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14. ABSTRACT *BRCA1* is a tumor suppressor gene for hereditary breast and ovarian cancers. In collaborating with various binding partners, BRCA1 protein participates in multiple cellular functions. Characterization of these binding proteins of BRCA1 is therefore key to the complete understanding of BRCA1's role in tumor suppression. Cofactor of BRCA1 (COBRA1) is a novel BRCA1-interacting protein and shares several functional commonalities with BRCA1 in regulating expression of genes involved various types of cancers. During the past year, we made significant progress in elucidating the role of COBRA1 in breast cancers. By using the xenograft model, we found that COBRA1 reduction led to increased estrogen sensitivity of breast cancer cells and elevated growth potential in the presence of limited amount of estrogen. In addition, we found that COBRA1 reduction in breast cancer cells promoted tumor recurrence after estrogen ablation. Thus, our work uncovered a unique function of COBRA1 in breast cancer. It also provides a solid and logical foundation for exploring a synergistic relationship between BRCA1 and COBRA1 in future studies.

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BRCA1, COBRA1, tumor suppression, transcriptional regulation

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INTRODUCTION

Breast Cancer 1 (BRCA1) is a tumor suppressor, whose mutations are responsible for about 45% of the hereditary breast and ovarian cancers (1). In light of this high incidence of cancer occurrence in *BRCA1* mutation carriers, it is a conundrum that BRCA1 mutations are rarely found in sporadic breast cancers. While reduced BRCA1 expression that has been found in 20-30% sporadic breast cancers may play a similar role as *BRCA1* mutation in sporadic cancers, it is equally possible that comprised BRCA1-dependent biological pathways with no alteration to BRCA1 expression may also contribute to the development of breast cancers. BRCA1 is a multifunctional protein and interacts with numerous binding partners that are implicated in various cellular functions, such as DNA damage response, transcriptional regulation, ubiquitination and cell cycle regulation (2). And several of these proteins are indeed well-known players that either suppress or promote breast cancer development. Therefore, deregulation of their expression and activity may act as the functional substitutes for BRCA1 mutations in the sporadic breast cancers, and in-depth and thorough characterization to the interplay between BRCA1 and its binding partners will improve our understanding to the mechanisms of BRCA1's tumor suppression function in breast cancers.

Cofactor of BRCA1 (COBRA1) is a novel BRCA1-interacting partner that has been isolated from a yeast two-hybrid screening from our laboratory (3). At the molecular level, COBRA1 and BRCA1 share several functional similarities. First of all, both proteins are preferentially expressed in luminal epithelial cells (4). Secondly, COBRA1 and BRCA1 can both induce large-scale chromatin reorganization (3). Thirdly, these two proteins have been demonstrated to interact with estrogen receptor α (ERα) and served as corepressors in modulating estrogen-dependent gene expression (4, 5). Last but not least, our work from the first year of this funding period has identified a large number of genes that are co-regulated by COBRA1 and BRCA1 through an unbiased screening with the microarray technology (6). Importantly, many of those genes, such as S100P, TIMP-1, and GABBR1, have been previously implicated in various types of cancers. Taken together, these findings suggest that COBRA1 and BRCA1 may indeed cooperate with each other to modulate gene expression in breast cancer cells.

In light of the physical and functional interactions between BRCA1 and COBRA1, we have decided to combine the studies of BRCA1 and COBRA1 in gene regulation and in breast cancer. Our work during the second year of the current funding period has explored a potential function of COBRA1 in breast cancer. First we analyzed COBRA1 expression in clinical samples from both normal and tumor tissues and found that COBRA1 expression was extremely low in breast tumors that were associated with local recurrence and metastasis. To discriminate a causal or bystander effect of COBRA1 in breast cancer progression, we then carried out a xenograft study by comparing tumor growth of control or COBRA1 knockdown ZR-75-1 cells in athymic nude mice. Interestingly, we found that COBRA1 reduction significantly promoted tumor growth in the absence, but not presence of exogenously supplied estrogen. While these clinical observation and functional data did suggest a unique function of COBRA1 in breast cancer progression, several questions remained to be addressed with regard to the underlying mechanisms. First of all, since the nude mice used in our study have intact ovaries that produce low level of estrogen to the circulation, it is unclear whether the growth

phenotype associated with COBRA1 reduction is estrogen dependent or not. Secondly, although estrogen ablation is generally effective in treating both primary and advanced breast cancers, it is often followed by disease relapse when tumors become refractory to hormonal therapy (7). Given the fact that COBRA1 reduction promotes tumor growth under low estrogen condition, it will be interesting to examine whether COBRA1 is also involved in disease recurrence after estrogen ablation treatment by using the same xenograft model. Thirdly, ERα plays a central role in the initiation and progression of breast cancers. Since COBRA1 represses the transcriptional activity of ERα, it will be important from the mechanistic point of view to determine whether deregulation of ER-dependent gene expression in COBRA1 knockdown cells is involved in the growth phenotype. In the current report, I will address these questions with data from both in vivo xenograft studies and in vitro tissue culture experiments.

