 0.7, 95% confidence interval from 0.53 to 0.93, p = 0.01) and relapse free survival (n = 155, hazard ratio [HR]: 0.71, 95% confidence interval from 0.54 to 0.93, p = 0.01) in the full cohort (Fig.4.). By PROGgeneV2 - Pan Cancer Prognostics Database, survival analyses showed that the high expression of EMP2 has higher survival expression.

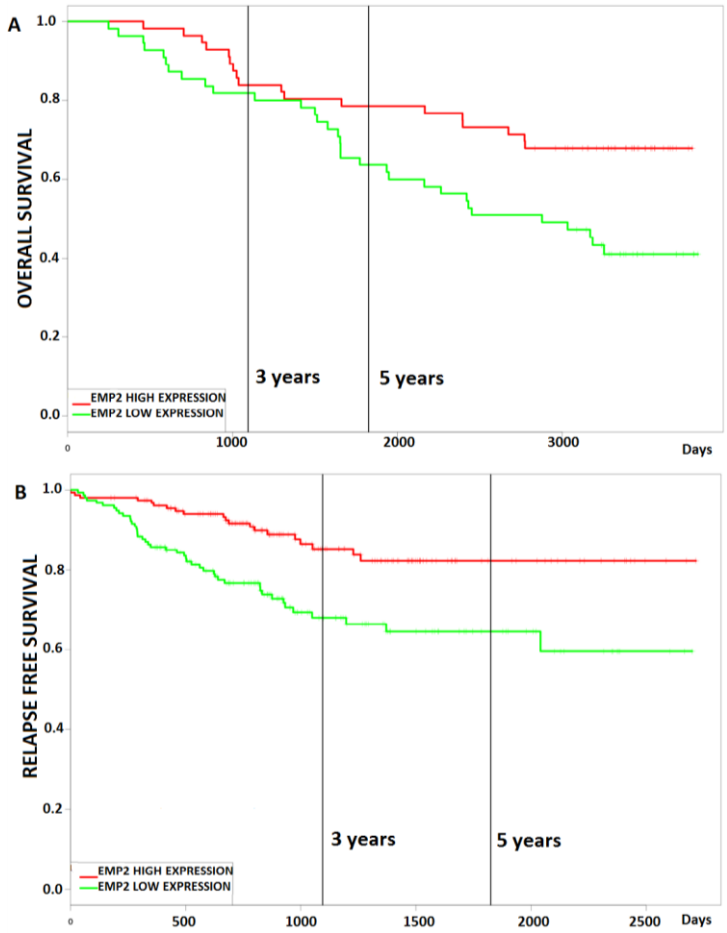

Figure 4. Effects of EMP2 on overall survival and relapse free survival curves of breast cancer patients.

(A) KM plot created with PROGgeneV2 for overall survival (n = 56, HR: 0.7, 95% CI: 0.53-0.93, p = 0.01) is plotted for breast cancer patients (GSE19783-GPL6480) (B) KM plot created with PROGgeneV2 for relapse free survival curves (n = 155, HR: 0.71, 95% CI: 0.54-0.93, p = 0.01) is plotted for breast cancer patients (GSE25055).

3.2. EMP2 inhibits lung metastasis in the MMTV-PyMT mouse

To investigate the *in vivo* role of Emp2 in breast cancer, MMTV-PyMT mouse model of breast cancer and Emp2 knockout mice ($Emp2^{-/-}$) were used. Tumor-bearing MMTV-PyMT breast cancer mice were observed pulmonary metastasis at 15 weeks of age [29]. Lung tissues were obtained by sacrificed mice when aged at 12 weeks and 16 weeks (Fig. 5. and Fig. 6.). The data which described from MMTV-PyMT/$EMP2^{-/-}$ and MMTV-PyMT/$EMP2^{+/+}$ mice (n=10) were showed in these figures. The average tumor area of lung lesion and the number of lung nodules were increased in MMTV-PyMT/$EMP2^{-/-}$ mice. Notably, MMTV-PyMT/$EMP2^{-/-}$ lung tumors area was increased 3-fold and MMTV-PyMT/$EMP2^{-/-}$ lung nodules increased 22-fold at 12 weeks of age. Actually in 12 weeks of age data, p value was still bigger than 0.05 so these data were non-significant. I continued with MMTV-PyMT 16 weeks of age mice experiment, MMTV-PyMT/$EMP2^{-/-}$ lung tumors area significantly increased 8-fold and MMTV-PyMT/$EMP2^{-/-}$ lung nodules increased 25-fold at 16 weeks. Taken together, these findings suggest that loss of EMP2 could increase breast cancer metastasis.

