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Targeting Thromboxane A2 Receptor for Antimetastasis Therapy of Breast Cancer

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Dissemination and metastasis of tumor cells are major causes of morbidity and mortality in breast cancer patients. Therefore it is of vital importance to identify druggable targets to inhibit breast tumor invasion and metastasis. A critical component in the invasive growth, dissemination, and metastasis of cancer is acquisition of motility by tumor cells. Our preliminary studies suggest a novel role for the thromboxane A2 receptor (TP) in controlling breast tumor cell motility via regulating cytoskeleton reorganization. The objective of this proposal is to define the function of TP in tumor cell motility and to validate TP as a target for anti-metastasis therapy of breast cancer. In the first aim, the role of TP in breast tumor cell motility will be determined in the presence or absence of TP activation or inhibition. The isoform(s) of TP involved in tumor cell contraction and motility will be identified in the second specific aim. In the third aim, an orthotopic mouse model will be used to assess whether TP can be targeted to reduce breast cancer metastasis. The proposed studies will be highly significant toward the goals of developing mechanism-based interventions for cancers.

Breast cancer, metastasis, thromboxane, G protein coupled receptor, migration, invasion
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Introduction

Major causes of death in breast cancer patients are the spread and metastasis of tumors. “Metastasis” means that the cancer cells have detached from the original tumor site and started to grow in another part of the body. The goal of this research project is to find out how breast cancer cells disseminate and move so that new methods can be developed to identify and treat breast cancer. For a tumor cell to move, it must push forward in the front and contract in the rear to power ahead. The main idea of the proposed project is that tumor dissemination and migration can be inhibited by blocking tumor cell contraction. Our laboratory has identified a cell receptor (TP) that receives information (“receptor”) in breast tumors and then participates in cell contraction and migration. This receptor can be activated by a lipid called thromboxane A₂. A published study suggests that the level of this receptor expressed in breast tumor tissues have been linked with poor prognosis and a significant decrease in disease free survival. Our preliminary studies suggest that when thromboxane A₂ receptor is activated, breast cancer cells immediately contract. If the activation of this receptor is blocked, breast cancer cells cannot move and spread. We hypothesize that TP (TxA₂ receptor) regulates the motility of carcinoma cells by elaborating the reorganization of cytoskeleton during migration and that inhibition of TP activation can reduce the motility, invasion, and metastasis of breast carcinoma cells. The objective of this proposal is to define the function of TP in tumor cell motility and to validate TP as a target for anti-metastasis therapy of breast cancer with the following aims:

Aim 1. Define the role of TP activation in breast cancer cell motility.

Aim 2. Determine the isoform(s) of TP involved in cytoskeleton reorganization in motility of tumor cells.

Aim 3. Validate TP(s) as a target for treatment of breast cancer metastasis.
Task 1. Define the role of TP activation in breast cancer cell motility (Months 1 – 12).

This task has been completed. The major findings are summarized as below:

In order to study whether and how TP contribute to the progression of breast cancer, we examined whether TP is expressed at protein level in breast cancer cells. By immunohistochemistry, it was found that infiltrating breast carcinoma presented strong staining for TP (Figure 1A, left), while in matched normal tissue a much weak staining was noticed (Figure 1A, right).

Figure 1. A. Expression of TP in breast carcinoma tissue as revealed by immunohistochemistry. Brown color indicates positive TP immunoreactivities. Right, a stage II non-dpecific infiltrating duct carcinoma; left, matched normal tissue. Magnification= 100 X. B. Expression of TP in MDA-MB-231 cells as revealed by immunocytochemistry. Specificity of antibody was confirmed by incubation with block peptide for the antibody. Magnification= 400 X. C. Live cell staining. MDA-MB-231 cells were grown on coverglass and stained with the antibody against the N-terminal of TP followed by AlexaFluor 488 conjugated secondary antibody. Cells without primary antibody were used as negative control.

