METCAM/MUC18 promoted tumorigenesis of human breast cancer SK-BR-3 cells in a dosage-specific manner

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Abstract

Objective: Overexpression of METCAM/MUC18, an immunoglobulin-like cell-adhesion molecule, promotes tumorigenesis and progression of human breast cancer cells. We also observed an intriguing phenomenon that a high-expressing SK-BR-3 clone manifested a transient tumor suppression effect in vivo. The purpose of this study was to understand if this was caused by clonal variation, METCAM/MUC18-dosage effect, or the number of cells injected.

Materials and Methods: Several G418-resistant clones of SK-BR-3, expressing different levels of METCAM/MUC18, were obtained for testing effects of human METCAM/MUC18 on in vitro motility, invasiveness, and anchorage-independent colony formation (in vitro tumorigenicity) and in vivo tumorigenesis in female Balb/C athymic nude mice. Tumor sections were made for histology and immunohistochemistry analyses, and tumor lysates for Western blot analysis to determine the effects of human METCAM/MUC18 expression on levels of various downstream effectors.

Results: METCAM/MUC18 promoted in vitro motility, invasiveness, and in vitro tumorigenicity of SK-BR-3 cells in a dosage-specific manner. Overexpression of METCAM/MUC18 could promote in vivo tumorigenesis of SK-BR-3 cells even when one tenth of the previously used cell number (5 \times 10^5) was injected and in vivo tumorigenesis of SK-BR-3 cells was directly proportional to the dosage of the protein. The previously observed transient tumor suppression effect from the same clone was no longer observed. The downstream effector, such as phospho-AKT/AKT ratio, was elevated in the tumors.

Conclusion: Transient suppression observed previously in the clone was caused by injection of a high cell number (2 \times 10^6–5 \times 10^6). METCAM/MUC18 positively promotes tumorigenesis of SK-BR-3 cells by increasing the survival and proliferation pathway.

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Introduction

Breast cancer is still the most prevalent and deadly cancer in women worldwide, as well as in Taiwan [1]. The cause of death from recurrent breast cancer is distant metastasis. After many years of extensive studies, the mechanism and biology of breast cancer tumor formation and metastasis is still poorly understood [2]. METCAM/MUC18, an immunoglobulin (Ig)-like cell-adhesion molecule, plays an important role in promoting angiogenesis, tumorigenesis, and metastasis in melanoma and prostate cancer [3–12]. Likewise, overexpression of METCAM/MUC18 promotes tumorigenesis and progression of human breast cancer cells [13–15]. We also observed an intriguing phenomenon that a high-expressing SK-BR-3 clone manifested a transient tumor suppression effect in vivo, but not the other high-expressing clone [14]. The purpose of this study was to further understand if this was due to clonal variation, METCAM/MUC18-dosage effect, or the number of cells injected.

For comparison, several G418-resistant (G418R) clones of SK-BR-3, expressing different levels of METCAM/MUC18, were obtained for re-examination in animal tests. Two million cells per clone were injected in athymic mice from the National Laboratory Animal Center (Taipei, Taiwan), and tumorigenicity was determined. We found that suppression was again observed for one high-expressing clone and two moderate-expressing clones but not the low-expressing clone. These G418R-clones of SK-BR-3, expressing different levels of METCAM/MUC18, were used for testing effects of
human (hu)METCAM/MUC18 on their in vitro behaviors and in vivo tumorigenesis in female athymic nude mice (Balb/cAnN.Cg-Foxn1nu/CrlNarl) from the National laboratory Animal Center (Taipei, Taiwan). Tumor sections were taken for histological and immunohistochemical analyses and tumor lysates for Western blot analysis to determine the effects of huMETCAM/MUC18 expression on levels of various downstream effectors. We observed that METCAM/MUC18 promoted in vitro motility and invasiveness in a dosage-specific manner. The extent of formation of anchorage-independent colony (in vitro tumorigenicity) of SK-BR-3 cells was directly proportional to the dosage of the protein. Overexpression of METCAM/MUC18 promoted tumorigenesis of SK-BR-3 cells when one tenth of the previously used cell number ($5 \times 10^5$) was injected into female athymic nude mice. Furthermore, the extent of in vivo tumorigenesis of SK-BR-3 cells was directly proportional to the dosage of the protein. The transient tumor suppression effect from the same clone, which we previously observed in athymic nude mice (Hsd: Athymic Nude-nu) from Harlan Company, Indianapolis, IN, USA, was no longer found.

