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14. ABSTRACT The majority of breast cancer mortality is caused by metastatic relapse, which requires the reactivation and outgrowth of tumor cells after they have disseminated to pre-metastatic sites and undergone prolonged dormancy. Understanding the mechanisms underpinning dormancy, reactivation, and outgrowth will provide novel approaches for the prevention and treatment of metastatic diseases. We discovered the TGFβ signaling antagonist Coco as a mediator of the reactivation of lung-disseminated breast cancer cells. We also made contributions to the mechanistic understanding and cancer relevance of autophagy, a catabolic response sustaining cancer cell survival under stress and therapeutic treatment. In this proposal, we investigate the role of autophagy and its interplay with Coco signaling in breast cancer dormancy and metastatic relapse, and the therapeutic potential of targeting autophagy for the treatment of breast cancer metastasis. Over the last reporting period, we have further deepened our understanding of the Coco signaling in dormancy reactivation, and the mechanisms and tumorigenic role of autophagy. We have developed a series of genetically controlled cancer cell models for in vivo mouse studies to define the roles and interplay of Coco signaling with autophagy in breast cancer dormancy and reactivation, as well as the therapeutic effect of targeting Coco and autophagy. Monoclonal antibodies against Coco have also been developed for future Coco-targeted therapies.					
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Introduction

The goal of this project is to determine the role of autophagy and its interplay with Coco signaling in breast cancer dormancy and metastatic relapse, and the therapeutic potential of targeting autophagy and Coco for the treatment of breast cancer metastasis. The majority of breast cancer mortality is caused by metastatic relapse, which requires the reactivation and outgrowth of tumor cells after they have disseminated to pre-metastatic sites and undergone prolonged dormancy. Therefore, understanding the mechanisms underpinning dormancy, reactivation, and outgrowth will provide novel approaches for the prevention and treatment of metastatic disease. Recently, we developed a novel mouse model for breast tumor dormancy, and using this model we discovered that a signaling molecule called Coco mediates the reactivation of lung-disseminated breast cancer cells. Analysis of breast cancer patient samples revealed a significant correlation of Coco expression with lung metastasis and poor prognosis, suggesting that Coco expression can be used as a diagnostic tool. Furthermore, Coco signaling may regulate autophagy, a survival response that has been frequently implicated in cancer cell survival under stress and therapeutic treatment. In this research project, we not only investigate fundamental questions about the biology of breast cancer metastasis, but also test our clinically-relevant hypothesis that targeting autophagy is a viable strategy for eradicating the disseminated breast cancer cells that are responsible for metastatic relapse, and that combined inhibition of autophagy and Coco can induce the death of dormant cells and prevent their reactivation. Over the last year (the reporting period), we have made notable progress in keeping with the Statement of Work of the grant. Further, to translate this research into breast cancer treatment, the two laboratories involved in this project are also actively developing small molecule inhibitors targeting specific autophagy proteins and humanized antibodies blocking Coco. These inhibitors will be the drug leads for further medicinal chemistry and preclinical/clinical studies.

Body

Overview Over the first year of the grant period, we have made significant progress largely in keeping with the approved Statement of Work (SOW). In addition to the original plan, we have also made significant progress in generating monoclonal antibodies for Coco, which may be a promising tool for future Coco-targeted therapies. There is one area of experiments that are lagging though, which is the area of xenograft mouse model experiments, largely due to unexpected half-year delay of the official approval of our ACURO animal protocol. Below we report our progress, starting with the SOW check list below followed by detailed description.

Statement of Work (SOW):

Progress/Status

Task-1: To determine the effect of autophagy in the dormancy of breast cancer cells in xenograft mouse models (months 1-12)

1a. To establish 4T07 cell lines harboring triple modality reporter gene and (1) tetracycline (tet)-inducible LacZ shRNA as control, (2) tet-inducible ATG7-shRNA (two constructs), or (3) tet-inducible ULK1-shRNA (two constructs); confirm the knockdown effect on autophagy-block (**months 1-3; Jiang Lab**).

Completed as planned

1b. To establish breast cancer cell dormancy at the lung in nude mice using the cell lines established in task-1a, induce autophagy-RNAi by doxycycline-feeding, and prepare lung sections. Each condition needs 10 mice, 50 mice total (**months 4-8; Giancotti Lab**).

