

# Anti-cancer Effect of Unique Cartilage Matrix-associated Protein in Breast Cancer Cells Depends on $\gamma$ -Carboxylation

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**Abstract.** *Background/Aim:* Unique cartilage matrix-associated protein (UCMA), a recently discovered vitamin K-dependent protein (VKDP) with a large number of  $\gamma$ -carboxyglutamic acid (Gla) residues, is associated with ectopic calcifications. Although the function of VKDPs is related to their  $\gamma$ -carboxylation status, the carboxylation status of UCMA in breast cancer is still unknown. Here, we investigated the inhibitory effect of UCMA with differing  $\gamma$ -carboxylation status on breast cancer cell lines, such as MDA-MB-231, 4T1, and E0771 cells. *Materials and Methods:* Undercarboxylated UCMA (ucUCMA) was generated by mutating the  $\gamma$ -glutamyl carboxylase (GGCX) recognition sites. The ucUCMA and carboxylated UCMA (cUCMA) proteins were collected from culture media of HEK293-FT cells that had been transfected with mutated GGCX and wild-type UCMA expression plasmids, respectively. Boyden Transwell and colony formation assays were performed to evaluate cancer cell migration, invasion, and proliferation. *Results:* Culture medium containing cUCMA protein inhibited the migration, invasion, and colony formation of MDA-MB-231 and 4T1 cells to a greater degree than medium containing ucUCMA protein. Significant reductions in the migration, invasion, and colony formation were also observed in cUCMA-treated E0771 cells compared to those in ucUCMA-treated cells. *Conclusion:* The inhibitory role of UCMA in breast cancer is closely related to its  $\gamma$ -

carboxylation status. The results of this study may be a basis for the development of UCMA-based anti-cancer drugs.

Vitamin K-dependent proteins (VKDPs) undergo post-translational carboxylation via the addition of a COO-molecule to the  $\gamma$ -carbon of glutamic acid (Glu) by  $\gamma$ -glutamyl carboxylase (GGCX) to form  $\gamma$ -carboxyglutamic acid (Gla), which is the biologically active form (1). Although this carboxylation is common to all VKDPs, including coagulation factors, the production and activation processes vary. Coagulation factors and other hepatic VKDPs are produced and carboxylated in the liver, whereas extrahepatic VKDPs, such as unique cartilage matrix-associated protein (UCMA), matrix Gla protein (MGP), and osteocalcin (OC), are carboxylated in other tissues, like the bone and vascular tissues.

UCMA is a newly discovered VKDP, also termed Gla-rich protein (GRP), that contains a higher number of Gla domains (16 Gla residues across 74 amino acids) than other VKDPs, such as MGP and OC (2). UCMA was first detected in the cartilaginous tissues of mice, rats, and sturgeon. It accumulates in the bone, skin, and vascular tissues, where it is secreted into the extracellular matrix (3). UCMA is highly conserved in humans, mice, rats, dogs, and zebrafish (4). UCMA plays a role in promoting osteoblast differentiation and calcium deposition in bone and inhibiting ectopic calcification in the vascular system (5, 6). We recently reported the inhibitory effect of UCMA on the migration, invasion, and *in vivo* tumor growth of triple-negative breast cancer (TNBC) (7).

The carboxylation status of VKDPs correlates with its biological activity. Various coagulation factors need to be carboxylated to functionally bind to calcium and phospholipid membranes (8). MGP requires carboxylation to exert its inhibitory function in vascular calcification (9). The carboxylation of OC increases its calcium binding and enhances hydroxyapatite incorporation to form bone matrix and to promote bone mineral density (2). The carboxylation of UCMA also plays a role in inhibiting aortic calcification

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(6), reducing mineral deposition in osteoarthritis (10, 11), and blocking the release of inflammatory cytokines (12). Interestingly, several studies have reported that vitamin K-dependent carboxylation plays a role in liver cancer (13), and that the accumulation of UCMA in cancer tissues depends on its carboxylation (14), suggesting UCMA carboxylation as a potential marker for various pathological conditions, including cancer. However, the role of VKDP carboxylation in cancer development remains unclear. In this study, we investigated the role of UCMA carboxylation on its inhibitory effects on breast cancer cells, and our results highlight that targeting UCMA carboxylation in breast cancer cells can be a novel therapeutic strategy for breast cancer.