We concluded that the transient suppressive effect from the clone was not due to clonal variation, or METCAM/MUC18-dosage effect, or the different genetic background of the mice, but rather to the effect of a high number of injected cells ($2 \times 10^6 - 5 \times 10^6$). The downstream effector, such as the phospho-AKT/STAT ratio, was elevated in the tumors, suggesting that METCAM/MUC18 positively promotes tumorigenesis of SK-BR-3 cells by augmenting the survival and proliferation pathway.

Materials and methods

Cell lines and culture conditions

Human breast cancer cell line SK-BR3 from the American Type Culture Collection (ATCC) was grown and maintained in McCoy’s 5A medium with 10% fetal bovine serum (FBS) as previously described [14]. A human melanoma cell line, SK-Mel-28, and three human prostate cancer cell lines, DU145, PC-3, and LNCaP, from ATCC were grown and maintained as previously described [14]. All G418-KR SK-BR-3 clones were grown in the same medium of parent SK-BR-3 cells (McCoy’s 5A medium supplemented with 10% FBS plus 0.8 mg/mL G418 (Cellgro/MediaTech (Manassas, VA, USA) and Hyclone (Logan, UT, USA)) in a humidified 37°C incubator with 5% CO2.

Lipofection of SK-BR-3 cells and selection for METCAM/MUC18-expressing G4188-clones

Lipofection was carried out according to a published procedure [13,14], except that FuGene HD (Roche, Indianapolis, IN, USA) was used as the transfecting agent and 0.8 mg/mL G418 (active component ~75%) was added to the growth medium after transfection. G4188 clones were transferred and expanded sequentially from 24-well to six-well culture plates. Cell lysates of each clone were made from each well of six-well plates, boiled, and kept frozen at $-20^\circ$C. The METCAM/MUC18-positive clones were further expanded, processed, and frozen in liquid nitrogen for preservation as stocks. After 24 colonies were picked, the remaining majority of colonies in the 60-mm plates were trypsinized, mixed, and seeded to two T-25 flasks. Cells grown in one flask were expanded, made into stock, and designated as a pooled clone; those in another flask were made into Western blot lysates and designated as a pooled clone lysate.

Cell motility assay

A cell motility assay was carried out according to a published method [16] with minor modifications [5–8,13,14]. After 18 hours, cells migrating to the bottom wells were detached with trypsin treatment, concentrated by centrifugation, and counted with a hemocytometer. The experiments were repeated three times and means and standard deviations of triplicate values were indicated.

Cell invasiveness assay

A cell invasiveness assay was carried out according to a published method [16] with minor modifications [5–8,13,14]. All procedures were similar to cell motility assays except that before seeding cells to the top wells, the porous polycarbonate membrane (pore size 8 μm) at the bottom of each top well was coated with 70 μg or 140 μg of the diluted Matrigel (growth-factors-reduced and phenol-red-free grade; Culltrex, Trevigen, Gaithersburg, MD, USA, cat. # 3431-005-01, 12.8 mg/mL diluted to 1 mg/mL). After 24 hours, cells migrating to the bottom well were processed and counted. Means and standard deviations of triplicate values were indicated.

Anchorage-independent colony-formation assay

The published procedures [17] were followed with slight modifications [13,14]. The number of colonies (20–50 cells per colony) was counted after 14 days in a humidified 37°C CO2 incubator.