In progress

1c. To determine the role of autophagy in dormancy by immuno-histological analysis of the lung sections (**months 9-12; Giancotti Lab**).

In progress

Task-2: To examine the interplay of BMP signaling, autophagy, and cell death in breast cancer cells in cultured cells (months 4-14)

2a. To establish 4T07 cell lines expressing GFP-LC3 that can be used for monitoring autophagy by immunofluorescence. These cell lines will also harbor (1) tet-inducible LacZ shRNA as control, (2) tet-inducible ATG7-shRNA, or (3) tet-inducible ULK1-shRNA (**Jiang Lab, months 4-5**).

Completed as planned

2b. To establish the potential role of the canonical TGF β signaling pathway in BMP-induced autophagy in 4T07 cells by Smad6 overexpression and Smad1 RNAi (**Jiang Lab, months 5-8**).

Completed as planned

2c. To determine whether Coco can antagonize BMP-induced autophagy (**Jiang Lab, months 9-10**).

Completed as planned

2d. To determine whether tet-inducible, RNAi-mediated autophagy-block can result in an increase of cell death upon BMP treatment. Both apoptotic and non-apoptotic cell death, as well as long-term viability of the cells will be assessed (**Jiang Lab, months 11-14**).

In progress as planned

Task-3: To determine the role of autophagy in Coco-induced reactivation of lung-disseminated breast cancer cells in mouse models (months 1-15)

3a. To establish 4T07 cell lines expressing triple modality reporter gene, tet-inducible Coco expression, and tet-inducible shRNA against autophagy genes or LacZ control. Totally 5 cell lines (**Jiang Lab, months 1-3**).

Completed as planned

3b. To establish breast cancer cell dormancy at the lung in nude mice using the cell lines established in task-3a; induce Coco expression (thus reactivation) and/or autophagy-RNAi by doxycycline-feeding; and prepare lung sections. Each condition needs 10 mice, 50 mice total (**Giancotti Lab, months 4-9**).

In progress

3c. To determine the role of autophagy in Coco-induced exit from dormancy by immuno-histological analysis of the lung sections (**Giancotti Lab and Jiang Lab, months 10-15**).

In progress as planned

Continued in the next page

Continued --- Statement of Work (SOW):

Progress/Status

Task-4: To determine the effect of autophagy on BMP/Coco-regulated cancer cell stemness/self-renewal capability (months 6-15)

4a. In a 4T07 cell culture model, determine the role of autophagy in BMP4/Coco-regulated tumor sphere formation (**Jiang Lab, months 6-12**).

In progress

4b. In a 4T07 cell culture model, determine the role of autophagy in BMP4/Coco-regulated expression of multiple stem cell markers (**Jiang Lab, months 6-12**).

In progress

4c. Using 4T07 cell lines harboring tet-inducible Coco expression and autophagy-RNAi or control-RNAi (5 genetic conditions, each with or without tet induction), determine the role of Coco and autophagy in tumor initiation ability of the cancer cells in vivo. Each condition needs 10 mice, 100 mice total (**Giancotti Lab and Jiang Lab, months 9-15**).

In progress as planned

Task-5: To study the function of autophagy in lung-metastatic breast cancer outgrowth (months 13-20)

5a. To establish 4T1 cell lines harboring triple modality reporter gene and (1) tet-inducible LacZ shRNA as control, (2) tet-inducible ATG7-shRNA, or (3) tet-inducible ULK1-shRNA (**month 13; Jiang Lab**).

5b. Using cell lines established in 5a and xenograft models, determine the role of autophagy in lung-metastatic breast cancer cell outgrowth at the pre-angiogenesis stage. Each condition needs 10 mice, 50 mice total (**months 14-20; Jiang Lab**).

5C. Determine the role of autophagy in lung-metastatic breast cancer cell outgrowth after exiting dormancy but at the post-angiogenesis stage in xenograft mouse models. Each condition needs 10 mice, 50 mice total (**months 14-20; Giancotti Lab**).

Tasks 5 and 6 are for the second year of the grant period, thus we have not worked on them yet.