## Materials and Methods

**Cell culture.** As TNBC cell lines, the mammary gland/breast metastatic site-derived human breast epithelial cell line MDA-MB-231 and the mammary gland tissue-derived mouse breast cancer cell line 4T1 were used (15, 16). E0771 (CH3 BioSystems, Amherst, NY, USA) was used as an estrogen-receptor-positive luminal type B mouse breast cancer cell line (17). MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose (Hyclone, Rockford, IL, USA), and 4T1 and E0771 cells were cultured in RPMI-1640 medium (HyClone). All culture media were supplemented with 10% fetal bovine serum (FBS, HyClone), 100 U/ml penicillin, and 100 µg/ml streptomycin (Thermo Scientific, Waltham, MA, USA). HEK293-FT cells were cultured in DMEM-high (Hyclone) supplemented with 10% FBS and appropriate antibiotics.

**Preparation of culture medium containing secreted UCMA protein.** Generation of the construct harboring mutated  $\gamma$ -glutamyl carboxylase (mGGCX) recognition sites in UCMA has been described previously (18). Briefly, three nucleotide substitutions, T208A, C227T, and C238A, were generated at the GGCCX recognition site in UCMA, designated as UCMA/mGGCX, to mutate three amino acids - phenylalanine (F) to isoleucine (I) at amino acid position 48 (F48I), alanine (A) to valine (V) at position 54 (A54V), and leucine (L) to isoleucine (I) at position 58 (L58I), respectively - in UCMA protein. To obtain culture medium containing secreted UCMA protein, the UCMA or UCMA/mGGCX expression plasmid was transiently transfected into HEK293-FT cells at a density of  $3 \times 10^5$  cells/well in 6-well culture plates. The cells were incubated in serum-free medium for 4-6 h, recovered in 10% FBS for 5 h, and then transferred to fresh serum-free medium. After overnight incubation, the culture medium was collected and centrifuged at 100 g for 5 min to remove cell debris and dead cells. Culture media harvested from UCMA- and UCMA/mGGCX-transfected cells were designated as  $\gamma$ -carboxylated UCMA (cUCMA) and undercarboxylated UCMA (ucUCMA), respectively.

**Boyden Transwell chamber migration and invasion assay.** Cell migration and invasion assays were conducted in a modified Boyden chamber system using a Transwell chamber with a cell growth area of 0.3 cm<sup>2</sup> and membrane pore size of 8 µm (Falcon, Corning, NY, USA), as described previously (7). For the migration assay,  $5 \times 10^4$  MDA-MB-231, 4T1, or E0771 cells were transferred

to the upper chamber with 200 µl of serum-free ucUCMA or cUCMA medium. A 700-µl of growth medium containing 2% FBS was placed in the lower chamber. After 6 h incubation, the cells remaining on the upper surface of the Transwell chamber were removed, and the migrated cells on the lower surface of the membrane were stained with crystal violet and counted. For the invasion assay, 100 µl of a 1:2 mixture of Matrigel and serum-free ucUCMA or cUCMA medium was placed in the upper chamber, and 700 µl growth medium containing FBS was added to the lower chamber. After overnight starvation,  $5 \times 10^4$  cells were seeded in the upper chamber. After 36 h incubation, the number of invaded cells was counted following visualization using crystal violet staining.

**Colony formation assay.** For the colony formation assay, 50 MDA-MB-231 or E0771 cells, and 1,000 cells of 4T1 were seeded in separate wells of 6-well plates. The cells were cultured in medium supplemented with 10% FBS and 10% ucUCMA or cUCMA medium. After 14 days incubation for MDA-MB-231 and E0771 cells and 9 days incubation for 4T1 cells, colonies were fixed, stained with 4% crystal violet, and counted.

**Statistical analysis.** All data are presented as means  $\pm$  standard deviation (SD) and were analyzed using Student's *t*-test. Cell culture experiments were performed independently at least twice, and each experiment was performed in duplicate. A *p*-value <0.05 was considered statistically significant.

