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Version: 3 Date: 22 June 2007

Author's response to reviews: see over
Cross-linking of CD24 inhibits growth of MCF-7 breast cancer cells

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Short Title: Effects of CD24 cross-linking on MCF-7

Key Words: Breast cancer, MDA-MB-231, MCF-7, CD24, cross-linking
Abstract

**Background:** The effects of neutralization of CD24 via cross-linking on different aspects of breast tumour biology are yet to be established. We analyzed the impact of cross-linking CD24 on human breast cancer cell lines MCF-7 and MDA-MB-231.

**Methods:** MCF-7 and MDA-MB-231 were cross-linked with anti rabbit polyclonal IgG or anti-human CD24 rabbit polyclonal antibodies and then proliferated. Changes in cell characteristics such as, cell cycle, degree of apoptosis, survival in three-dimensional cultures, adhesion, and migration ability were assayed in MCF-7 after CD24 cross-linking.

**Results:** Expressions of CD24 analyzed by flow cytometry in MDA-MB-231 and MCF-7 were 2% and 65%, respectively. After cross-linking CD24 with antibody of 500 ng/ml concentrations, the time dependent reduction in proliferation was shown in MCF-7, in contrast to no change in MDA-MB-231. In MCF-7, survival rate decreased by 15% on MTT assay using three-dimensional culture system by CD24 cross-linking. Increased apoptosis determined through annexin V staining was shown, while there was no cell cycle arrest in CD24 cross-linked MCF-7. The migration capacity of MCF-7 was significantly diminished by 30% after cross-linking CD24.

**Conclusion:** These results showed that cross-linking of CD24 could inhibit growth and migration in MCF-7. It suggests that CD24 might be considered as a novel therapeutic target for breast cancer expressing this protein.
Background
CD24 is expressed in hematopoietic cell subpopulations, including B-cell precursors and neutrophils [1], and conventionally used as a differentiation marker for B-cell and T-cell ontogeny [2]. Accumulating evidence supports the presence of CD24 in a variety of malignancies, including B-cell lymphoma, renal cell carcinoma, small-cell and non-small-cell lung carcinoma, nasopharyngeal carcinoma, hepatocellular carcinoma, bladder carcinoma, as well as epithelial ovarian and breast cancers [3].

CD24, designated ‘heat-stable antigen’ (HSA) in mice, is a glycosylated cell-surface protein linked to the membrane by a glycosyl-phosphatidylinositol (GPI) anchor [4]. CD24 has several potential N- and O-linked glycosylation sites, which act as ligands for P-selectin [4]. Through interactions with P-selectin, CD24 is involved in cellular adhesion processes and signalling pathways in cancer cells [4]. Moreover, CD24-mediated binding to P-selectin on endothelial cells and platelets possibly facilitates the exit of tumour cells from the bloodstream, hence favouring metastasis [3]. In P-selectin-deficient mice, diminished tumour growth and metastasis is observed, compared with wild-type animals [5]. Moreover, CD24 over expression augments cell proliferation, adhesion, and motility in rat carcinoma cell line [2], and induces migration and invasion of gliomas [6].

These studies collectively imply that CD24 might play an important role in tumorigenesis and progression of human cancer. Moreover, CD24 expression is suggested to be a marker of poor prognosis in various cancers, including breast carcinoma [7]. In breast cancer, CD24 mediates progression, metastasis, and rolling of tumour cells through interactions with P-selectin [8]. Additionally, CD24 may be related to tamoxifen resistance [9]. In this study, we sought to further clarify the role of CD24 in breast cancer by blockage via cross-linking the protein in MDA-MB-231 and MCF-7. Changes in viable cell number, adhesion and invasion abilities, and effects on cell cycle and apoptosis are assessed.

Methods
Cell culture
Unless otherwise specified, all reagents were purchased from Sigma Corporation. (St. Louis, MO). MDA-MB-231 and MCF-7 were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). For the anchorage-dependent culture, $5 \times 10^5$ cells were seeded on a tissue culture dish (Falcon, San
Jose, CA). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Photographs were obtained with an inverted system microscope (IX51 model) equipped with a DP50 camera system (Olympus, Tokyo, Japan).