RESULT

I. Confirmation of COBRA1-Knockdown Effect on Tumor Growth with Independent shRNA

Our original observation that COBRA1 knockdown led to increased tumor growth under low level of estrogen condition was based on ZR-75-1 cells that have been engineered to

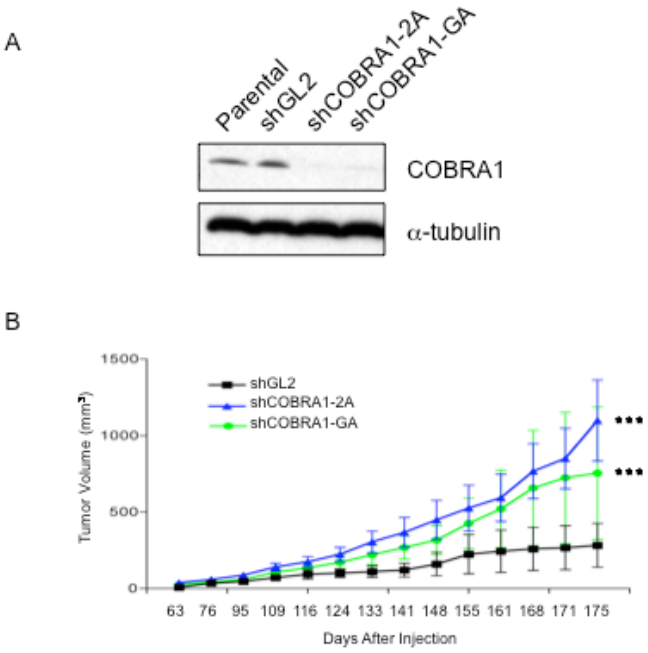

Figure 1 COBRA1-Knockdown effect on tumor growth with independent shRNA against COBRA1. A Western blot analysis of COBRA1 level in parental, control, shCOBRA1-2A, and shCOBRA1-GA expressing ZR-75-1 cells. α-tubulin was measured as loading control. **B.** Growth curve of tumors derived from control and COBRA1 knockdown ZR-75-1 cells. Cells were inoculated in inguinal mammary gland. Tumor volume was measured two weeks after cell injection and monitored every two to three days from then on. *** P<0.001 by paired T test. N=5 for each group.

stably express one shRNA sequence (shCOBRA1-2A) targeting COBRA1. While the shRNA based technology is powerful in reducing the expression of endogenous genes, we are fully aware of the caveats inherent to this approach. To ascertain the conclusion, we made a second COBRA1 stable knockdown cell line with an independent shRNA sequence (shCOBRA1-GA) and tested its growth phenotype in nude mice together with the newly prepared control and shCOBRA1-2A cells. As shown in Figure 1A, both 2A and GA efficiently reduced COBRA1 level in ZR-75-1 cells with GA having a smaller knockdown effect compared with the 2A sequence. We then inoculated the control along with the two knockdown cell lines in nude mice at the mammary gland with no supplementation of estrogen pellet. Consistent with our previous finding, COBRA1 reduction by both 2A and GA sequences led to significantly larger tumor than the control cells after prolonged growth in nude mice, thus confirming our original conclusion.

II. COBRA1-Knockdown Associated Growth Phenotype is Estrogen Dependent.

The nude mice we have been using in our study carry the intact ovaries and produce the endogenous level of estrogen, although the estrogen production in nude mice is known to be extremely low. Therefore, the COBRA1-knockdown resulted elevation in tumor growth could represent the activation of estrogen-independent pathway that sustained tumor growth under low level of estrogen condition. Alternatively, COBRA1 reduction might increase the estrogen sensitivity of the ZR-75-1 cells so that they could live and grow even with no exogenous estrogen supplied. To discriminate these two possibilities, we tested the effect of tamoxifen, a

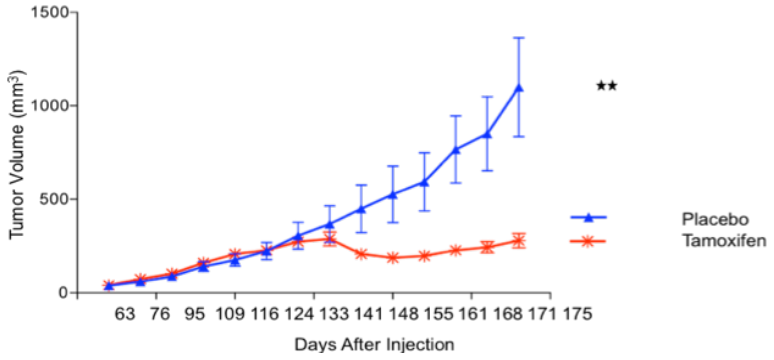

Figure 2 Effect of Tamoxifen on tumor growth of COBRA1 knockdown cells. Control and shCOBRA1-2A cells were inoculated as Figure 1. When average tumor size reached to 300mm3 (day 133), tamoxifen or placebo pellet was embedded and tumor growth was monitored thereafter. ** P<0.01, paired T test. N=5 for each group.