Task-6: To examine the therapeutic potential of combination targeting of autophagy and Coco (months 13-24)

6a. To establish a collection of 4T1 cell lines harboring triple modality reporter gene, and/or tet-inducible Coco-shRNA, and/or tet-inducible autophagy-shRNA (**months 13-14; Jiang Lab**).

6b. In xenograft mouse models, establish breast cancer lung metastasis using these cell lines. Upon dormancy establishment or reactivation/outgrowth from dormancy, induce autophagy-RNAi, Coco-RNAi, or their combination by doxycycline-feeding. Subsequently, assess the therapeutic effect of autophagy-targeting, Coco-targeting, or their combination, by monitoring metastatic tumor growth, immunohistology, and Kaplan-Meier animal survival. Each condition needs 10 mice, 80 mice total (**months 15-24; Giancotti Lab and Jiang Lab**).

Detailed description This project aims to dissect the potential role of autophagy in three distinctive stages of Coco-induced breast cancer metastatic relapse at the lung: dormancy, reactivation from dormancy, and outgrowth. We are using inducible RNAi to regulate autophagy in breast cancer cells with defined Coco expression, thus dormancy status. Using these cells and our newly developed mouse model for breast tumor dormancy and outgrowth, we will also determine the therapeutic potential of targeting autophagy and Coco.

(1) Genetic engineer of multiple tool tumor cell lines (Tasks-1a, 2a, 3a). Up to now, we have accomplished the development of various breast cancer cell lines with defined Coco expression status that harbor GFP-LC3 (to monitor autophagy) and doxycycline (DOX)-inducible shRNA of autophagy genes (ULK1 and ATG7, as well as LacZ control). In these cells, we can manipulate autophagy in a DOX-induced manner. Further, we introduced triple modality reporter gene into these cells, enable live animal imaging of tumor cells in the in vivo experiments. These cells are currently used in xenograft experiments to examine the role of autophagy in

Coco-mediated reactivation of dormant breast cancer in lung. Fig. 1 gives an example of such genetically engineered cell line in which autophagy can be controlled by DOX. In these experiments, autophagy was monitored by LC3-I/LC3-II conversion (Fig. 1A-C) and GFP-LC3 translocation (Fig. 1D).

(2) The potential interplay between TGF β signaling and autophagy We examined the possibility that TGF β signaling and its antagonist Coco are important regulators of autophagy in breast cancer cells (Tasks-2b, 2c). However, the effect of TGF β signaling on nutrient starvation-induced autophagy is rather modest, if any, thus ruling out the possibility. We will examine the opposite possibility: since pre-angiogenic stress triggers autophagy in the metastatic tumor cells, is autophagy able to modulate TGF β signaling and thus reactivation of breast cancer cells from dormancy?

(3) The role of autophagy in metastatic dormancy, reactivation from dormancy, and outgrowth. This study is ongoing using the novel Coco-dictated xenograft mouse models and the

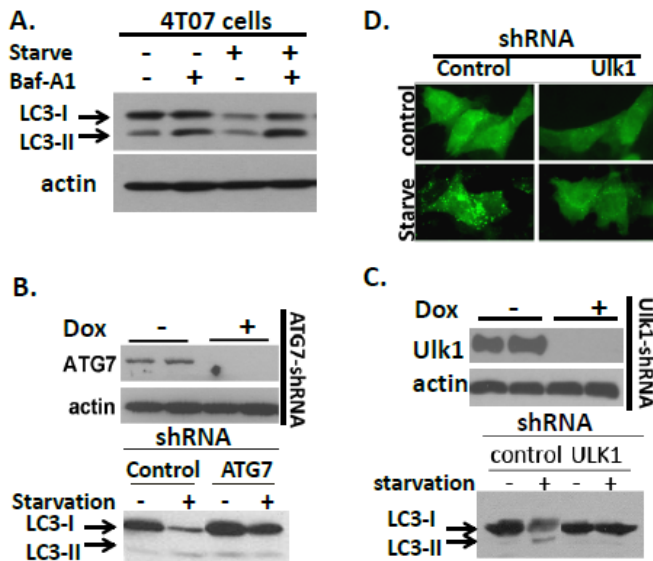


Fig. 1. Breast cancer cells harboring GFP-LC3 and DOX-inducible shRNAs targeting ATG genes. A) 4T07 cells possess intact autophagy response upon starvation. B) DOX-inducible ATG7-shRNA blocks autophagy as monitored by LC3-I/LC3-II conversion. C) DOX-inducible ULK1-shRNA blocks autophagy. D) same as C) but monitored by GFP-LC3 translocation.

inducible autophagy RNAi breast cancer cell lines we have developed.