**CD24 expression in breast cell lines**

To detach MDA-MB-231 and MCF-7 from the adhesive membrane of the culture dish, cells were treated with diluted trypsin-EDTA solution. Detachment was monitored with a phase-contrast microscope, and accomplished within 3 minutes to minimize damage to growth of cells. After visual identification of detached cells, 5 ml of medium containing serum was added to the culture for trypsin inactivation. Cells were subjected to centrifugation, and washed with 5 ml of PBS. Following a second centrifugation step, pellets were resuspended in a total volume of 500 µl with the appropriate amounts of antibodies and supplement of PBS. The PE anti-human CD24 antibody (BD Pharmingen, NJ, USA) was employed according to the manufacturer’s manual. After incubation with the antibody for 25 min at room temperature, cells were rinsed three times with PBS. Flow cytometry analysis was performed on a FACSCalibur system (Becton & Dickinson, San Jose, CA). All experiments were performed in triplicate.

**Assessment of changes in proliferation of MDA-MD-231 and MCF-7 cells upon CD24 cross-linking**

5×10⁵ cells were seeded on a tissue culture dish with DMEM containing 10% FBS. After 24 h, they were washed twice with PBS, same medium (DMEM) was added to the dish, and cross-linked with anti rabbit polyclonal IgG and anti-human CD24 rabbit polyclonal antibodies (Santa Cruz Biotechnology, Franklin Lakes, NJ). For each condition, the number of viable cells was estimated with 0.4% trypan blue dye in a Neubauer counting chamber.

**Three-dimensional cultures using matrigel culture and MTT assay**

For three-dimensional cultures, millicell (millipore, Billerica, MA, USA) with 3 um pore size was coated with 5 ug/ml matrigel (BD Pharmingen, NJ, USA) on a 6-well plate at 37°C for 1 h. MCF-7 was harvested with diluted trypsin-EDTA and was seeded to millicells with dose dependent manner of each on 1×10⁵, 3×10⁵, and 5×10⁵ cells at 37°C for 24h. After 24h, cells were cross-linked with anti-rabbit IgG (500 ng/ml) or anti-human CD24 rabbit polyclonal antibody (500 ng/ml) at 37°C for 72h. After 72h, media was removed. 100 ul MTT reagents (5mg/ml) add to each millicell and incubated at 37°C for 3h, after the media removed. 200 ul
dimethylsulphoxide (DMSO) was added to each millicell and were shaken for 10 min. The absorbance of the resulting solution was measured at 595 nm.

**Cell cycle analysis and Annexin V staining**

After cross-linking for 72h, MCF-7 was harvested from the culture dish. Cells were fixed in 70% ethanol for 1 h, and washed with PBS. Next, cells were treated with 100 µg/ml RNase A for 1 h at 37°C, followed by 25 µg/ml propidium iodide solution. Flow cytometry analysis was performed on a FACSCalibur system (Becton & Dickinson, San Jose, CA). All experiments were performed in triplicate. Annexin V staining was performed according to the manufacturer's manual (BD Pharmingen, NJ, USA). MCF-7 was washed with PBS, followed by treatment with diluted trypsin-EDTA solution for detachment from the adhesive membrane of the culture dish. After centrifugation, MCF-7 was washed twice with cold PBS, and resuspended in binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂) at a concentration of 1 × 10⁵ cells/ml. An aliquot (100 µl) of the solution comprising 1 × 10⁵ cells was transferred to a 5 ml culture tube, and 5 µl of Annexin V-FITC and 5 µl of PI were added. After vortexing, cells were incubated for 15 min at room temperature (25°C) in the dark, and 400 µl of 1X binding buffer was added to each tube. Flow cytometry analysis was performed on a FACSCalibur system (Becton & Dickinson, San Jose, CA) within 1 h.

**Adhesion assay**

For adhesion assay, a 96-well plate was coated with type I collagen (10 µg/ml) at 37°C for 1h. Coated wells were washed twice with PBS, and blocked with 10% FBS in PBS. MCF-7 was seeded and incubated with anti-rabbit IgG (500 ng/ml) or anti-human CD24 rabbit polyclonal antibody (500 ng/ml) at 37°C for 24h. After 24h, MCF-7 was washed twice with PBS and then treated with diluted trypsin-EDTA solution to separate from each culture condition. Cells were seeded 2×10⁴/well and incubated at 37°C incubator for 24h. Adherent cells were fixed with 3.7% paraformaldehyde at room temperature for 20 min. After fixation, cells were air-dried for 5 min, followed by staining with 0.1% crystal violet in methanol at room temperature for 45 min. Cells were washed three times with PBS, and rinsed with 0.1 M sodium citrate solution at room temperature for 30 min. The absorbance of the resulting solution was measured at 595 nm.