## Results

Previously, we observed that UCMA over-expression in the TNBC cell lines MDA-MB-231 and 4T1 inhibited migration, invasion, and colony formation, suggesting that UCMA is a promising therapeutic agent for TNBC (7). UCMA is a VKDP, and its carboxylation status correlates with various biological activities. cUCMA has been reported to be biologically active and to accumulate under pathological conditions (8, 10, 13). Based on this information, we investigated the carboxylation dependence of the effect of UCMA on breast cancer cells, including TNBC cells. Cell migration, invasion, and colony formation assays were performed using breast cancer cells treated with either cUCMA or ucUCMA, which is culture medium containing a carboxylated form of the wild-type UCMA protein or an undercarboxylated form of the UCMA protein, respectively. Cell migration was dramatically decreased in MDA-MB-231 cells treated with cUCMA compared to those treated with ucUCMA (Figure 1). The number of migrated MDA-MB-231 cells was significantly reduced by cUCMA treatment (Figure 1). Further, 4T1 and E0771 breast cancer cells treated with cUCMA exhibited significant reduction in cell migration compared to those treated with ucUCMA (Figure 1). The number of migrated 4T1 and E0771 cells that had been treated with cUCMA was also significantly lower than the number of migrated ucUCMA-treated cells (Figure 1). Compared to cells treated with ucUCMA, treatment with cUCMA reduced the migration of MDA-MB-231, 4T1, and