Determination of tumorigenicity of SK-BR-3 clones/cells in athymic nude mice

All animal tests were approved by the Institutional Animal Care and Use Committee (IACUC) with an approval #10012 at Chung Yuan Christian University, Taoyuan City, Taiwan. The guidelines of IACUC were strictly followed for the animal studies. Five 7-week-old female Balb/cAnN.Cg-Foxn1nu/CrlNarl athymic nude mice from the National Laboratory Animal Center were used for the subcutaneous injection of cells from each clone. A single cell suspension from monolayer cultures of SK-BR3 cells of clones 2F-2 (p28), 2F-3 clone (p28), 2F-7N (p33), 2F-18N (p32), 2F-19N (p20), 2F-20N (p36), 2F-22N (p36), or

Figure 1. Expression of METCAM/MUC18 in G418KR SK-BR-3 clones. Human METCAM/MUC18 expression in lysates prepared from various clones/cells, 5 μg proteins per lane, was determined by western blot analysis. Cell lysates from SK-Mel-28 (Lane 1) and that from the parental SK-BR-3 (Lane 2) were used as a positive and a negative controls, respectively. METCAM/MUC18 expression in cell lysates from seven different clones (2F-1, 2F-2, 2F-3, 2F-7N, 2F-18N, 2F-20N, and 2F-22N) and two pooled clones (pooled 2F and pooled 3F) of SK-BR-3 is shown in Lanes 3–11. The number under each lane indicates the relative level of METCAM/MUC18 in each cell line/clone, assuming that in SK-Mel-28 as 100%. α-Actin was the loading control.
the vector clone 3F (p20 or p28) were prepared by treatment with trypsin, washed with phosphate-buffered saline, and \(2 \times 10^6\) or \(5 \times 10^5\) cells were resuspended in cold 0.05 mL cold McCoy’s 5A medium without FBS, mixed with an equal volume of 15.44 mg/mL Matrigel (Cultrex; Trevigen) [18], and subcutaneously injected with a gauge #301/2G needle under the left third nipple. After injection, the size of the tumor was measured with a digital caliper every week until the endpoint of the experiment. Tumor volume was calculated by using the ellipsoid formula \(V = \frac{4}{3} \pi d_1 d_2 d_3 (\text{mm})^3\) [16]. At the endpoint (86 days), mice were killed and tumors from each mouse were excised, weighed, and a portion was used for making cell lysates for Western blot analysis [19] to determine the levels of METCAM/MUC18 and its downstream effectors, such as B-cell lymphoma 2 (Bcl2), Bcl-2 associated X protein (Bax), poly (ADP-ribose) polymerase (PARP), phospho-AKT (phosphorylated AKT)/pan AKT (all three AKTs), vascular endothelial growth factor (VEGF), VEGF receptor 2 (VEGFR2), lactate dehydrogenase A (LDH-A), that may affect the process of tumorigenesis. The rest of the tumor was fixed in formaldehyde (Fisher, Waltham, MA, USA), paraffinized, and sectioned for histology and immunohistochemical staining.