known ER antagonist, on the growth potential of COBRA1 knockdown cells. We first inoculated shCOBRA1-2A cells to the mammary gland of nude mice, and tamoxifen pellet or the placebo pellet was then embedded subcutaneously when the average tumor size reached to 225mm^3. As shown in Figure 2, addition of tamoxifen immediately stalled the tumor growth and the tumor started to regress about two weeks after treatment. Therefore, the COBRA1-knockdown associated growth phenotype was estrogen dependent, which suggested that COBRA1 reduction might indeed increase the estrogen sensitivity of the ZR-75-1 cells.

III. COBRA1-Knockdown Promotes Recurrence after Estrogen Ablation.

As a malignancy of hormone responsive organ, ER positive breast cancers rely on estrogen signaling pathway for tumor to grow and survive. Consequently, therapies aiming to inhibit the activity of estrogen are among the most effective approaches for treating breast cancers. However, disease recurrence does occur in many cases because of the development drug resistance to hormonal therapy, and the mechanisms of which are still largely unknown. To this end, it is of great interest to note that reduction of COBRA1 expression is indeed associated with increased local recurrence and metastasis as we have shown in previous report. To test whether COBRA1 reduction would have any effect on disease recurrence, we compared the tumor growth of control and shCOBRA1-2A cells after estrogen ablation in nude mice. Briefly, we inoculate both cell lines to nude mice and the initial tumor formation and growth was allowed in the presence of exogenously supplied estrogen pellet. As expected, COBRA1 reduction had no effect on tumor growth in this phase. When the average tumor size in both groups reached 500mm^3, the mice in each group were rearranged and estrogen pellet was replaced with placebo pellet in half of the mice to mimic the estrogen ablation therapy applied for human patients. As shown in Figure 3, tumors from mice retaining the estrogen

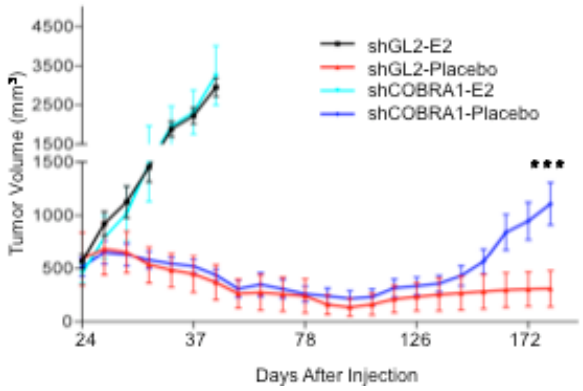

Figure 3 Effect of estrogen withdrawal on tumor growth of COBRA1 knockdown cells. Control and shCOBRA1-2A cells were inoculated as Figure 1 in the presence of exogenous estrogen. When average tumor size reached to 500mm^3 (day 24), mice were regrouped and estrogen pellet was replaced with placebo pellet in half of the mice from each group. Tumor growth was monitored thereafter. *** P<0.001, paired T test. N=5 for each group.

pellet continued to grow robustly, whereas estrogen withdrawal drastically reversed the growth curve and the tumor shrunk significantly. Interestingly, after growing for extended period of time in nude mice, the COBRA1 knockdown tumors, but not the controls, started to grow again in the absence of estrogen pellet. Thus, this data suggest that COBRA1 reduction in breast cancer cells did promote recurrence after estrogen ablation.

IV. Effect of COBRA1-Knockdown on Estrogen-Dependent Gene Expression

Our previous studies have demonstrated that COBRA1 was a corepressor of ERα in T47D cells and attenuated estrogen-dependent gene expression by inhibiting transcription elongation. The fact that COBRA1 reduction led to elevated tumor growth under low level of

estrogen condition might therefore result from increased estrogen-dependent gene expression in COBRA1 knockdown cells. To test this possibility, we first performed luciferase reporter assay with an ERE-containing TK promoter in control and COBRA1 knockdown cells under various concentrations of estrogen. In contrast to our speculation, estrogen-dependent transcription from this ERE-responsive promoter was not significantly affected in COBRA1