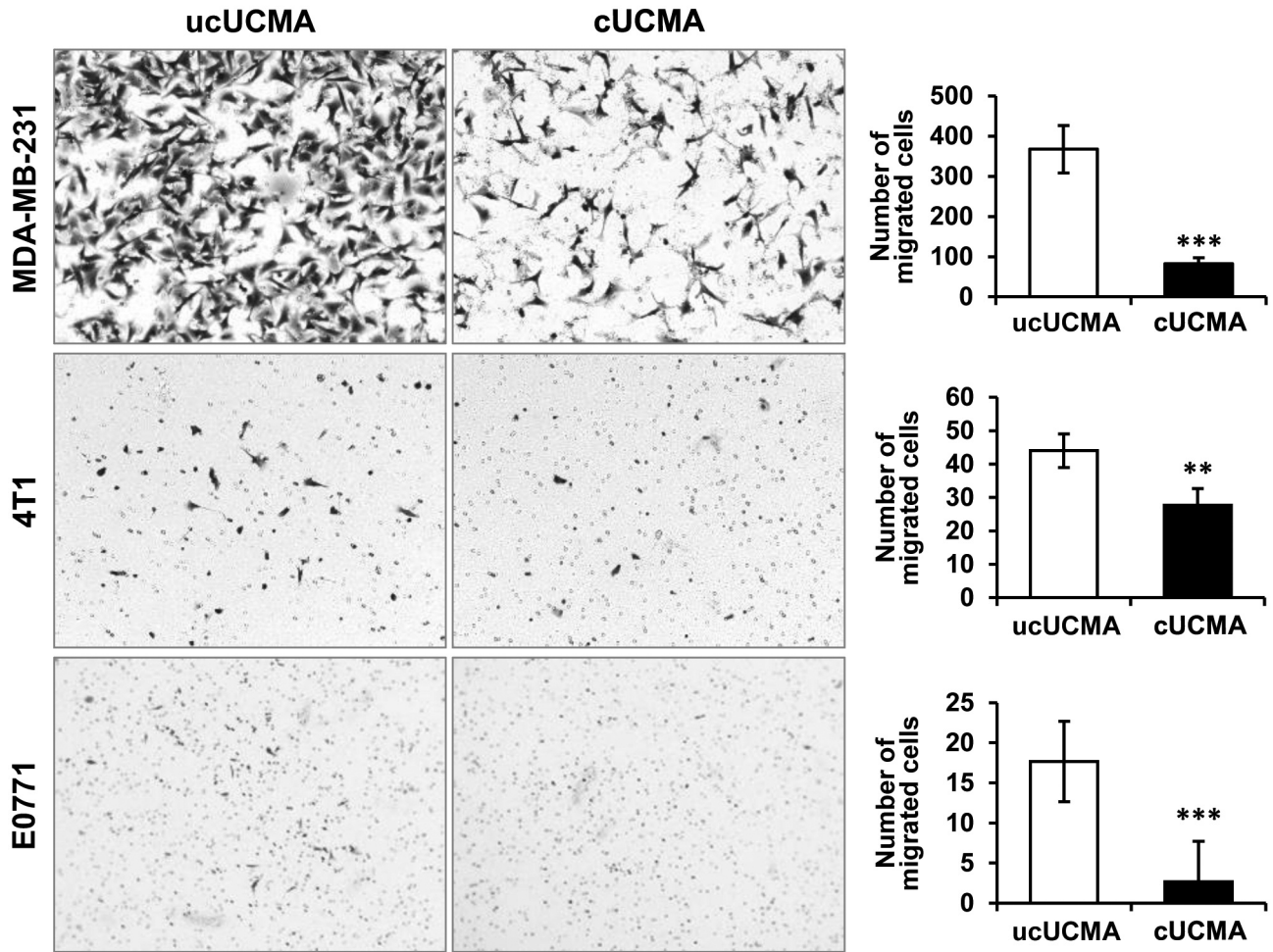


Figure 1. Inhibition of migration of breast cancer cells by  $\gamma$ -carboxylated UCMA (cUCMA). In the migration assay, three breast cancer cell lines (MDA-MB-231, 4T1, and E0771) treated with culture medium containing undercarboxylated UCMA (ucUCMA) or cUCMA protein were placed in the upper chamber, and growth medium containing FBS was placed in the lower chamber. Quantitative analyses were performed by cell counting, and the data are presented as mean $\pm$ SD. \*\* $p$ <0.01 and \*\*\* $p$ <0.001 versus ucUCMA,  $n$ =3.

E0771 cells by 77%, 37%, and 84%, respectively (Figure 1). Similar to the inhibitory effect of cUCMA on cell migration, the invasiveness of MDA-MB-231, 4T1, and E0771 cells treated with cUCMA was also decreased (Figure 2). The number of invasive MDA-MB-231, 4T1, and E0771 cells reduced by 70%, 74%, and 42%, respectively, following treatment with cUCMA when compared to cells treated with ucUCMA (Figure 2). Colony formation of cUCMA-treated MDA-MB-231, 4T1, and E0771 cells was reduced compared to that of ucUCMA-treated cells, and the number of cUCMA-treated colonies was significantly lower by 21%, 23%, and 21% in MDA-MB-231, 4T1, and E0771 cells, respectively (Figure 3). Collectively, these results indicate that culture medium containing cUCMA protein significantly

inhibits the migration, invasion, and colony formation of breast cancer cells, including TNBC cells.

## Discussion

Vitamin K is essential for the biological activity of VKDPs as it is involved in catalyzing  $\gamma$ -carboxylation (1, 2). Vitamin K deficiency inactivates VKDPs, which increases the risk of a bleeding disorders, cardiovascular diseases, and bone fractures (1, 2). The functions of three major extrahepatic VKDPs, MGP, OC, and UCMA, in bone homeostasis and ectopic calcification have been explored; additional functions of these proteins are now being investigated, with a focus on cancer pathogenesis (19, 20). Results thus far suggest that the carboxylation status