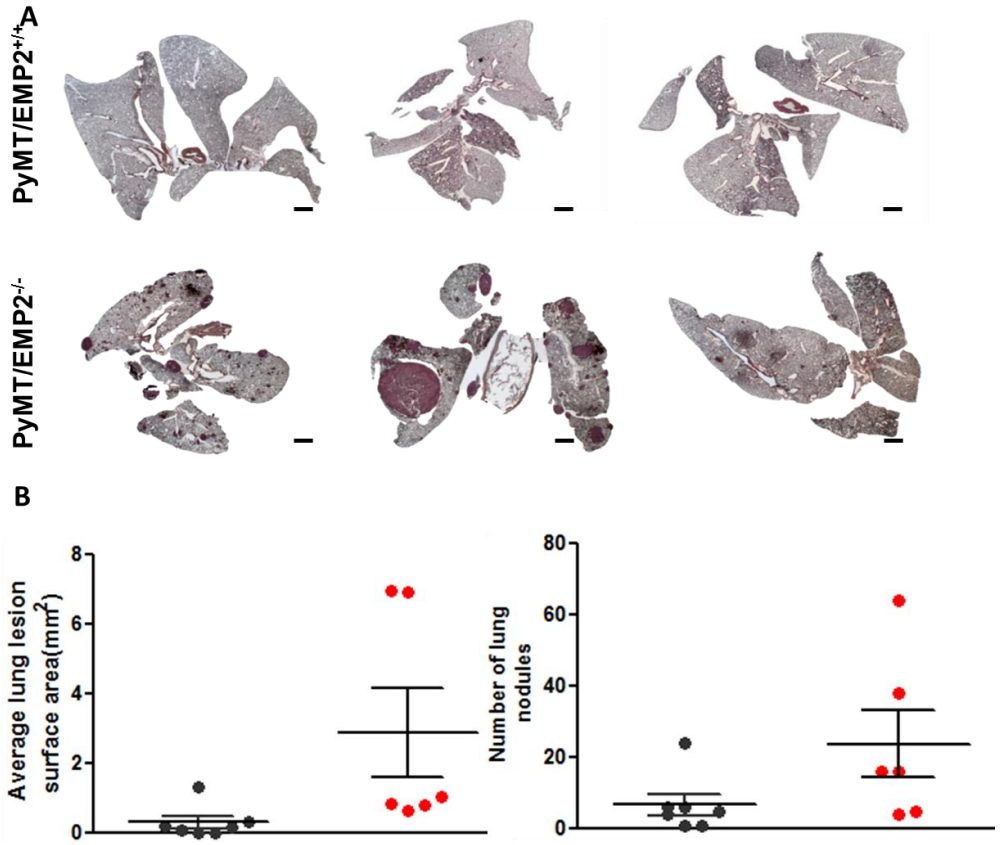

Figure 5. Loss of EMP2 promotes lung metastasis in 12 weeks MMTV-PyMT mouse model.

(A) Representative images of H&E-stained lung tissue in $EMP2^{+/+}$/MMTV-PyMT and $EMP2^{-/-}$/MMTV-PyMT mouse (scale bar: 1mm). (B) The number of lung nodules and the average lung lesions in $EMP2^{+/+}$/MMTV-PyMT and $EMP2^{-/-}$/MMTV-PyMT mouse.