**Western blot analysis**

Cell and tumor lysates were prepared by adding a Western blot lysis buffer containing anti-proteolysis cocktails as previously described [5–8,13,14]. The protein concentration of each lysate was determined and verified by gel electrophoresis and staining as previously described [5–8,13,14]. Western blot analysis was carried out as previously described [5–8,13,14]. Our chicken anti-METCAM/MUC18 IgY [5,20] (1/300 dilutions) was used as the primary antibody and the alkaline phosphatase (AP)-conjugated rabbit anti-chicken IgY (AP162A; Chemicon, Dublin, OH, USA) as the secondary antibody (1/2000 dilution) to detect huMETCAM/
MUC18 expression. Rabbit polyclonal antibodies were used as the primary antibodies to detect expression levels of Bcl2 (N-19, SC-492; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bax (N-20, SC-493; Santa Cruz Biotechnology), VEGF (SC-152; Santa Cruz Biotechnology), and LDH-A [21]. A rabbit monoclonal antibody was used to detect the expression level of VEGFR2 (53B11, 2479; Cell Signaling Technology, Danvers, MA, USA). Primary antibodies for detecting housekeeping genes actin, GAPDH, and β-tubulin were goat polyclonal antibody (SC-1615; Santa Cruz Biotechnology), goat polyclonal antibody (SC-20358; Santa Cruz Biotechnology) and rabbit polyclonal antibody (SC-9104; Santa Cruz Biotechnology), respectively, and secondary antibodies were rabbit anti-goat (AP106 A; Chemicon) and goat anti-rabbit (AP132A; Chemicon), respectively. The substrates BCIP (5-bromo-4-chloro-3-indolyl-phosphate)/NBT (nitro blue tetrazolium) (S3771; Promega, Madison, WI, USA) were used for color development. The image of the specific protein band corresponding to METCAM/MUC18 or other proteins on the nitrocellulose membrane was scanned with an Epson Scanner model 1260 (Epson, Long Beach, CA, USA) and its intensity was quantitatively determined by a NIH software program (Image J version 1.31, National Institutes of Health, Bethesda, MD, USA).

**Histology and immunohistochemistry staining of tumor tissue sections**

Paraffin-embedded tissue sections (5 μm) were used. A tissue section of the subcutaneous tumor derived from the LNCaP-expressing clone, LNS239 [6,7,10], was used as a positive external control for the immunohistochemical staining. Tissue sections were deparaffinized, rehydrated with graded alcohol and phosphate-buffered saline, and used for histological staining [22] and immunohistochemical analyses as previously described [6,7,13,14,23]. Dilutions (1/200 to 1/300) of the chicken anti-huMETCAM/MUC18 IgY were used as the primary antibody and a 1/250 dilution of the biotinylated anti-chicken IgY antibodies (G2891; Promega) as the secondary antibody [6–8,10,13,14]. A streptavidin-conjugated horseradish peroxidase complex (LSAAB-2 system; Dako, via Real Carpinteria, CA, USA) and diaminobenzidine were used for color development. Hematoxylin was used for counter staining. Negative

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**Figure 3.** Effects of METCAM/MUC18 expression on **in vitro** invasiveness. (A) Invasiveness of the SK-BR-3 clones of 2F-2, 2F-3, 2F-18N, and 3F, and (B) that of 2F-1, 2F-7N, 2F-19N, 2F-20N, 2F-22N, and 3F clones were also determined in the presence of 75–150 μg/mL anti-huMETCAM/MUC18 antibody (open columns) or control chicken immunoglobulin Y (filled columns), as indicated. Means and standard deviations of six repeated values of invasiveness tests are indicated. The p values were determined by analyzing two sets of data with the Student t test by using one-tailed distribution-type 1 method.
controls had the primary antibody replaced by the nonfat milk or the control isotype chicken IgY.

**Statistical analysis**

The Student t test was used to analyze the statistical significance of the data in all figures. Two corresponding sets of data were considered significantly different if the p value was < 0.05.

**Results**

**METCAM/MUC18 expression in G418R-clones derived from SK-BR-3 cells**

Previously, we observed an intriguing phenomenon that a high-expressing SK-BR-3 clone manifested transient tumor suppression in vivo, but not the other high-expressing clone [14]. To understand if this was due to clonal variation, dosage effect, or simply an artifact, we obtained several G418-resistant clones of SK-BR-3 that expressed different levels (24–84%) of METCAM/MUC18 and compared them to the two previous clones, 2F-2 (118%) and 2F-3 (94%), as shown in Figure 1.