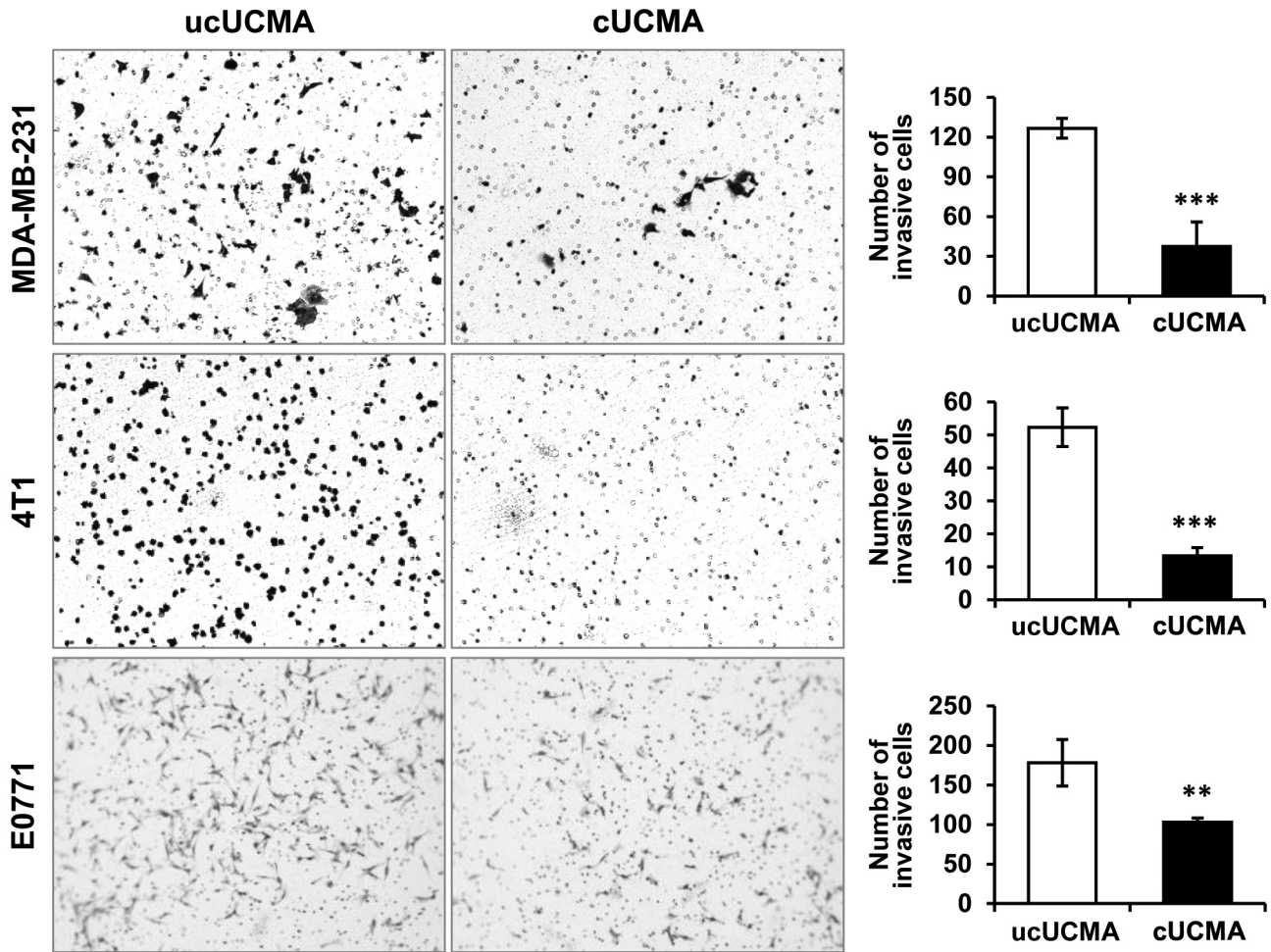


Figure 2. Inhibition of invasion of MDA-MB-231, 4T1, and E0771 breast cancer cells by cUCMA. For the invasion assay, the three breast cancer cell lines were serum-starved overnight and seeded in the upper chamber of a Transwell with Matrigel mixed with serum-free medium containing ucUCMA or cUCMA; growth medium containing FBS was placed in the lower chamber. Quantitative analyses were performed by cell counting, and the data are presented as mean±SD. \*\* $p < 0.01$  and \*\*\* $p < 0.001$  versus ucUCMA,  $n = 3$ .

of VKDPs may be important for its anti-cancer effects. MGP regulates colon cancer proliferation by upregulating the calcium signaling pathway (21) and promotes liver metastasis of colon cancer through activation of NF- $\kappa$ B signaling (22). Vitamin K-dependent carboxylation of MGP plays a key role in inhibiting vascular calcification and ectopic tissue calcification (9). However, whether the carboxylation status of MGP is important but its role in cancer has not been elucidated. OC regulates the development of metastatic lung cancer in bone tissue, which is associated with OC-expressing osteoblasts in the bone microenvironment (23). The growth of prostate cancer cells is accelerated by carboxylated OC, which is essential for promoting bone mineralization, and is inhibited by undercarboxylated OC (2, 24). Additionally, undercarboxylated OC promotes proliferation and metastasis of breast cancer cells

(25). These results suggest that the carboxylation status of OC is essential for its role in regulating cancer growth. Our recent functional study on UCMA in TNBC cells revealed its anti-cancer effects, specifically in inhibiting cell migration, invasion, and colony formation (7). Survival analysis using the Kaplan–Meier plotter database confirmed that high UCMA expression was significantly correlated with high survival rates in patients with TNBC (7).