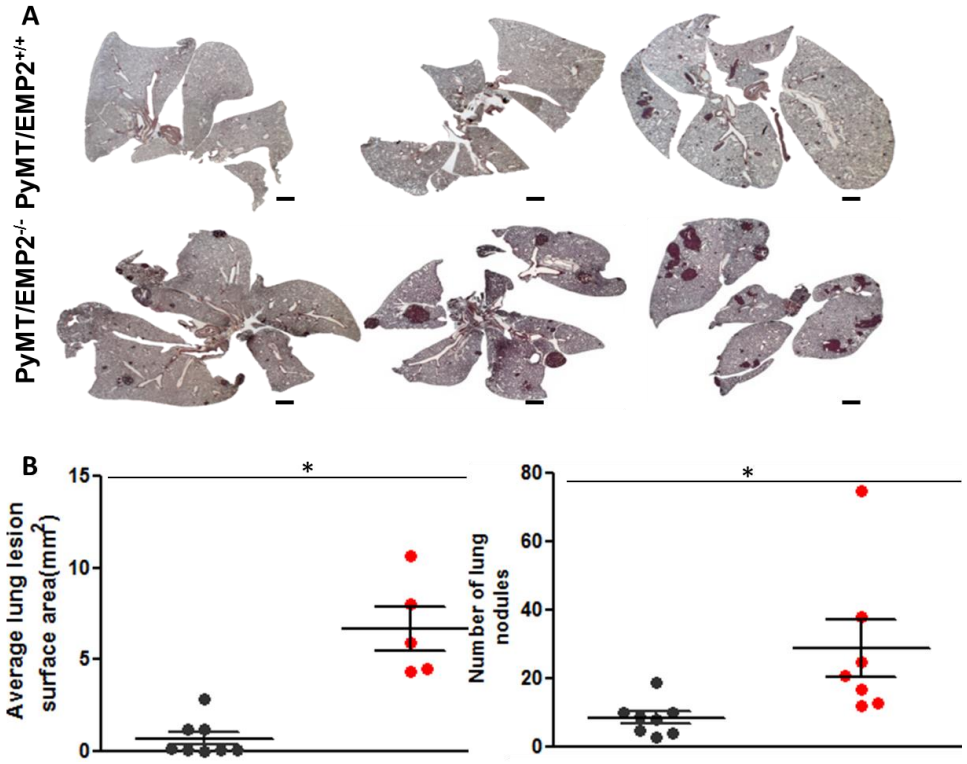

Figure 6. Loss of EMP2 promotes lung metastasis in 16 weeks MMTV-PyMT mouse model.

(A) Representative images of H&E-stained lung tissue in $EMP2^{+/+}$/MMTV-PyMT and $EMP2^{-/-}$/MMTV-PyMT mouse (scale bar: 1mm). (B) The number of lung nodules and the average lung lesions in $EMP2^{+/+}$/MMTV-PyMT and $EMP2^{-/-}$/MMTV-PyMT mouse.

3.3. Loss of EMP2 induces EMT in breast cancer cells SKBR3 and MMTV-PyMT mouse

To determine the role of Emp2 in breast cancer, I confirmed the expression of EMT markers in mammary gland tumor tissue. The analysis of the mouse mammary gland from MMTV-PyMT/EMP2$^{+/+}$ and MMTV-PyMT/EMP2$^{-/-}$ mice revealed that the expressions levels of mesenchymal markers N-cadherin, vimentin, and ZEB1 were increased. Besides, the expression of the epithelial marker E-cadherin was decreased in tumor tissues from MMTV-PyMT/EMP2$^{-/-}$ mice. Because some study revealed that MMTV-PyMT mice also developed multifocal adenocarcinoma at 5 weeks of age [29]. Mice were divided to 2 groups, MMTV-PyMT/EMP2$^{+/+}$ (n=4) and MMTV-PyMT/EMP2$^{-/-}$ (n=4) at 4 weeks and 6 weeks of age, respectively (Fig. 7. and Fig. 8.). In addition, the quantification of data from NIH ImageJ program and the image from DAB staining of mammary glands have been demonstrated the morphological changes during cancer progression when knocking out EMP2 gene.

To validate the IHC results, the same antibodies were also used in western blot RT-PCR, qPCR analysis. The expression of the epithelial marker E-cadherin had also decreased beside the levels of the mesenchymal markers N-cadherin, and vimentin and related transcription factor such as ZEB1, Snail had increased in shEMP2 SKBR3 cells (Fig. 9. and 10.). Similar result, loss of EMP2 also results in decreasing the level of epithelial marker E-cadherin and increasing the levels of mesenchymal markers and transcription factors in primary cells from

MMTV-PyMT mouse breast tumors (Fig.11. and Fig. 12.). So, loss of EMP2 induces EMT in breast cancer cells SKBR3 and MMTV-PyMT cells. Therefore, loss of EMP2 induces EMT in breast cancer.