**Migration assay**
The migration assay was performed using the manufacturer’s manual. MCF-7 was pre-treated with anti-rabbit IgG (500 ng/ml) or anti-human CD24 rabbit polyclonal antibody (500 ng/ml) at 37°C for 24h. After 24h, $2\times10^4$/well cells were seeded to the upper side of a transwell chamber (BD Pharmingen, NJ, USA) and incubated at 37°C incubator for 24h. Cells of the upper side of the transwell were scraped off mechanically, and fixed with 100% methanol at room temperature for 2 min. Cells were stained with 1% toluidine blue in 1% borax solution at room temperature for 2 min. The number of stained cells was counted with an inverted system microscope.

**Statistical analysis**
All data were produced after replication of more than a minimum of three times. Data for statistical analysis were expressed as the mean ± standard error of the mean. Comparison results of treated cells versus the control cells were analysed using the t-test. p value of less than 0.05 was considered statistically significant.

**Results**

**CD24 expression in the MDA-MB-231 and MCF-7**
Expression of CD24 was analyzed in MCF-7 and MDA-MB-231 by flow cytometry. CD24 was expressed on 2% and 61% respectively in MDA-MB-231 and MCF-7 (Fig. 1).

Based on the result, we selected MDA-MB-231 as negative control and MCF-7 as positive control for cross-linking CD24 on breast cancer growth.

**Proliferation of MDA-MD-231 and MCF-7 cells upon CD24 cross-linking**
72h after cross-linking, the number of viable cells was counted to analyze the effects of cross-linking on cell growth. MCF-7 viability did not change after cross-linking with 500 ng/ml anti-rabbit IgG. However, cell viability after 72h was reduced by 75% and 96% at antibody concentrations of 1 ug/ml and 2 ug/ml, respectively (Fig. 2A). Accordingly, 500 ng/ml was selected as the dose of rabbit IgG antibody that exerted no effects on viability. MDA-MB-231 and MCF-7 were separately cross-linked with 500 ng/ml rabbit IgG or 500 ng/ml CD24 antibody. Proliferation did not change in MDA-MB-231 (Fig. 2B). In contrast to MDA-MB-231, proliferation in MCF-7 decreased 4%, 14%, 46%, 50%, and 62% at time-points of 24h, 48h, 72h, 96h, and 120h, respectively, compared to cells cross-linked with the rabbit IgG antibody (Fig. 2C). Our data clearly indicate that CD24 cross-linking has an inhibitory effect on growth of cells expressing CD24.
Inhibition of growth after CD24 cross-linking in three-dimensional culture

Three-dimensional culture using matrigel was suggested as a useful method to conform the matrigel effect of antibody blocking in vitro. We looked for inhibition of growth on CD24 cross-linking in an organized three-dimensional culture using matrigel containing ECM. MCF-7 treated with rabbit IgG antibody and CD24 antibody at doses 500ng/ml for 72h. When cultured on top of three-dimensional matrigel, MCF-7 cross-linked with CD24 antibody, compared to cells cross-linked with the rabbit IgG, has decreased about 15% in proliferation (Fig. 3A, B).

Cell cycle and apoptosis analysis after cross-linking of CD24

We observed decrease in cell number after cross-linking with CD24. To determine whether the cause of decreased cell number was cell cycle arrest or apoptosis, we analyzed cell cycle using DNA content assay with propidium iodide solution and apoptosis assay using staining of annexin V and flow cytometry. Stained DNA contents after CD24 antibody were similar in each cell cycle compared to those after treatment with rabbit IgG antibody (Fig. 4). Annexin V-unstained survived cell population decreased by 10% and 26% at 72h and 96h, respectively, exhibiting increased apoptosis by CD24 cross-linking (Fig. 5. A, B).

Effects of CD24 cross-linking on the adhesion and migration capacity of MCF-7

We assumed the role of CD24 as a cell adhesion molecule in MCF-7. MCF-7 was pre-treated to block function of CD24 with isotype control antibody or CD24 antibody (500 ng/ml) for 24 h. The adhesion capacity of harvested MCF-7 with CD24 pre-treatment was reduced a little but not significantly by statistic analysis (Fig. 6. A). In contrast to the adhesion ability, the number of migrating cells was reduced by 30% after cross-linking with anti-CD24 antibody (Fig. 6B).