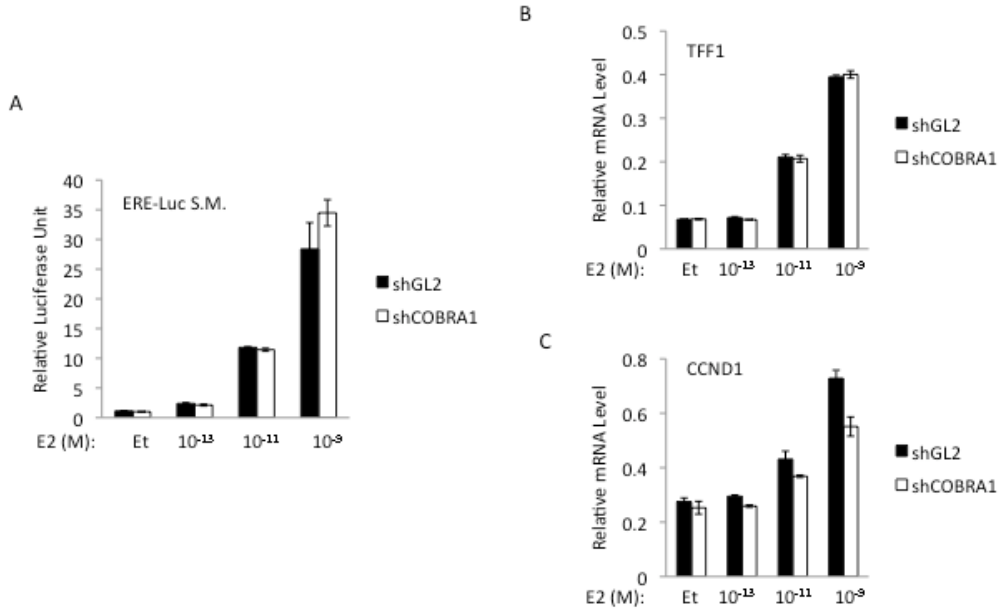

Figure 4 Effect of COBRA1-Knockdown on estrogen-dependent gene expression. A Luciferase reporter assay to measure estrogene-dependent gene expression in control and shCOBRA1-2A cells. The cells were starved in estrogen-free medium for 3 days and then plated to N unc plate for transfection with Lipofectamin Plus. 250ng of ERE-TK-luciferase (shGL2 silent mutant) along with 50ng of Renilla luciferase reporter vector was transfected for 24 hours followed by treatment with various concentrations of estrogen for another 24 hours. Cells were then harvested for luciferase assay with the Dual luciferase reporter system. Data shown was mean from triplicate samples, error bar representing S.D. **B, C** Expression of estrogen regulated genes in control and shCOBRA1-2A cells. Cells were starved in estrogen-free medium for 3 days and then treated with various concentrations of estrogen for 24 hours before harvest for total RNA isolation. c DNA was synthesized with random primer and real-time PCR was performed to measure expression of TFF1 (B) and CCND1 (C), two well-known estrogen regulated genes. β-actin was measured as internal control and used for normalization. Data shown was mean from duplicated PCR reactions, error bar representing S.D.

knockdown cells (Figure 4A). We then tested expression of several endogenous estrogen-responsive genes by using similar estrogen stimulation conditions. In concert with results from the luciferast reporter assay, expression of these genes was not affected either. Collectively, our result suggested that the growth advantage of COBRA1-knockdown was not associated with alteration in estrogen-dependent gene expression.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of the potential mechanisms of COBRA1's effect on tumor growth in xenograft model.

REPORTABLE OUTCOMES

Sun J, Watkins G, Blair AL, Moskaluk C, Ghosh S, Jiang WG, Li R. Deregulation of cofactor of BRCA1 expression in breast cancer cells. (2008) J Cell Biochem. 103(6) 1798-1807

CONCLUSION

In the last year of the current funding period we have explored the mechanisms of COBRA1's effect in breast cancer progression. By using xenograft model, we demonstrated that COBRA1 reduction in ER positive breast cancer cells increased tumor growth in the presence of limited amount of estrogen. Since this growth was effectively reversed by inhibiting the estrogen signaling pathway with the ER antagonist tamoxifen, the COBRA1-reduction associated growth phenotype is likely a reflection of elevated estrogen sensitivity of the COBRA1 knockdown cells in vivo. In addition, we found that COBRA1 reduction in breast cancer cells promoted tumor recurrence after estrogen withdrawal, suggesting the functional involvement of COBRA1 in breast cancer progression and its potential implication as a prognosis marker for the breast cancers. Taken together, our studies have uncovered an interesting tumor suppressor-like function of COBRA1 in breast cancer progression. These work also justify the further exploration of the functional cooperation of COBRA1 and BRCA1 in suppressing tumor initiation and progression in breast cancers.

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