(4) Monoclonal Antibodies Blocking Coco and their Preclinical Efficacy Because Coco promotes dormant breast cancer cell outgrowth from lung metastatic sites, it is a possible therapeutic target. As an extracellular factor, Coco can be targeted by specific monoclonal antibodies. For this reason, we developed monoclonal antibodies that block Coco's binding to BMP proteins and evaluate their preclinical efficacy, as single agents or in combination with Lapatinib, in mouse models of ErbB2-mediated mammary tumorigenesis. Our preliminary results are exciting. We are aware that this direction is not in our original plan, but it is in line with our ultimate goal of developing Coco- and autophagy-targeted therapies to treat lung-metastatic breast cancer. Therefore, below we describe **the progress as well as the immediate future plan** of this direction.

The atomic structure of the prototypical BMP inhibitor Noggin in complex with BMP7 indicates that these inhibitors act as ligand traps, preventing ligand binding to their cognate receptors (Fig. 2A, next page)¹. We plan to test the pre-clinical efficacy of MAbs that block Coco's binding to BMP proteins. We anticipate that these MAbs will inhibit the self-renewal capacity of metastasis-initiating cells. Since these cells exhibit properties of tumor progenitor cells and may therefore be partially resistant to chemotherapy or oncogene-targeted therapy², we hypothesize that inhibition of Coco will significantly enhance their sensitivity to these agents. In agreement with this hypothesis, we have observed that silencing of Coco potentiates the ability of Lapatinib to inhibit the proliferation of primary ErbB2-transformed mammary tumor cells (Fig. 2B). To evaluate the potential biological efficacy of antibody-mediated inhibition of Coco, we have examined if affinity-purified goat polyclonal antibodies to full-length human Coco reverse Coco's inhibition of Smad signaling in LM2-4175 lung-metastatic derivative of MDA-MB231 cells. The results indicated that these antibodies function as

stoichiometric inhibitors and can fully reverse the effect that Coco exerts on BMP signaling in highly metastatic breast cancer cells (Fig. 2C). Notably, these antibodies enhanced the ability of Lapatinib to induce growth arrest and apoptosis in ErbB2-transformed cells (Fig. 2D, E). Since these experiments were performed in defined medium devoid of BMP, these results imply that MAb to Coco may cooperate with HER2-targeted therapies to prevent metastatic reactivation in multiple organs, not just in the lung.

We have recently generated a panel of mouse Mabs using antigen epitopes of mouse Coco that were located on the BMP binding surface and were conserved between the mouse and human protein. Hybridoma

supernatants were tested by ELISA on replicate plates containing mouse or human Coco. Ascites were further tested for their ability to bind to Coco and block Coco's binding to BMP4 by ELISA. The results indicated that several antibodies bind to both human and mouse Coco and block their ability to bind to BMP4 (Fig. 3). We will verify the ability of these antibodies to reactivate BMP signaling in human MDA-MB231 and mouse ErbB2 cells, as shown above. Function-blocking Mabs will be further characterized for their ability to disrupt Coco binding to BMP proteins in vitro. Two antibodies possessing the class, isotype, and affinity characteristics required to inhibit Coco in vivo will be further evaluated for their pre-clinical efficacy as single agents as well as in combination with Lapatinib. Initial experiments will focus on assessing the ability of these MABs to inhibit the capacity of mouse and human ErbB2-transformed cells to survive and proliferate in vitro, either alone or in combination with Lapatinib. We have shown that silencing of endogenous Coco completely suppresses the ability of mouse 4T1 mammary carcinoma cells and human MDA-MB231 breast cancer cells to colonize the lung upon tail vein injection. To optimize dose scheduling of the anti-Coco MABs, we will therefore use these two xenograft models. Mice will be injected intraperitoneally with different amounts of anti-