**METCAM/MUC18 expression increased in vitro motility and invasiveness of SK-BR-3 cells**

Motility of SK-BR-3 cells was proportional to expression of METCAM/MUC18 in the clones (Figure 2A). Invasiveness of SK-BR-3 cells was proportional to expression of METCAM/MUC18 in the clones (Figure 3A). These cellular behaviors were reduced by the presence of anti-METCAM/MUC18 (Figures 2B and 3B).
Figure 5. Effects of METCAM/MUC18 expression on (A) in vivo tumorigenesis, (B) tumor-take, (C) tumorigenicity, and (D) mean final tumor weights of SK-BR-3 clones/cells in female athymic nude mice. All the mice were injected with \(2 \times 10^6\) cells per mouse. (A) The left-most panel shows the mice bearing tumors from the 2F-20N clone; the middle left panel the mice bearing tumors from the 2F-22N clone; the middle central panel the mice bearing tumors from the 2F-7N clone; the middle right panel the mice bearing tumors from the pooled 3F clone. The actual size of excised tumor is shown under each mouse. The bar shows the scale of 1 cm. (B) Tumor-take shows number of mice bearing tumors for each of the four clones at the end point of the experiment. (C) Tumorigenicity of the five SK-BR-3 clones in athymic nude mice is shown by plotting mean tumor volumes/weights versus days after injection. The \(p\) values were determined by analyzing all the data with the Student t test. The \(p\) values between tumor volumes through the time course of one SK-BR-3 clone (2F-20N), which expressed a high level of METCAM/MUC18, and two SK-BR-3 clones (2F-22N and 2F-7N), which expressed a moderate level of METCAM/MUC18, and that of the vector control SK-BR-3 clone (3F) were >0.05. This indicates that the differences among the three sets of data were not significant. The \(p\) value between tumor volumes through the time course of the 2F-19N clone, which expressed a low level of METCAM/MUC18, but still was slightly higher than that of the vector control clone (as shown in Figure 7), and that of the 3F clone was 0.08, indicating that the difference between the two sets of data may be statistically significant. (D) The mean final tumor weights of these four clones in athymic nude mice were compared at the endpoint. The mean final tumor weights from the 2F-20N, 2F-22N, and 2F-7N clones were not significantly heavier than the mean tumor weight from five mice of the vector control clone, 3F, since the overall \(p\) value analyzed by the Student t test was >0.05. The mean final tumor weight from the 2F-19N clone was significantly heavier than the mean tumor weights from five mice of the 2F-20N, 2F-22N, 2F-7N, and the vector control clone, 3F, since the overall \(p\) values analyzed by the Student t test were <0.03.
conclude that these effects were directly induced by METCAM/MUC18.

**METCAM/MUC18 expression increased in vitro anchorage-independent colony formation**

Anchorage-independent colony formation in soft agar has been successfully used to assess the tumorigenicity of cancer cells in vitro, which has been positively correlated to in vivo tumorigenicity in animal models [17]. The ability of SK-BR-3 cells to form anchorage-independent colony in soft agar was dependent upon expression of METCAM/MUC18 (Figures 4A and 4B). We conclude that the ability of SK-BR-3 cells to induce in vitro tumorigenicity was dependent upon the expression levels of METCAM/MUC18.

**METCAM/MUC18 expression increased tumorigenicity and final tumor weight of SK-BR-3 cells in athymic nude mice**

To re-examine if the previously observed transient tumor suppressive effect of the one high-expressing clone was reproducible in the female athymic nude mice from National Laboratory of Animal Center with a genetic background (Balb/cAnN.Cg-Foxn1n/u/CrlNarl) different from those from Harlan (Hsd: athymic nude-nu), a high number of cells (2 × 10⁶/mouse) from four METCAM/MUC18-expressing clones, 2F-20N, 2F-22N, 2F-7N, and 2F-19N, and a vector control clone (clone 3F) were injected. Figures 5A and 5B show that tumor-take was similar in all the clones, as shown in each mouse injected with 5 × 10⁶ cells per mouse [14]. Tumor suppression was shown in the one high-expressing clone, 2F-20N, and two moderate-expressing clones, 2F-22N and 2F-7N, but not the low-expressing clone, 2F-19N, which had better tumorigenicity than all the above three clones and the vector control clone (Figure 5C). This was further supported by the results of final tumor weight (Figure 5D). However, we did not keep the mice for a longer period to examine if the tumor suppression was transient.