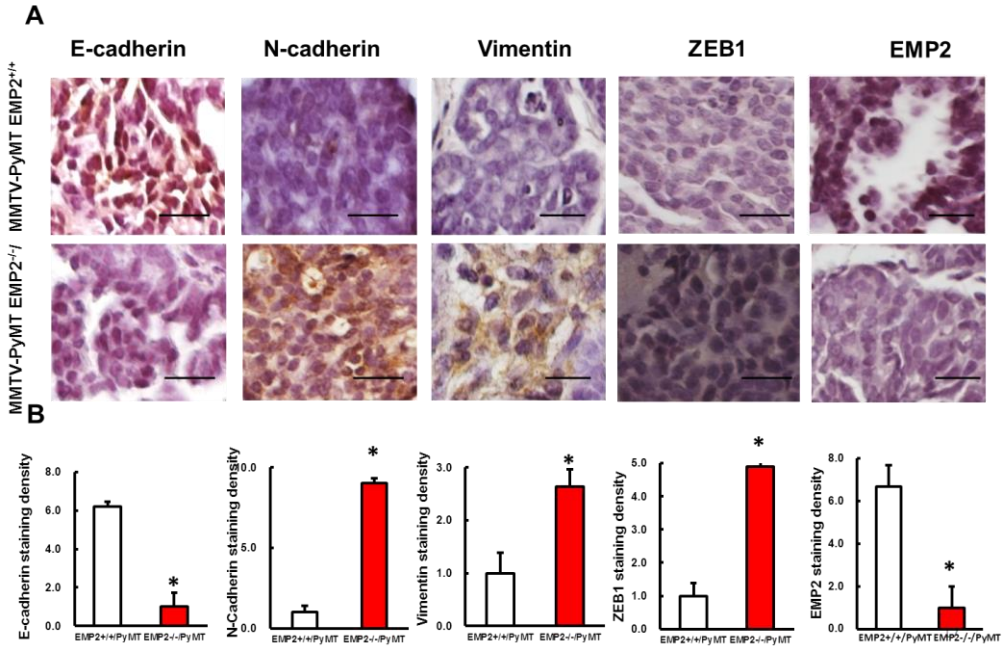

Figure 7. Loss of EMP2 induces EMT in 4 weeks MMTV-PyMT mouse

Histological image of EMT markers in mammary gland tissue of $EMP2^{+/+}$/MMTV-PyMT and $EMP2^{-/-}$/MMTV-PyMT mouse in 4 weeks of age (A). The staining density of each markers was quantified by the percentage of staining area by NIH ImageJ program (B). All experiments were performed in triplicate. All quantitative data are depicted as mean ±S.E. per group. *P<0.05. Scale bars: 20 μm.

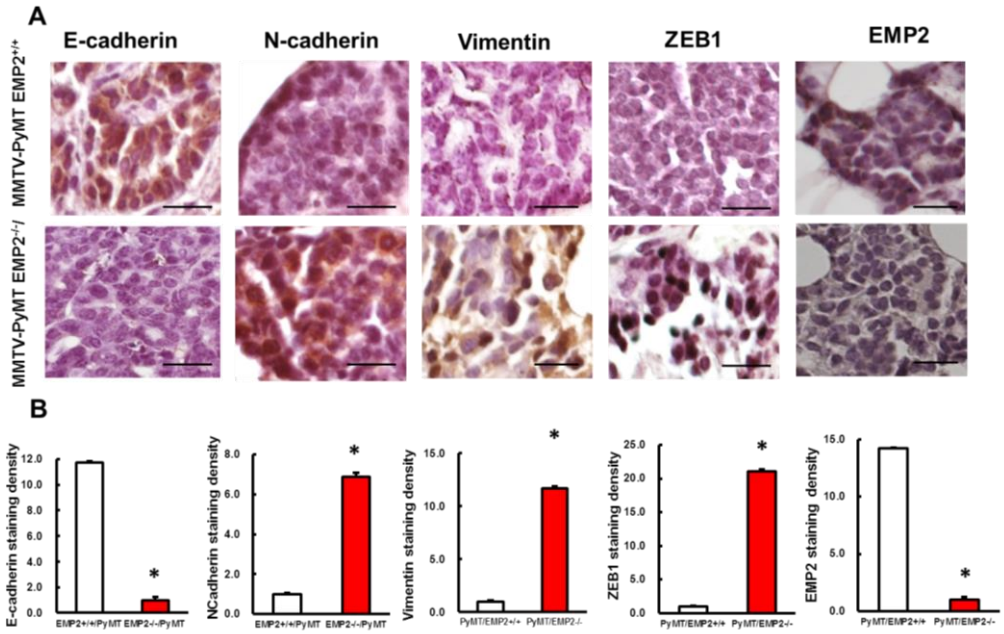

Figure 8. Loss of EMP2 induces EMT in 6 weeks MMTV-PyMT mouse.

Histological image of EMT markers in mammary gland tissue of $EMP2^{+/+}$/MMTV-PyMT and $EMP2^{-/-}$/MMTV-PyMT mouse in 6 weeks of age (A). The staining density of each markers was quantified by the percentage of staining area by NIH ImageJ program (B). All experiments were performed in triplicate. All quantitative data are depicted as mean ±S.E. per group. *P<0.05. Scale bars: 20 μm.