A previous study investigated the possible role of UCMA carboxylation in pathological mineralization, including in cancer (14). cUCMA was predominant in healthy tissues, whereas ucUCMA accumulated in tumor cell cytoplasm, suggesting UCMA carboxylation status as a potential marker of cancer (14). In this study, we investigated the  $\gamma$ -carboxylation dependence of UCMA function in breast

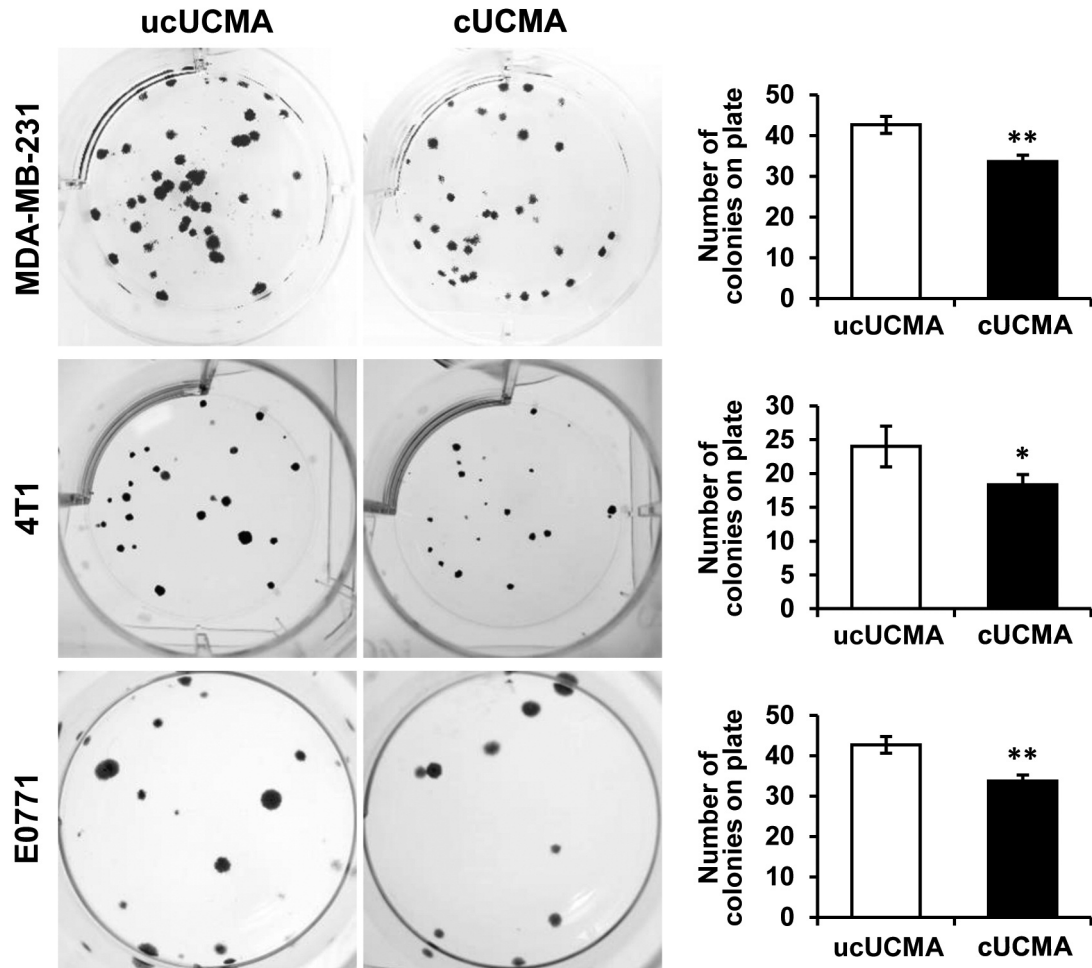


Figure 3. Inhibition of colony formation of MDA-MB-231, 4T1, and E0771 breast cancer cells by cUCMA. MDA-MB-231 and E0771 cells were seeded at 50 cells per well and cultured for 14 days; 4T1 cells were seeded at 1,000 cells per well and cultured for 9 days in the presence of 10% culture medium containing ucUCMA or cUCMA. Quantitative analyses were performed by colony counting, and the data are presented as mean $\pm$ SD. \* $p$ <0.05 and \*\* $p$ <0.01 versus ucUCMA,  $n$ =3.