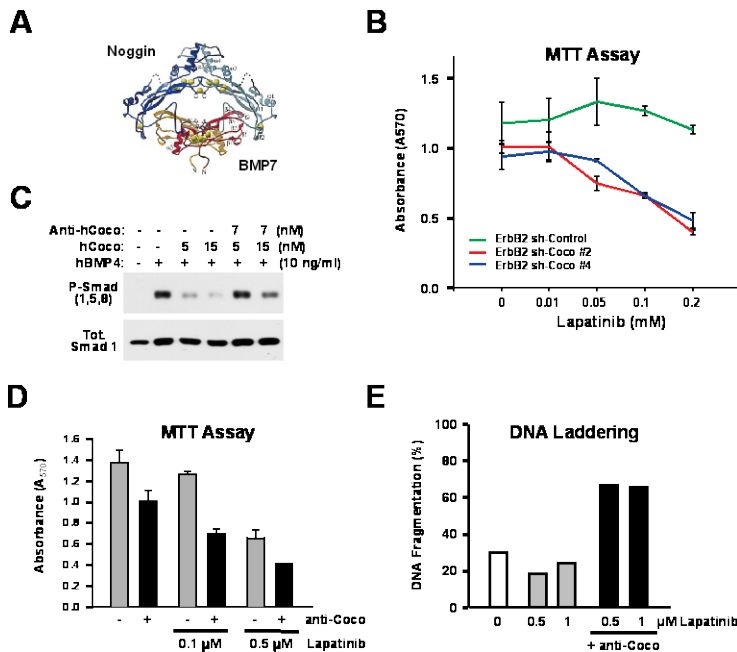


Fig 2. Genetic or antibody-blockade of Coco increases the efficacy of Lapatinib in vitro. (A) Control and Coco-silenced ErbB2-transformed mammary tumor cells were treated with the indicated concentrations of Lapatinib and subjected to MTT assay. (B) Structure of Noggin in complex with BMP7. (C) MDA-MB231 cells were treated with human Coco, polyclonal antibodies to human Coco, and BMP4 as indicated and blotted. (D) ErbB2-transformed cells were treated as indicated and subjected to MTT assay. (E) ErbB2-transformed cells were treated as indicated and DNA laddering was evaluated by electrophoresis and densitometry.

Coco MAB (5-20-50 mg/kg) starting 24 hours before injection of the cancer cells and every other day thereafter. 4T1 and MDA-MB231 cells expressing the bioluminescent imaging vector TGL will be injected in the tail vein of syngeneic or nude mice, respectively, and their ability to outgrow in the lung parenchyma will be monitored by bioluminescent imaging. If antibody-mediated inhibition replicates the effect of silencing, we will observe that an empirically determined dose schedule of anti-Coco MAB suppresses lung metastasis in both models. Confocal imaging on lung sections taken at various times after tail vein injection will enable us to verify that the anti-Coco MAB does not affect extravasation or homing but profoundly inhibits the outgrowth of the cells into macroscopic metastases, as anticipated. Target inhibition will be verified by staining extravasated cancer cells for P-SMAD 1/5/8.

If the studies outlined above provide evidence that the anti-Coco MABs suppress lung colonization by at least 75%, we will examine if they can prevent the outgrowth or possibly induce regression of established lung metastases in combination with oncogene-targeted therapy. To this end, we will use the MMTV-*Neu* (YD) mice, which express an activated form of rat ErbB2 from the mammary epithelium-specific MMTV promoter and reproducibly develop macroscopic lung metastases by 7.5 weeks of age (100% incidence)³. These mice represent an excellent model for HER2+ human breast cancer^{4, 5}. Since the HER1/2 kinase inhibitor Lapatinib effectively blocks rodent ErbB2⁶, we plan to test the effect of the anti-Coco MAB, alone or in combination with Lapatinib, in MMTV-*Neu* (YD) mice. Cohorts of at least 20 mice will be left untreated or treated with the anti-

Coco MAB, Lapatinib, or both (anti-Coco MAb 25-50 mg/kg 3d/week i.p.; Lapatinib 75 mg/kg twice daily 6 d/week by oral gavage) following two regimens. In the preventative regimen, the agents will be administered beginning at 1 month of age prior to the development of MIN lesions⁵. In the therapeutic regimen, primary tumor growth will be monitored by caliper and primary tumors will be surgically removed when they reach 0.5 cc (~0.5 gr). Metastatic burden will be assessed at 2, 4, and 6 weeks after beginning of treatment by histological examination and computer assisted image analysis of serial sections throughout one entire lung as

well as Q-PCR and ELISA quantification of rat Neu expression in the lysate from the other lung. Suppression of ErbB2 kinase activity will be demonstrated by staining for P-ErbB2 or by immunoblotting primary tumor lysates, as shown previously⁵. The results will indicate if the anti-Coco MABs reduce the growth or induces regression of established lung metastases, alone or in combination with inhibition of ErbB2, in a mouse model of HER2+ breast cancer.