Next, we examined the expression of TP in breast carcinoma cells by immunocytochemistry. As shown in the Figure 1B left, MDA-MB-231 cells showed strong cytoplasmic staining for TP. The specificity of staining was confirmed by incubation with blocking peptide for TP antibody (Figure 1B, right). The predominant localization of TP in the cytosol of MDA-MB-231 cells led us to examine whether a subset of TP is expressed at cell surface. A polyclonal antibody raised against the N-terminal 120 amino acid residues of TP was used to stain live, non-permeabilized cells. As shown in the Figure 1C, MDA-MB-231 cells had positive staining at cell surface, suggesting that a subset of TP was localized at plasma membrane.

We observed that when cultured in serum free RPMI-1640 medium, MDA-MB-231 cells presented a spindle-like morphology (Figure 2A, EtOH). Treatment of a thromboxane A2 mimic, U46619 (200nM), caused the cells to contract and present “round” shape within 5 to 15 minutes (Figure 2A, U46619). To confirm the contraction of MDA-MB-231 cells was induced by TP activation, we pretreated the cells with a high affinity TP antagonist, SQ29548 (10μM), and found the contraction of MDA-MB-231 cells by U46619 could be blocked (Figure 2A, SQ29548+U46619).
Further, we generated MDA-MB-231 cells with TP expression stably ablated by using shRNA constructs. Two stable clones with down-regulated TP level (denominated MDA-MB-231-58-1-1 and MDA-MB-231-58-1-3) as well as one vector control (denominated MDA-MB-231-2003) (Figure 2B) were applied in the following experiment. As shown in the Figure 2C, both 58-1-1 and 58-1-3 cells failed to contract upon U46619 and I-BOP treatment when compared with the cells in vector control. These data suggest that thromboxane A₂ mimic U46619 induced cell contraction through TP activation and the induction of cell contraction by U46619 could be blocked by TP antagonist SQ29548.

![Image](86x404 to 526x629)

**Figure 2.** A. Induction of cell contraction by TP agonist, U46619, and the inhibition of cell contraction by TP antagonist, SQ29548. B. Generation of stable transfectants with TP expression ablated using shRNA constructs in MDA-MB-231 cells. 58-1-1, 58-1-3: shRNA constructs, 2003: vector control. C. Blockade of U46619-induced cell contraction by down regulation of TP in MDA-MB-231 cells. 1-1 and 1-3 are two stable transfectants with TP expression ablated. **, P<0.01 when compared with vector control. D. Blockade of U46619-induced cell contraction by CAY10535, antagonist of TPβ. Cells were pretreated with CAY10535 at 100nM for 15 min before the treatment with 200 nM U46619. ***, P < 0.001, when compared with its vehicle control [ethanol (EtOH)].

As a member of G-protein coupled receptor, binding to its ligands can induce the coupling of α subunit of the G protein with downstream effector proteins, for example the Gₐs, Gai, Gaq, Gα12/13. To study which G protein is involved in TP activation induced cell cytoskeleton reorganization, we transfected MDA-MB-231 cells with Gα12 shRNA construct 910 to silence the expression of Gα12. A control construct 30007 was also introduced into MDA-MB-231 cells. As shown in Figure 3A and Figure 3B, down regulation of Gα12 blocked U46619 induced cell contraction.
**Figure 3.** A, MDA-MB-231 cells were transfected with Ga12 shRNA 910 or vector control 30007. 24h post transfection, the cells were serum starved for 12h then treated with either EtOH or U46619 for 15min. The cells were then fixed and stained with TRITC-Phalloidin. B, The total green positive cells and the round-shaped green positive cells in each well were counted. ***, P<0.001 when compared with vector control 30007.

Gα12 is known to regulate RhoA activity, which plays a critical role in cell migration and invasion. To study the involvement of small Rho GTPase in TP agonist induced cell contraction, we treated MDA-MB-231 cells with a pharmacological inhibitor for Rho kinase, Y27632. It was found that pretreatment with Y27632 (10μM) blocked U46619 induced contraction in MDA-MB-231 cells as shown in the **Figure 4A**.