Discussion

CD24 plays important roles in progression, migration, and metastasis of human breast cancer. Here, we showed that blocking CD24 through cross-linking is sufficient to inhibit tumour growth, adhesion and invasion in MDA-MB-231 and MCF-7. Additionally, apoptosis is increased upon CD24 cross-linking. Our results are in agreement with previous findings. Suzuki et al. [10] reported that cross-linked CD24 induced apoptosis in a human B-cell subset during the early activation stage through interactions with glycolipid-enriched membrane domains. Taguchi et al. [11]
demonstrated that apoptosis observed upon cross-linking CD24 did not result from non-specific binding of either mouse immunoglobulin or secondary rabbit polyclonal anti-mouse immunoglobulin antibody, but from treatment with a combination of anti-CD24 and rabbit anti-mouse immunoglobulin antibodies in KM-3 pro-B cells. Several analogous reports are documented on proliferation and apoptosis in human B cells [10] and murine thymocytes [12] after cross-linking of CD24. However, the effects of cross-linking CD24 in human breast cell lines have not been investigated to date. Interestingly, in contrast to our findings, Schabath and colleagues showed that MDA-MB-231 transfeted to express CD24 had reduced migration and tumour growth in NOD/SCID mice [13]. Initially, the effects on cell growth after cross-linking anti-rabbit IgG and anti-human CD24 rabbit polyclonal antibody were observed through cell counting. Consistent with previous studies showing that polyclonal immunoglobulin inhibits growth in cancer cells [14], the number of viable cells did not change after cross-linking with 500 ng/ml anti-rabbit IgG, but was reduced by 75% and 96% at concentrations of 1 ug/ml and 2 ug/ml antibody, respectively, after 72 h. Thus, 500 ng/ml was selected as the dose of rabbit IgG antibody that had no effect on cell viability (Fig. 2 A). MDA-MB-231 and MCF-7 were separately cross-linked with either 500 ng/ml rabbit IgG or CD24 (FL-80) antibody. In our experiments, growth rate of MDA-MB-231 were not altered by cross-linking with either 500 ng/ml rabbit IgG or CD24 antibody (Fig. 2 B). But MCF-7 decreased in a time-dependent manner (Fig. 2 C). To study directly cross-linking effect of CD24 in the inhibition of growth to approximate in vivo situation, we applied the there-dimensional culture system using matrigel. MCF-7 also showed inhibition of growth in the three-dimensional culture system (Fig. 3. B). These result implies that cross-linking of CD24 has an inhibitory effect on breast cancer cell growth on MCF-7. In agreement with our findings, Wang et al. [15] reported a reduction in T cell proliferation upon blockage with an anti-HSA antibody. Moreover, cross-linking of CD24 induced apoptosis in several cells, including human T cells [15] and murine thymocytes [12]. Jung and colleagues [12] demonstrated that apoptosis triggered by cross-linking of CD24 results in the generation of reactive oxygen species (ROS), and that the release of apoptosis inducing factor (AIF) does not lead to caspase activation in murine thymocytes. Migration capacities of MCF-7 were reduced to 30% after cross-linking with the CD24 antibody. Our results confirm that CD24 plays an important role in migration in MCF-7, and that tumour progression can be inhibited by cross-linking CD24 (Fig. 6. B). Using in vitro migration assays (matrigel) and in vivo immunohistochemical staining, Senner et al. [6] showed that CD24 induces migration of glioblastomas, in concordance with our data.
CD24 also is an adhesion molecule [16]. During tumour progression, adhesion to the extracellular matrix (ECM) is the initial step for invasion and metastasis [2]. The adhesion of MCF-7 was reduced by 4%, which is not of significant value after cross-linking with the CD24 antibody. Furthermore, CD24 is expressed in most neuroendocrine carcinomas of the skin and can thus be applied as a diagnostic marker as well [17].

Interestingly, we observed variable expression rates of CD24 in MDA-MB-231 and MCF-7. Figure 1 depicts CD24 expression rate of 2% and 65% in MDA-MB-231 and MCF-7. However, expression of CD24 in MCF-7 ranged from 65% to 98%, depending on the passage and culture conditions, such as serum composition of culture media (data not shown). A number of reports show that alterations in protein expression are dependent on the culture environment. For instance, serum in MCF-10A culture plays an important role in CD24 expression [18]. ER expression is altered in MCF-7 and BT474 depending on the passage number, as shown by Lostumbo and colleagues [19]. Consistent with previous reports, our results indicate that the culture environment, such as serum concentration and passage, contributes to the degree of protein expression. CD24 is a prognostic indicator of poor survival in breast cancer due to its role in progression and metastasis [7]. In our experiments, functional blocking of CD24 through cross-linking in MCF-7 had anti-tumour effects, specifically, inhibition of proliferation and migration, as well as induction of apoptosis.