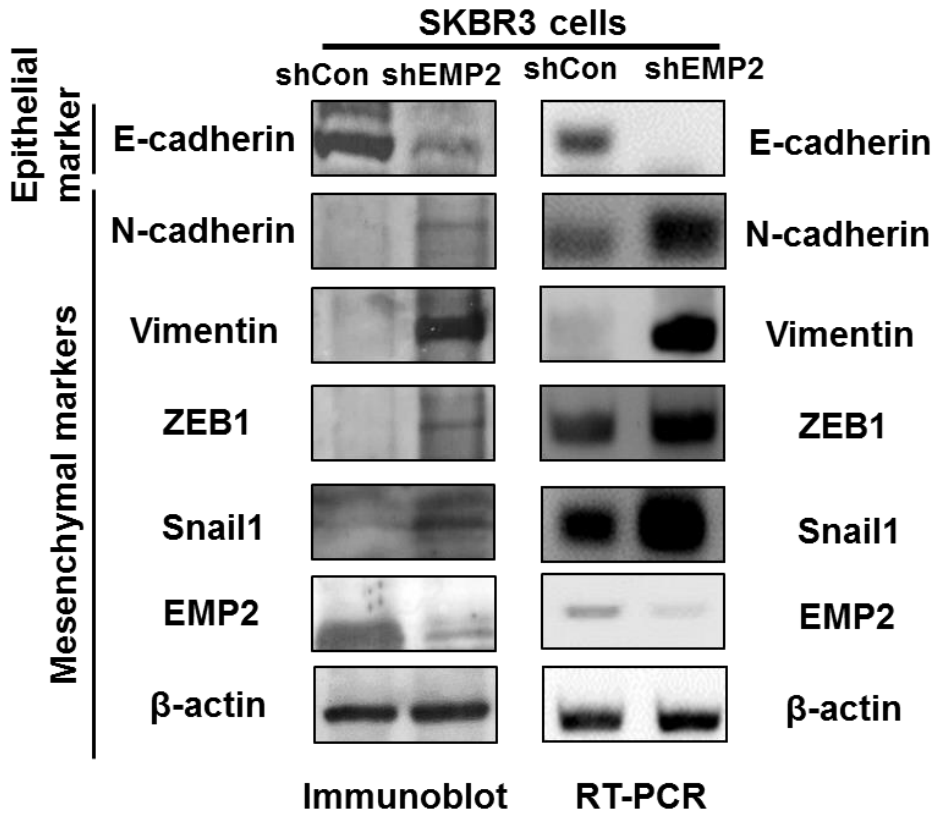

Figure 9. Loss of EMP2 induces EMT in breast cancer cells.

Western blot and RT-PCR analysis of EMT markers and EMT-related gene expression in $SKBR3_{shCon}$, $SKBR3_{shEMP2}$ cells. Cells were cultured in conditioned media. After incubation, cell lysates and total RNA from cells were subjected to Western blot and reverse transcription–PCR.

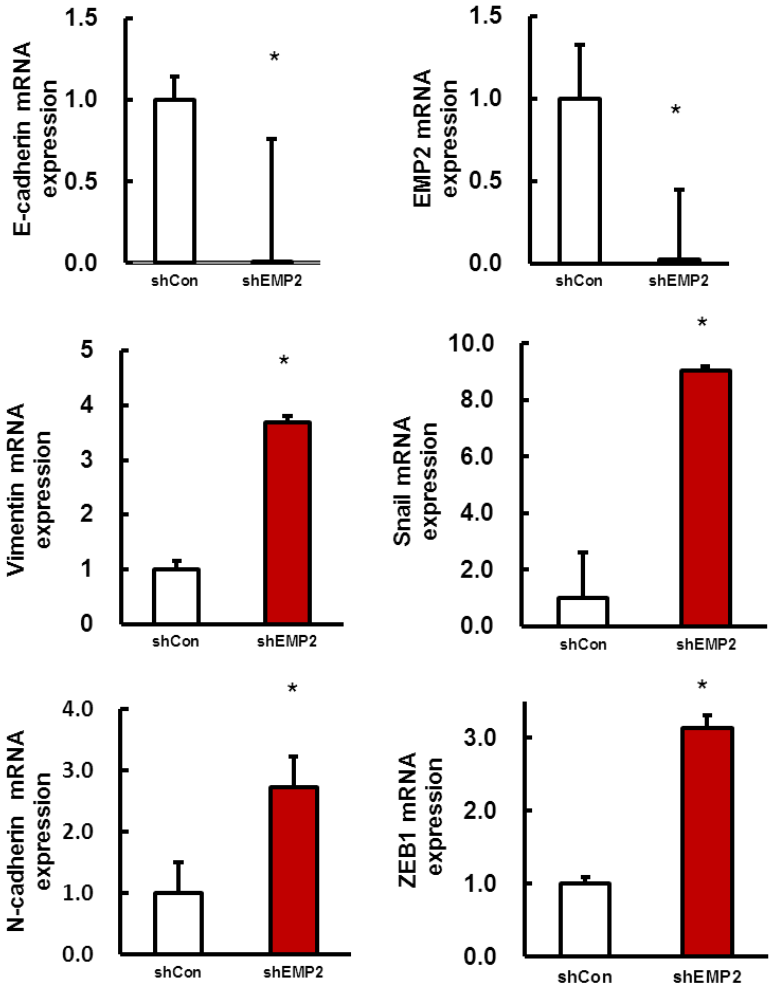

Figure 10. Loss of EMP2 induces EMT in breast cancer cells.

Real-time RT-PCR analysis of EMT markers markers and EMT-related gene expression in **$SKBR3_{shCon}$, $SKBR3_{shEMP2}$** cells . All experiments were performed in triplicate. All quantitative data are depicted as mean ±S.E. per group. *P<0.05.