To examine the involvement of RhoA in U46619 induced cell contraction, we transfected MDA-MB-231 cells with different RhoA constructs: wild type (**Figure 4B, top panel**), dominant negative form (**Figure 4B, bottom panel**) or constitutively active form (**Figure 4B, middle panel**) of RhoA and all of them had stable expression of green fluorescent protein (GFP). As shown in Figure 4B middle panel, constitutively active RhoA was sufficient to induce cell contraction, and it could not be blocked by pretreatment of SQ29548. On the other hand, as shown in the **Figure 4B bottom panel**, MDA-MB-231 cells transfected with dominant negative form of RhoA failed to contract upon U46619 treatment. U46619 induced significant increase of the contracted cells with or without SQ29548 pretreatment in wild type RhoA transfected cells (**Figure 4C**), whereas it had no effect on neither constitutively active nor dominant negative RhoA transfected cells, suggesting RhoA activation was required for cell contraction which is mediated by TP activation. Also, by using GST-pull down assay, we found out U46619 treatment did not induce CDC42 activation. (data not shown)

Further, we confirm the ability of TP in activating RhoA by G-LISA activation assay. A significant increase in absorbance was observed in the cells treated with U46619 when compared with control. Moreover, the pretreatment with TPβ specific inhibitor blocked U46619 induced RhoA activation.
Figure 4. A. Inhibition of U46619-induced cell contraction by Rho kinase inhibitor Y27632. B. Active RhoA was sufficient to cause cell contraction, whereas dominant negative RhoA blocked U46619-induced cell contraction. Green cells denote the transfected cells with the constructs indicated at the left. C. MDA-MB-231 cells were transfect with plasmid pcDNA3-EGFP-RhoA-Q63L with RhoA constitutively active, pcDNA3-EGFP-RhoA-T19N with RhoA dominant negative and pcDNA3-EGFP-RhoA-wt. The cells were treated with 300 nM U46619, and then scored for cell contraction. Columns: mean of percentage contracted; bars: standard deviations. D. RhoA activation by U46619 measured by G-LISA™ activation assay. MDA-MB-231 cells were serum starved for 20h and untreated or treated with CAY10535 (50nM) for 15min in serum free medium at 37°C, then activated with U46619 (300nM for 5 min) or EtOH only. 25 µg of cell lysates were subjected to the G-LISA™ assay. Absorbance was read at 490 nm.

To study the effect of TP activation or inhibition in breast cancer cell survival and proliferation, MDA-MB-231 cells were treated with two TP agonists and one antagonist. The number of viable cells was counted by trypan blue assay. As shown in the Figure 5C, the viability of MDA-MB-231 cells did not have significant changes upon treatment of neither TP agonists U46619/I-BOP nor TP antagonist SQ29548. In addition, down-regulation of TP in MDA-MB-231 cells did not affect the proliferation ability of the cells either (Figure 5D).

Cytoskeleton reorganization is required for many cellular processes, including cell migration, which is a coordinated multi-step process. To study the role of TXA2-TP signaling in tumor cell migration, we performed wound healing assay in MDA-MB-231 cells. As shown in the Figure 5A, PTA2, an antagonist of TP (23), dramatically reduced wound healing (average 13 cells per unit area), when compared to the control group (average 23 cells per unit area) (p < 0.05). Further, PTA2 blocked EGF-stimulated wound healing (average 34.6 cells per unit field for EGF treated group, vs. 4.2 cells per unit area for EGF+ PTA2 treated group, p < 0.01). Similar results were obtained with SQ29548 (data not shown). It should be noted that the tremendous effects of TP antagonists on cell migration is not due to inhibition of cell proliferation or induction of apoptosis by the previous trypan blue exclusion assay.