Conclusions
In conclusion, we demonstrated that CD24 cross-linking by anti-CD24 antibody led to decreased proliferation and migration ability, and induction of apoptosis of human breast cancer cell line MCF-7. Thus, the blockage of CD24 can be proposed as a novel therapeutic strategy for breast cancer treatment.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
All the authors contributed to the conception of the work during the initial stages and study design, analysis and interpretation of the data, as well as drafting and critical revision of the important intellectual content. All authors approve the final version of the manuscript to be published. JBK and EK have equally involved in all parts of this
study. DYN and WH were in charge of the general supervision of the research. The order of authorship was based on a joint decision.

Acknowledgements
This study was supported by grants No. 03-2005-008-0 from the Clinical Research Institute, Seoul National University Hospital, and SC-3180 from the Stem Cell Research Center, 21st Century Frontier R&D Program, funded by the Ministry of Science & Technology of Korea.

References

**Figures**

Fig 1. CD24 expression in MDA-MB-231 and MCF-7 by FACS (%). They were cultured on a tissue culture dish with DMEM containing 10% FBS for 72h. Flow cytometry analysis was performed with PE anti-human CD24 antibody on a FACSCalibur system. One of three the representative experiments are shown in the result.
Fig 2. The cell viability after CD24 cross-linking in MDA-MB-231 and MCF-7. 
A) MCF-7 was cross-linked with control Ab (anti-rabbit polyclonal IgG) on dose dependent manner for 72h. B) MDA-MB-231 was cross-linked with control Ab 500 ng/ml or CD24 Ab 500 ng/ml for 72h. C) MCF-7 was cross-linked with control Ab 500 ng/ml or CD24 Ab 500 ng/ml on time dependent manner for 120h. Relative survival cell rate in the data was shown as percent survivals versus the control cells. Data represent the means of at least three independent experiments and standard errors of the means. *: p value of less than 0.05 * *: p value of less than 0.01.

Fig 3. Growth in three-dimensional culture system in MCF-7.
1×10^5, 3×10^5, and 5×10^5 cells were cultured with DMEM containing 10% FBS in the three-dimensional matrigel (5 mg/ml) for 24h and then cross-linked with control Ab 500 ng/ml or CD24 Ab 500 ng/ml for 72h. A) Phase microscope picture of cells cultured on the three-dimensional matrigel (1×10^5 cells). Scale bar 200 X. 50um. B) Absorbance by MTT assay in cells cultured on the three-dimensional matrigel plated with 1×10^5, 3×10^5, and 5×10^5 cells. The absorbance of the resulting solution was measured at 595 nm. The data was shown as the absorbance of survivals versus the control cells. Data represent the means of at least three independent experiments and standard errors of the means. *: p value of less than 0.05.

Fig 4. Changes in cell cycle after CD24 cross-linking for 72h. 
The cell cycle was analyzed using FACS after DNA staining by treatment of propidium iodide. One of three representative experiments is shown in the result.

Fig 5. Changes in the apoptosis after CD24 cross-linking. 
A) cross-linking for 72h. B) cross-linking for 96h. Cells were stained with FITC-conjugated annexin V in a buffer containing propidium iodide and analyzed by flow cytometry. For each group of cells, the percentage of survival cells is shown in the lower left quadrant where both in annexin V low and propidium iodide levels are low. One of three representative experiments is shown in the result.

Fig 6. Effects of CD24 cross-linking on the adhesion and migration capacity of MCF-7. 
A) Adhesion assay. For adhesion and migration assay, MCF-7 were pretreated with control Ab 500 ng/ml or CD24 Ab 500 ng/ml for 24h. 24h after seeding, the absorbance of the resulting solution was measured at 595 nm. B) Migration assay. For the migration assay, MCF-7 was seeded to a transwell chamber and only migrating cells were counted after 24h. A) Relative adhesion rate in the data was shown as percent adhesion cells versus the control cells. B) Relative migrating cell
rate in the data was shown as percent migrating cells versus the control cells. *: \( p \) value of less than 0.05.