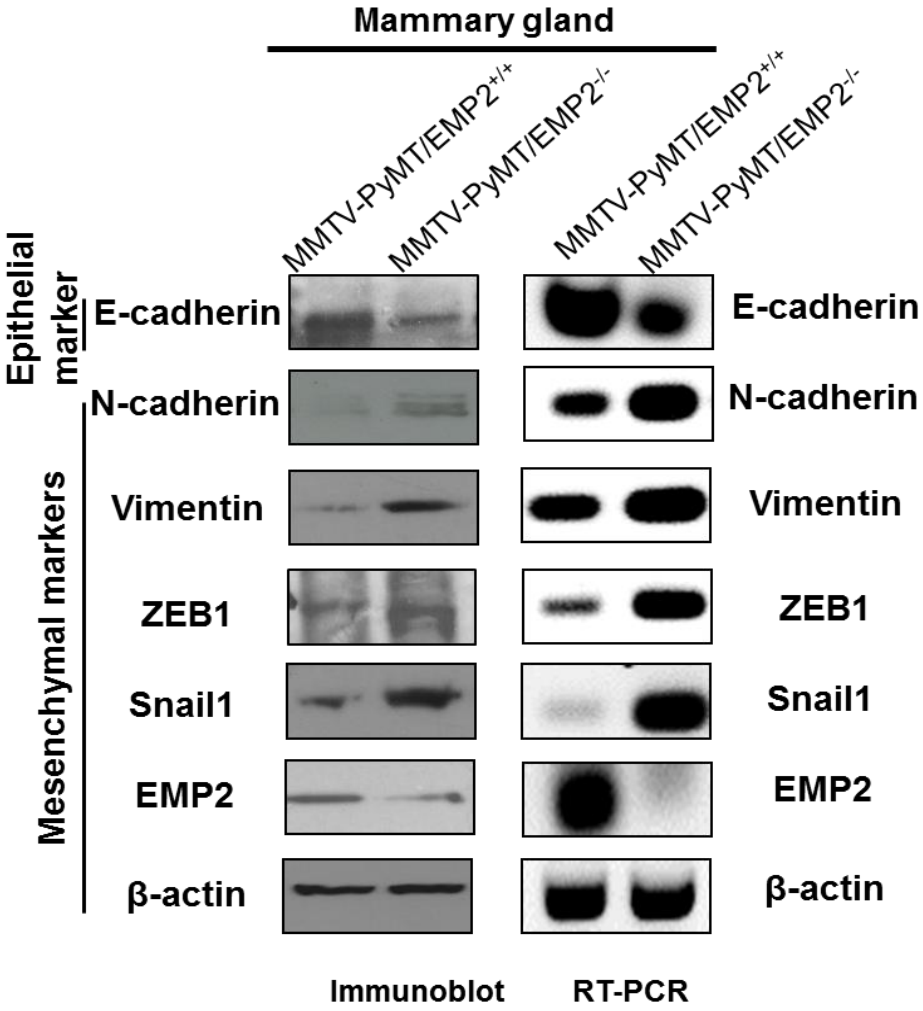

Figure 11. Loss of EMP2 induces EMT in primary MMTV-PyMT cells.

Western blot and RT-PCR analysis of EMT markers and EMT-related gene expression in primary MMTV-PyMT cells. Cells were cultured in conditioned media. After incubation, cell lysates and total RNA from cells were subjected to Western blot and reverse transcription–PCR.