Further, using modified Boyden Chamber assay, we noticed that MDA-MB-231 cells with down regulated TP expression level showed decreased invasion capability when compared with vector control cells (Figure 5B).
The results suggest a pivotal role for TP activation in tumor cell motility and that inhibition of cytoskeleton reorganization by either treatment of TP antagonist or ablation of TP expression in breast cancer cells may prevent cells to detach from substrata and move forward.

Figure 5. A. Inhibition of cell migration in a wound healing assay. A wound in confluent MDA-MB-231 cells was created and then treated with EGF (5ng/ml), with or without PTA2 (3μM), in serum free medium for 16h. White lines denote the original wound area. Cells were stained with H&E. note the stimulation of cell migration into denuded area by EGF and inhibition by PTA2. B. Inhibition of cell invasion in a Boyden Chamber Assay. C. MDA-MB-231 cells (2 X 10⁵) were plated out in RPMI1640 medium with 5% FBS. 12 hours later, the cells were treated with U46619 (300 nM), or IBOP (300 nM), or SQ29548 (10 µM). Cells were harvested with trypsin-EDTA, neutralized, and counted using Backman Cell Counter. D. MDA-MB-231 cells with TP expression ablated and the vector control cells were plated out in 5% FBS containing RPMI1640 at 2 X 10⁵. Cells were applied to trypan blue assay at the indicated time points.
Task 2. Determine the isoform(s) of TP involved in cytoskeleton reorganization in motility of tumor cells (Months 13-24): The studies in this aim have been completed.

Human thromboxane A2 receptor (TP) is expressed as two different isoforms, TPα and TPβ, that arise by alternative mRNA splicing. To examine the expression of TPs in human breast carcinoma cells, we used isoform-specific PCR primer sets to amplify a 268-bp fragment of TPα and a 330-bp fragment of TPβ. We choose MDA-MB-231 cells in our study due to their highly metastatic ability. As shown in Figure 6, MDA-MB-231 cells express both TPα and TPβ, indicating the co-expression of both isoforms of TP in carcinoma.

**Figure 6.** Expression of TPα and TPβ in breast carcinoma cancer cells as revealed by RT-PCR using isoform specific primers. a: TPβ band, target size 330 bps; b: TPα band, target size 268 bps.

We further examined the expression of TPalpha and TPbeta in a number of breast cancer cell lines and found that while TPalpha is expressed in all cell lines examined, TPbeta is expressed in more malignant cells such as MDA-MB-231 cells, and Rao3 and Rao 4 cells, which are H-Ras transformed, highly malignant triple negative breast cancer cells (Figure 7).

**Figure 7.** Expression of TPα and TPβ in breast carcinoma cancer cells as revealed by RT-PCR using isoform specific primers.

To detect TPbeta expression in breast cancers, we used multi pronged approaches. First, we have generated rabbit polyclonal antibody against TPbeta specific peptide sequences. Currently we are validating the specificity of the polyclonal antibody.

The antibody developed was not working well as expected.
Second, we initiated collaboration with Dr. Kinsella in Ireland, who has TPalpha and TPbeta specific antibodies. Using these isoform specific antibodies, we performed IHC analysis of TP expression in breast cancer tissues and analyzed their correlations with clinical and pathological parameters.

We made the following findings:

1) TPbeta expression is significantly increased in breast cancer patients with distant metastasis when compared with metastasis free patients (Figure 8).

2) TPbeta expression is significantly increased in breast cancer patients with larger tumor size (> 3cm) compared with those have smaller tumor size (< 3cm), and in tumors with high grades (Figure 9).

3) Neither TPalpha nor TPbeta expression level correlates with patients’ age/ clinical outcome/ No. of positive lymph nodes/ ER/ PR/ p53 status/ tumor grade, according to the specimens we analyzed so far.

We have made recombinant protein covering the unique sequence of TPbeta isoform. We have developed monoclonal antibodies against TPbeta. The antibody can be used as a tool to valuate TP expression in breast tumors.