To examine if the previously observed transient tumor suppressive effect of 2F-2, which expressed a level of METCAM/MUC18 as high as the positive control SK-Mel-28, was due to an artifact of injecting a high number of cells, we performed the same experiment by injecting the same previous three clones plus one more high-expressing clone, 2F-18N, with 5 × 10⁵ cells/mouse, which was one tenth of the cell number previously used. Overexpression of METCAM/MUC18 promoted tumorigenesis of SK-BR-3 cells even when the injected cell number was one tenth (5 × 10⁵) of that in the previous experiment (Figure 6A). The tumor-take was dependent upon the expression levels of METCAM/MUC18 (Figure 6B),
Figure 7. Expression of METCAM/MUC18 antigens in tumor lysates (A) and (B) quantitative results of (A), and (C) the histology and immunohistochemistry of the SK-BR-3 tumors. (A) Expression of human (hu)METCAM/MUC18 in the tumor lysates was determined by Western blot analysis. The lysate from tissue cultured SK-BR-3-2F-2 clone (Lane 1) was used as the positive control for comparison with those in the tumor lysates (Lanes 2–20). huMETCAM/MUC18 expression levels in two tumors from the 2F-2 clone, one tumor from the 2F-3 clone, three tumors from the 2F-19N, 2F-20N, and 2F-22N, four tumors from the 2F-7N clone, and three tumors from the pooled 3F clone are shown. β-Tubulin was the loading control. (B) shows the quantitative results of (A). (C) Panels A–D show histology of the tumor sections from the two SK-BR-3 clones, 2F-2 and 2F-3. Panels E–L show the immunohistochemistry of these tumor sections. Panels E–H show the anti-huMETCAM/MUC18 antibody staining of the cells in the tumor sections. A tumor section from LNCaP clone LNS239 was used as a positive control for the immunohistochemistry (Panel E). Both tumor sections from 2F-2 (Panels F and G) showed stronger brown staining in immunohistochemistry than that from 2F-3 (Panel H) when the antibody was added, however, the tumor section from the clone 3F only showed a weak background staining (data not shown). Panels I–L show the corresponding negative controls, which showed no staining in the adjacent sections when no antibody or control immunoglobulin Y was added.
Transient tumor suppression was not observed in the same clone (2F-2), in which the transient suppression was previously observed when 5 × 10^6 cells were used for injection (Figure 6C). Final tumor weight, although only about one-third of that when a high number of cells (2 × 10^6–5 × 10^6/mouse) were injected, was proportional to the dosage of METCAM expressed in the clones (Figure 6D). We conclude that in vivo tumorigenesis of SK-BR-3 cells was dependent upon the expression levels of METCAM/MUC18, thus, the previously observed transient suppression was due to the injection of a high number of cells.

**METCAM/MUC18 expressed in tumors derived from different clones of SK-BR-3 cells**

To examine if METCAM/MUC18 was not altered or modified during tumorigenesis and the tumors were indeed from the injected clones, Western blot analysis of the METCAM/MUC18 expressed in tumor lysates was performed. METCAM/MUC18 in different tumors from different clones were expressed at different levels in tumor lysates, and METCAM/MUC18 had electrophoretic mobility similar to the positive control, as expected (Figures 7A and 7B). The level of METCAM/MUC18 was not significantly different among the three high-expressing clones, 2F-2, 2F-3, and 2F-22N (Figure 7B; p = 0.07). Figure 7C shows the histology and immunohistochemistry of these tumors. Tumor sections induced by the METCAM/MUC18-expressing clones, 2F-2 and 2F-3, were positively stained with the anti-METCAM/MUC18 antibodies with a stronger staining of the two tumor sections from 2F-2 clone than that from 2F-3 clone, as shown in panels F and G versus panel H in Figure 7C. Thus the immunohistochemistry results (Figure 7C) were consistent with the Western blot results (Figures 7A and 7B), and support the notion that these tumors originated from the injected SK-BR-3 clones, and METCAM/MUC18 was not altered or modified during tumorigenesis, indicating that it directly mediated the process.