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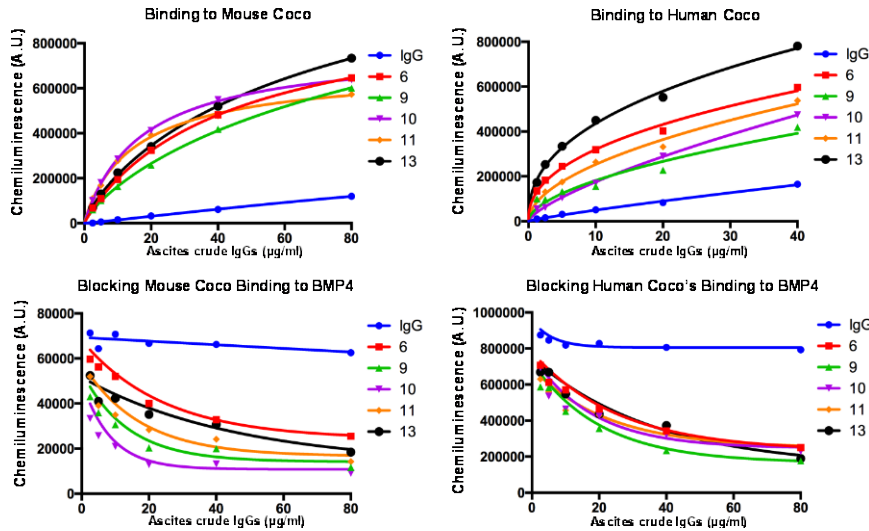


Fig. 3. Characterization of function-blocking monoclonal antibodies to human and mouse Coco. We generated a panel of mouse monoclonal antibodies by using as antigen 10-mer synthetic peptides modeled after mouse epitopes that displayed significant homology to the corresponding human epitopes. The top two graphs illustrate the ability of selected antibodies to bind to mouse (left) and human Coco (right). The bottom two graphs illustrate the ability of selected antibodies to block binding of mouse (left) and human Coco (right) to BMP4. Binding was estimated by ELISA.

Key research accomplishments

- Developed multiple breast cancer cell lines with controlled autophagy and Coco expression
- Determined the role of TGF β signaling in autophagy regulation
- In vivo study of the role of autophagy in breast cancer metastatic dormancy and reactivation in progress
- Developed preclinical monoclonal antibodies against Coco

Reportable Outcomes

- Developed multiple breast cancer cell lines with controlled autophagy and Coco expression
- Developed preclinical monoclonal antibodies against Coco
- One postdoctoral fellow involved in this project have obtained a faculty job
- Multiple research seminars presenting work partially supported by this grant

Conclusion

Metastatic relapse requires reactivation of the disseminated tumor cells after they survive prolonged dormancy. We posit that the survival of metastatic cells, both during dormancy and upon Coco-induced reactivation, requires regulated autophagy. Our experiments will put these potential mechanisms under test and thereby answer a fundamental question about the biology of breast cancer metastasis. Validation of our hypothesis has significant therapeutic impact: it will indicate that targeting autophagy is a viable strategy for eradicating the disseminated breast cancer cells that are responsible for metastatic relapse; and combined inhibition of autophagy and Coco could induce the death of dormant cells and prevent their reactivation.

The experiments are going smoothly as planned, with various genetically controllable breast cancer cells already developed as critical research tools and subsequent xenograft mouse modeling experiments ongoing to determine the role of autophagy and Coco (and thus therapies targeting them) in metastatic breast cancer dormancy and reactivation. In addition to these cell culture and animal model studies as originally planned, we have also developed pre-clinical anti-Coco monoclonal antibodies, for the ultimate goal of the development of a novel Coco-targeted therapeutic product for breast cancer treatment.

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