Figure 8. Expression levels of TPalpha/TPbeta in human breast tissues array categorized according to TNM grading. M0 – no distant metastasis, M1 – distant metastasis. **, p < 0.01 when compared with M0 group.

Figure 9. TPalpha and TPbeta expression in breast cancer tissues.
Figure 9. Expression of TPalpha and TPbeta in breast tumors of different grades. Note the increased expression of TPbeta in high grade tumors.

With the availability of TPbeta selective inhibitor, CAY10535, which shows 20 fold selectivity for TPβ (IC₅₀=99nM) relative to TPα (IC₅₀=1,970nM), we determined the involvement of TPisoform involved in U46619 induced cell contraction. It was found that both low (25nM) and high (100nM) concentration of CAY10535 blocked U46619-induced cell contraction (Figure 10 and Figure 11). The data suggest that thromboxane A₂ mimetic U46619 induces cytoskeleton reorganization through activation of TPβ.
Further we confirmed that CAY10535 blocked the U46619 activation of RhoA using Rhotekin pulldown assay (Figure 12). With our previous data suggesting the important role of RhoA in cell contraction induced by Y46619, the data suggest that TPbeta activation is required for U46619 to activate RhoA and to induce cell contraction. Next we examined whether TPbeta activation is required for cell motility. As shown in Figure 13, CAY10535 inhibited tumor cell migration as assessed by Boyden Chamber assay. Taken together, the data suggest TPbeta activation is required for tumor cell motility.

The studies have been completed.

To elucidate the functions of TP in tumor growth, progression and metastasis, we generated MDA-MB-231 cells with TP expression stably knocked down by using retroviral based shRNA constructs. Two stable clones with down-regulated TP levels (denominated 58-1-1 and 58-1-3) as well as one vector control (denominated MDA-MB-231-2003) were generated for further characterization. The knockdown of TP expression was confirmed by Western blot and RT-PCR (Fig 14A). In contrast to the vector control cells, neither 58-1-1 nor 58-1-3 cells were able to contract significantly upon U46619 (Fig 14B) or I-BOP treatment (Fig 14C).

Next we injected the GFP-luciferase labeled MDA-MB-231 cells with TP stably knocked down (58-1-3), as well as vector control cells (MM231-2003) into the right axillary mammary fat pad of female SCID mice. Tumor growth was monitored weekly. As shown in Fig 15A, knockdown of TP did not affect either the primary tumor formation or the primary tumor growth rate. We measured luciferase activity in the lungs, livers, spleens and brains harvested from the mice. The micrometastasis burdens, as shown by the luciferase activities in the tissue extracts, were significantly higher in the organs from mice with tumors from vector control cells, than those injected with TP knockdown cells (Fig 15B). Taken together, the data suggest that TP-mediated signaling is required for tumor metastasis.

To further determine the functional roles of TP in breast cancer metastasis, we injected MDA-MB-231 cells with TP stably knocked down, or control cells, into SCID mice via tail vein. Those mice injected with TP knockdown MDA-MB-231 cells presented fewer lung metastasis nodules than those injected with vector
control cells (Fig. 16A, B). Moreover, we found a significant reduction in luciferase readout in the livers from the mice injected with TP knockdown MDA-MB-231 cells as compared to those injected with control cells by measuring luciferase activity in the organs harvested from the mice as an indicator for metastatic burdens (Fig. 16C).

**Fig. 16.** Inhibition of development of experimental metastasis by TP knockdown: (A) Gross morphology of resected mouse lungs 30 days after tail vein injection of MDA-MB-231-2003 (vector control), MDA-MB-231-58-1-1 and MDA-MB-231-58-1-3 (TP knockdown) cells. (B) Reduction of metastatic lesions by TP ablation. Columns, average number of metastatic lesions per mouse. (C) Metastasis to the liver as indicated by the normalized luciferase readings.