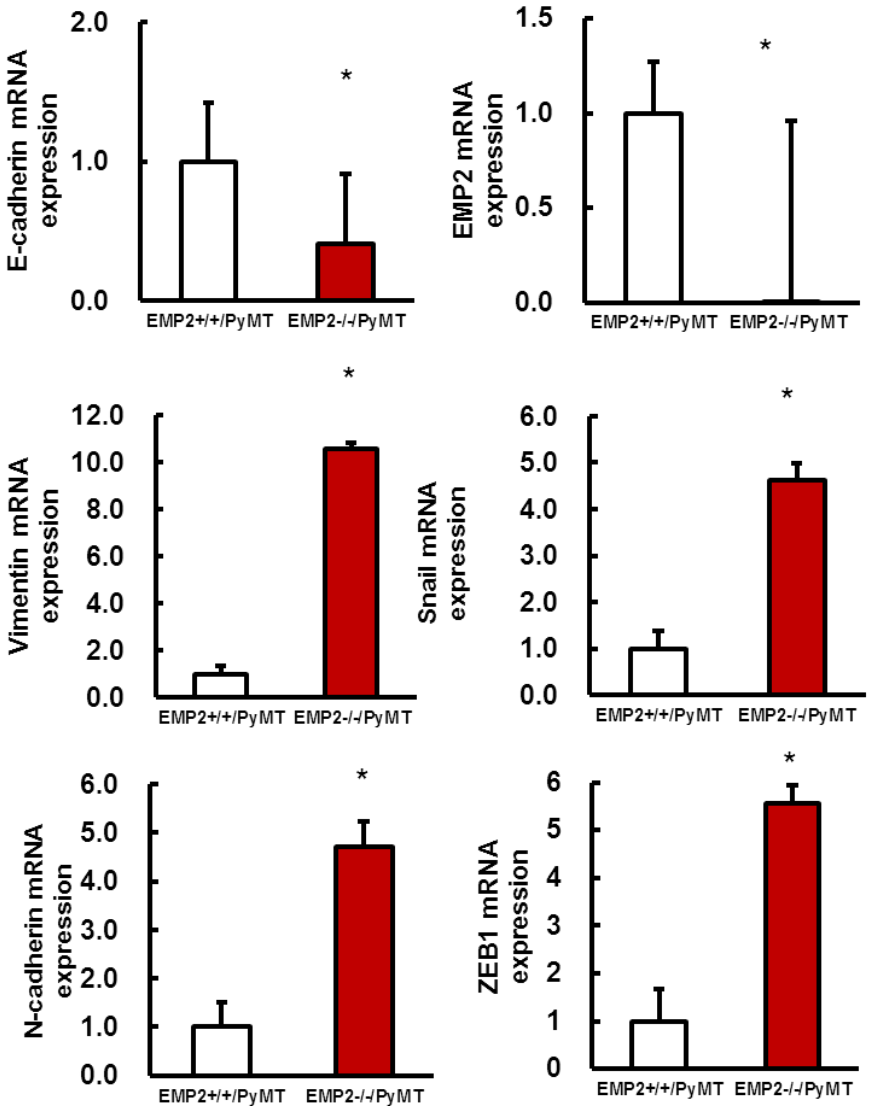

Figure 12. Loss of EMP2 induces EMT in primary MMTV-PyMT cells.

Real-time RT-PCR analysis of EMT markers markers and EMT-related gene expression in in primary PyMT cells. All experiments were performed in triplicate. All quantitative data are depicted as mean ±S.E. per group. *P<0.05

3.4. Loss of EMP2 promotes migration and invasion in breast cancer metastasis

Migration and invasion are typical characteristics of EMT. It is clearly to see that EMP2 knockdown results in increase of migration and invasion abilities in SKBR3 cells. Invasion ability of EMP2 knockdown increases 3-fold and migration ability increases 2-fold (Fig. 13.). Hence, I strongly confirmed that loss of EMP2 enhance migrated and invaded abilities of breast cancer cells.

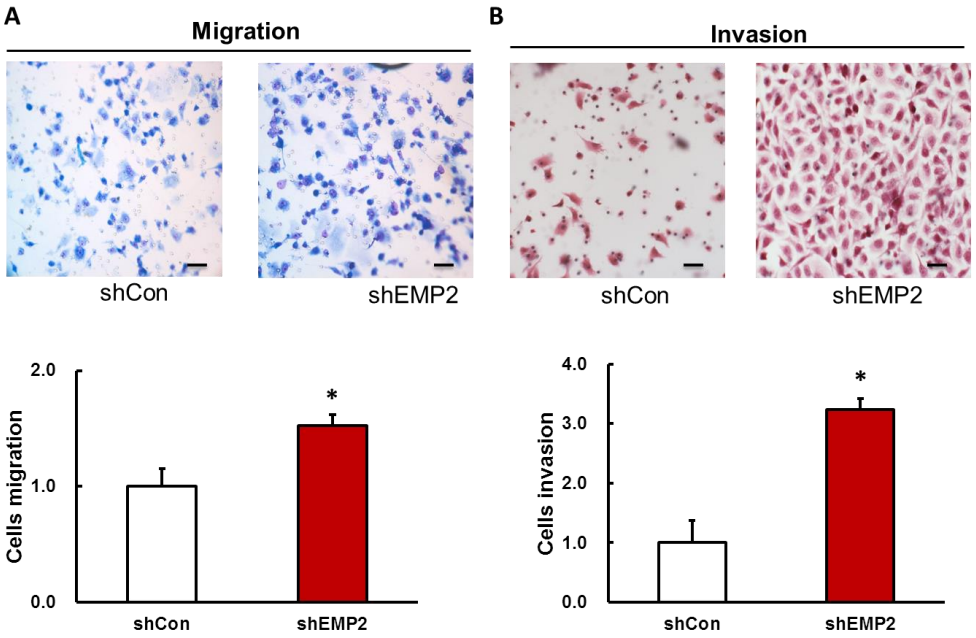

Figure 13. Loss of EMP2 increases the ability of migration and invasion.

(A) Migration (5×10^4 cells per well) and (B) invasion (2×10^5 cells per well) assays in SKBR3 cells. After incubation, cells were analyzed by counting the number of migrated cells in four randomly selected microscopic fields per well. All experiments were performed in triplicate. All quantitative data are depicted as mean ± S.E. per group. *P<0.05. Scale bars: 20 μm.