**Preliminary mechanisms of METCAM/MUC18-mediated tumorigenesis of SK-BR-3 cells**

HuMETCAM/MUC18 expression may increase tumorigenesis by crosstalk with many signaling pathways that affect survival, metabolism, and proliferation, and angiogenesis of tumor cells [24–26]. For this purpose, expression levels of an apoptotic index, an anti-apoptosis and survival index, phospho-AKT and pan-AKT, an index of aerobic glycolysis, and two angiogenesis indexes in tumors were determined (Figure 8A). After quantitative and statistical analysis, we found that levels of other parameters were not significantly elevated (data not shown). The ratio of phospho-AKT/AKT, the index for the survival and proliferative pathway, was significantly elevated in the tumors from METCAM/MUC18-expressing clones (Figure 8B).

**Discussion**

During our previous investigation of the effects of overexpression of METCAM/MUC18, an Ig-like cell-adhesion molecule, on tumorigenesis and progression of human breast cancer SK-BR-3 cells, we observed fortuitously an intriguing phenomenon that a high-expressing SK-BR-3 clone manifested transient tumor suppression in vitro, but not another high-expressing clone [14]. This phenomenon is intriguing in that it may reveal important mechanisms of METCAM/MUC18-mediated progression of human breast cancer cells, especially since one group had suggested that METCAM/MUC18 might have a tumor suppressor role in the tumorigenesis of one human breast cancer cell line, MCF-7 [27]. First, to re-examine if the above phenomenon was reproducible in nude mice with a different genetic background, we carried out in this study similar animal tests by injecting into each nude mouse 2 × 10^6 cells from four METCAM/MUC18-expressing clones and one vector control clone. We found that tumor suppression was reproducible in one high-expressing clone and two moderate-expressing clones when compared to the vector control clone, but not a low-expressing clone, which appeared to have a tumor-promoting effect. However, we did not know if this suppressive effect was transient because we did not keep the mice for a longer period. Then, to further explore the mechanisms of METCAM/MUC18-mediated tumorigenesis of human breast cancer cells and to understand if the above phenomenon was due to the effect of clonal variation, METCAM/MUC18 dosage, or injecting a high number of the SK-BR-3 cells, we also carried out the experiments reported here and showed that in vitro motility, invasiveness, formation of anchorage-independent colonies in soft agar (in vitro tumorigenicity) and in vivo tumorigenesis of SK-BR-3 were dependent upon the dosage of METCAM/MUC18 expression. Furthermore, overexpression of METCAM/MUC18 could promote tumorigenesis of SK-BR-3 cells even when the injected cell number was one tenth of that in previous experiments. Furthermore, the extent of in vivo tumorigenesis of SK-BR-3 cells was directly proportional to the dosage of the protein. The transient tumor-suppressive effect previously observed from the same clone was no longer observed. We thus conclude that the previously observed transient tumor suppression from one clone, which expressed 118% of METCAM/MUC18, as well as two other clones was reproduced again in this study, and was due to the injection of a high cell number. However, it was not due to an effect of clonal variation, indicating that precautions should be taken into consideration for injecting a high cell number, which appears to be a common practice in many animal studies by many research groups. The results also clearly ruled out the possibility that METCAM/MUC18 plays a tumor suppressor role and further supported the notion that METCAM/MUC18 plays a positive role in the progression of human breast cancer cells [2,13–15]. From preliminary mechanical studies, we found that METCAM/MUC18 positively promotes the tumorigenesis of human breast cancer SK-BR-3 cells via increasing the signal in the survival and proliferative pathway, the phospho-AKT/AKT ratio, which in turn increases the processes of survival and proliferation [28]. This is consistent with the notion that METCAM/MUC18 plays a reciprocal regulatory role with the AKT pathway in the tumorigenesis of human cancers [12,25,29].