It should be noted that that sizes of nodules formed by MDA-MB-231 cells with TP knocked down was comparable to those in the vector controls (Fig. 16A), consistent with the observations that TP knockdown in MDA-MB-231 cells did not affect either the primary tumor growth (Fig. 15A). The data suggest that TP-mediated signaling is required for the metastatic spread of tumor cells, but not for the growth of tumors.
KEY RESEARCH ACCOMPLISHMENT

1. Determination of the beta isoform of TP as required in cytoskeleton reorganization, small GTPase RhoA activation, and tumor cell motility.
2. Generation of TPbeta specific antibody. The polyclonal antibody did not work well in Western blot. Generation and characterization of monoclonal antibody are ongoing.
3. Animal experiments have validated TP as a target to prevent and reduce breast cancer metastasis
REPORTABLE OUTCOMES

Presentations:
Xuejing Zhang and Daotai Nie. Thromboxane A2 Receptor (TP) in Tumor Progression and Metastasis. G-Protein coupled receptor meeting, Madison, WI. September 8 - 9, 2008.

Xuejing Zhang, Man-Tzu Wang, Yong Tang, Yakun Chen, and Daotai Nie. Thromboxane A2 receptor (TP) in tumor cell motility and metastasis. 100th AACR Annual Meeting, Denver, CO. April 18-22, 2009.


Xuejing Zhang, Man-Tzu Wang, Yong Tang, Yakun Chen, and Daotai Nie. Thromboxane A2 receptor (TP) in tumor cell motility and metastasis. 100th AACR Annual Meeting, Denver, CO. April 18-22, 2009.


Abstracts published:


**Articles published:**


Robbins GT, Nie D. PPAR gamma, bioactive lipids, and cancer progression. Front Biosci. 17:1816-34, 2012.

**Grants:**


**Patents:**
Nie D and Zhang X. Use of Acetylsalicylic acid to reduce cancer metastasis. Disclosed to Technology Transfer office of Southern Illinois University School of Medicine.
CONCLUSIONS:

While the mRNA levels of receptor of thromboxane A₂ (TXA₂), TP, was found to correlate with a poor prognosis in breast cancer patients, it is unknown whether TP is expressed at protein levels, whether TP is functional, and whether TP regulates breast cancer progression that eventually impact on patient survival. In the past three years, we have obtained the following data suggesting the involvement of TP in breast cancer metastasis.

First, we found functional TP is expressed in breast cancer cells. Our studies identify TPbeta isoform as the receptor increasingly expressed in high grade tumors, especially in tumors with distant metastasis. TPbeta expression was found selectively limited to breast cancer cells of high malignancy as well. It is increasingly clear that TPbeta expression was stimulated as breast cancer cells become more invasive.

Second, TP activation led to cell contraction which required RhoA activation. We further determined that TPbeta is required for U46619 to induce cell contraction and RhoA activation. Identification of TPbeta in sytoskeleton reorganization and RhoA activation provides us a more specific target for possible future intervention to block breast cancer metastasis.

Third, TP activation is required for tumor cell motility. CAY10535, a select TPbeta inhibitor, inhibited tumor cell motility. The data, along with the data reported in the previous two annual reports, suggest the importance of TPbeta in tumor cell motility.

Fourth, TP is required for breast cancer metastasis. Depletion of TP reduced spontaneous metastasis in an orthotopic model, while had minimal effects on the growth of primary tumors. The results suggest TP as a potential target to reduce or block breast cancer metastasis.

The above data support the rationale of targeting TP to block breast cancer metastasis. We have found that aspirin can reduce breast cancer metastasis by reducing the production of thromboxane A₂.

So what? Our studies are significant in the following ways: 1) TP, especially TPbeta, can be a promising target to develop treatment to block breast cancer metastasis. 2) Inhibition of thromboxane A2 production, either using TX synthase inhibitor or aspirin or other cyclooxygenase inhibitors can reduce breast cancer metastasis. Further works are needed to translate the findings into clinical applications.
REFERENCES

N/A

APPENDICES

Presentations files: Not recorded

Patents: Form 882

SUPPORTING DATA

Embedded in the reporting body