4. Discussion

In this study, I demonstrated deficiency of EMP2 induced EMT in breast cancer, leading to more metastatic cancer progression. EMT contributes to cancer cell invasion and metastasis by allowing malignantly transformed epithelial cells to migrate, invade the surrounding stroma, and spread through the blood and lymphatic system to distant sites [33]. Loss of EMP2 in SKBR3 cells conferred enhanced motility and invasiveness, hallmarks of EMT. Furthermore, loss of Emp2 increases lung metastasis in PyMT breast cancer mouse model. These observations the idea that loss of EMP2 induces EMT. EMP2 seems to be similar to E-cadherin since loss of E-cadherin induces EMT [34]. So, EMP2 may be involved in maintaining epithelial characteristics.

My findings are to demonstrate loss of Emp2 promotes pulmonary metastasis in MMTV-PyMT/EMP2$^{-/-}$ mice. There are growing evidence that has shown EMP2 belongs to GAS3/PMP22 gene family that their trafficking to glycolipid-enriched lipid raft domains is important in receptor signaling. So, EMP2 is related with a variety of cellular processes, such as invasion, migration, proliferation. Collectively, that EMP2 affects the metastasis and tumor formation of breast cancer cell.

By the mechanism are signal transductions as NF-κB pathway, the EMP2 gene has been knocked out. After that, it promotes breast cancer and pulmonary metastasis. By the current studies from my laboratory, the MMTV-PyMT/EMP2$^{-/-}$ mice showed their phenotypes with increasing tumor amount compared to

MMTV-PyMT/EMP2$^{+/+}$ mice.

In this study, I found that both the expression changes in EMP2 knockdown SKBR3 cell lines and MMTV-PyMT/EMP2$^{-/-}$ primary cells could affect the invasion, proliferation and migration. It was clearly to see the upregulation in epithelial marker and downregulation in mesenchymal markers. In fact, MMTV-PyMT mice also developed multifocal adenocarcinoma at 5 weeks of age. Recent studies have highlighted the important role of EMT in tumor progression [35]. The transition from epithelial to mesenchymal promotes the tumorigenesis. Moreover, EMP2 knockdown in SKBR3 cells enhanced their migrated and invasive abilities. The results verified the expression in cancer patients when EMP2 high expression has higher survival rate than EMP2 low expression. Immunoblotting, RT-PCR and qPCR immunohistochemistry analysis results represent that EMP2 leads to increase mesenchymal markers such as N-cadherin, Vimentin, Snail and ZEB1 and decrease epithelial markers E-cadherin. So, I strongly confirm the importance of EMP2 gene in breast cancer as a metastasis suppressor. In summary, my data begin to clarify that the important role of EMP2 gene in breast cancer progression. The metastasis becomes faster by EMT in EMP2 knockdown human cancer cells and MMTV-PyMT/EMP2$^{-/-}$ mouse models.

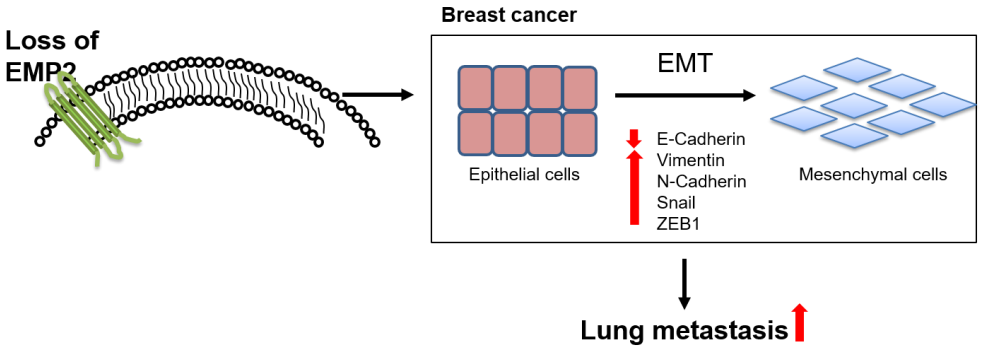

Figure 14. Effect of EMP2 on inducing EMT and increasing lung metastasis.

Loss of EMP2 results in decreasing the level of epithelial marker and increases the levels of mesenchymal markers then promotes pulmonary metastasis. Modified picture from [36]

5. Conclusion

In conclusion, my study has revealed that EMP2 in breast cancer play a role on suppressor gene. Loss of EMP2 induces EMT and lead to metastasis. It also provides useful knowledge for further investigations on metastasis study and dignose. Taken together, my results identify the function of EMP2 as a new biomarker in metastasis and demonstrate its prognostic value in cancer treatment.

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