We are not clear why injection of a high cell number of METCAM/MUC18-expressing SK-BR-3 clones caused a transient suppressive effect. Three possible interpretations are as follows. One possibility may be that when a high number of SK-BR-3 cells were injected, the transient suppression may have been due to the high compactness of the cells, which is supported by the recent study of Waclaw et al [30], who suggested that the spaces in the tumor mass can affect the growth rate of the tumor. When tumor cells are compacted in a tight mass, it decreases the growth rate, but when the tumor cells are loosely packed, the growth rate increases. Thus when a high number of cells were injected, a more tightly compacted initial tumor mass might have formed that transiently repressed tumor growth. Only after 3 weeks (or even longer), the tumor mass was somehow loosened by the high motility of cells (due to high expression of METCAM/MUC18). When fewer cells were injected, a more loose tumor mass was formed and cells were more freely mobile, thus transient suppression was not manifested. Alternatively, the transient suppression may have been due to tumor dormancy [31], which may be triggered by injection of a high number of cells from clones that highly express METCAM/MUC18. The transient dormancy may be turned on by the immediate negative response from the host innate immune system, natural killer (NK) cells, by a transiently dominant anti-angiogenic effect, or
Figure 8. Expression of signals downstream of METCAM/MUC18. (A) shows the summarized results of Western blot analysis of expression levels of various parameters (LDH-A, PCNA, VEGF, VEGFR2, Bcl2, Bax, PARP, pan-AKT, Phospho-AKT (Ser473)) in the SK-BR-3 tumors. Expression levels of housekeeping genes, actin and β-tubulin, were used as loading controls. The number below each lane shows the relative intensity. The numbers between the rows of actin and β-tubulin were the average relative intensities from the two housekeeping proteins. The quantitative results of the average relative intensity of the band corresponding to ratios of phospho-AKT/AKT in tumors are also shown in (B). The p values between those expressed in the tumor lysates derived from human METCAM/MUC18-expressing clones with a vector control clone, 3F, are shown. Bcl2, B-cell lymphoma 2; Bax, Bcl-2 associated X protein; PARP, poly (ADP-ribose) polymerase; VEGF, vascular endothelial growth factor; VEGFR2, VEGF receptor 2; LDH-A, lactate dehydrogenase A.

by an overwhelming apoptotic response over the anti-apoptosis pathway, thus, the growth was suppressed. The possibility of an immediate negative response from the host innate immune system, NK cells, which should be intact in athymic mice, may exist since METCAM/MUC18 has been shown to be a marker for maturation of mouse NK cells [32], although the effect of METCAM/MUC18 expression on the response of host NK cells to tumor cells has not been studied. The two other mechanisms of tumor dormancy may be supported by the results in Figure 8 that the Bax level was higher in tumor lysates than the Bcl2 level and VEGF and VEGFR2 levels were also low. These suppressive mechanisms may be gradually overcome after these cells have been tolerated for ≥3 weeks. The third possible interpretation may be that the tumor microenvironment needs to be conditioned first by the METCAM/MUC18-high expressing SK-BR-3 cells to become a favorable niche for tumor growth; this favorable niche may take ≥3 weeks to form. The above points to a new aspect of future research for us and the knowledge learned may reveal a new way to control the growth of breast cancer cells; hence the translational aspect of the current research.

We conclude that METCAM/MUC18-induced in vitro and in vivo tumorigenesis of SK-BR-3 cells was directly proportional to expression of METCAM/MUC18. We also conclude that the previously observed transient tumor suppression from one clone, which expressed 118% of METCAM/MUC18, was due to the injection of a high cell number. Thus, it was not due to an effect of clonal variation, pointing to caution when using a high cell number for injection. METCAM/MUC18 positively promotes tumorigenesis of human breast cancer SK-BR-3 cells via increasing the signal in survival and proliferation pathways, such as the phospho-AKT/AKT ratio, which in turn increases the processes of survival and proliferation.

Conflicts of interest

The authors declare no conflict of interest.

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