cancer cells. As shown in Figure 4, treatment with cUCMA led to dramatic suppression of the migration, invasion, and colony formation of breast cancer cells compared to treatment with ucUCMA, suggesting that the  $\gamma$ -carboxylation status of UCMA is critical for its anti-cancer effects. Although the concentration of ucUCMA or cUCMA protein present in the conditioned medium was not measured, our findings clarified the role of carboxylation status in the anti-cancer effect of UCMA in breast cancer cells. However, the precise molecular mechanism by which cUCMA inhibits breast cancer cell migration, invasion, and colony formation remains unclear. Since altered calcium signaling is crucial for cancer development and progression, many anti-cancer drugs, such as doxorubicin, paclitaxel, and docetaxel, have been developed to control calcium signaling (26). UCMA is

a GRP with a high affinity for calcium (2), suggesting that the anti-cancer effects of cUCMA may be related to calcium signaling, although further studies are needed to confirm this. Additional experiments using cUCMA protein are also necessary to determine the dose range of cUCMA required for exerting its anti-cancer effects; these studies would be useful for the development of anti-cancer drugs for breast cancer. We elucidated the importance of  $\gamma$ -carboxylation of UCMA in breast cancer, providing a basis for anti-cancer drug development and breast cancer diagnosis.

### Conflicts of Interest

All Authors have no competing interests to declare in relation to this study.

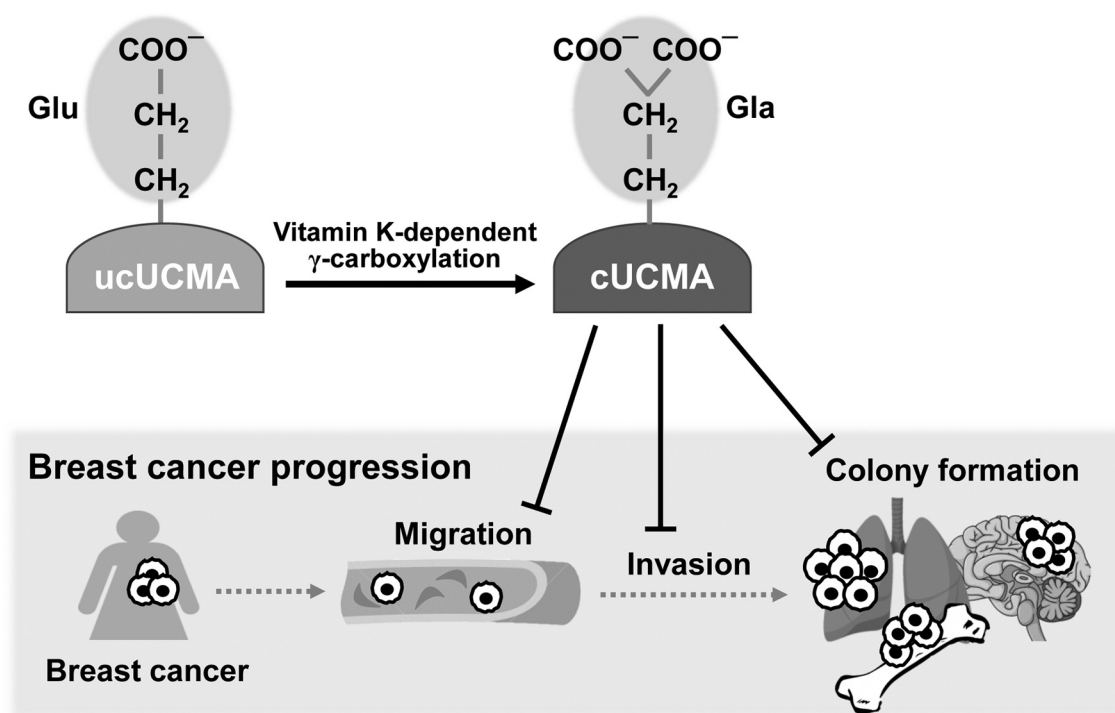


Figure 4. Schematic illustration of the inhibitory role of  $\gamma$ -carboxylated UCMA in breast cancer. Carboxylated UCMA (cUCMA) is generated by vitamin K-dependent  $\gamma$ -carboxylation of undercarboxylated UCMA (ucUCMA). During breast cancer progression, cUCMA inhibits breast cancer cell migration into blood vessels, invasion into other organs, and colony formation in metastasized organs. Glu: Glutamic acid; Gla:  $\gamma$ -carboxyglutamic acid.

### Authors' Contributions

Conception and design, NRP, YJL and JEK; Experiments, analysis, and interpretation of data, NRP, YJL and SHL; Writing, review, and revision of the manuscript, NRP and JEK.

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