Enforced Expression of MCAM/MUC18 Increases *In vitro* Motility and Invasiveness and *In vivo* Metastasis of Two Mouse Melanoma K1735 Sublines in a Syngeneic Mouse Model

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Abstract

Human MCAM/MUC18 has been shown to increase metastasis of human melanoma cells in xenograft mouse systems. To be more relevant to understanding the progression of clinical melanoma and for designing better preclinical therapeutic trials, it is highly desirable to establish a syngeneic mouse model for studying the mechanisms of MCAM/MUC18-mediated tumorigenesis and metastasis of melanoma cells. To reach this goal, we transfected the mouse MCAM/MUC18 (moMCAM/MUC18) cDNA into two MCAM/MUC18-minus, low-metastatic mouse melanoma K1735 sublines, K1735-10 (tumor$^-$/met^{low}) and K1735-3 (tumor$^+$/met^{low}), and selected for G418-resistant clones, which expressed different levels of moMCAM/MUC18, and used for testing the effect of MCAM/MUC18 overexpression on their *in vitro* growth rate, motility, and invasiveness and *in vivo* subcutaneous tumor growth and pulmonary metastasis in syngeneic mice. Enforced expression of moMCAM/MUC18 did not significantly affect *in vitro* growth rate, but it increased the *in vitro* motility and invasiveness of clones derived from both sublines. Ectopic expression of moMCAM/MUC18 did not alter the nontumorigenicity of the K1735-10 clones per cells nor significantly affect the subcutaneous tumor growth of the K1735-3 clones per cells. The moMCAM/MUC18-expressing K1735-10 clones were able to establish only microscopic lung modules in 86% of the mice. In contrast, the moMCAM/MUC18-expressing K1735-3 clones could induce numerous large lung nodules (3-4 mm in diameter) in all the mice. We concluded that increased moMCAM/MUC18 expression in the two K1735 sublines minimally affected their tumorigenicity, but it augmented their *in vitro* motility and invasiveness and increased their pulmonary metastasis in the syngeneic C3H mice. (Mol Cancer Res 2008;6(11):1666–77)

Introduction

Human MCAM/MUC18 (huMCAM/MUC18) was originally identified as a glycoprotein antigen, which is frequently and overly expressed on the surface of malignant human melanoma cells (1). Overexpression of huMCAM/MUC18 has been postulated to play a role in increasing the metastatic progression of clinical human melanomas (2). The hypothesis is further supported by that stable ectopic expression of huMCAM/MUC18 in a low-tumorigenic and nonmetastatic human cutaneous melanoma cell line, SB-2, significantly increases its metastatic capability in nude mice (3). A similar result is also obtained by using different human melanoma cell lines in a different immunodeficiency mouse model (SCID mouse; ref. 4). However, the effect varies between the two different low-tumorigenic, nonmetastatic human cutaneous melanoma cell lines, Xp-44 and SK-2. The ectopic expression of huMCAM/MUC18 in the human melanoma cell line XP-44 increases the lung nodules formation in experimental metastasis tests, but not the human melanoma cell line SK-2 (4). It is not clear if these results reflect the uniquely defective immune system of a xenograft mouse system or the different intrinsic properties of different cell lines. Furthermore, the knowledge learned from melanoma cell xenografts in immunodeficient mouse models may only be partially extrapolated to understanding the clinical progression of human melanomas.

Thus, a syngeneic mouse model with a complete immune system would greatly facilitate a more realistic understanding of the mechanism of the MCAM/MUC18-mediated metastasis of melanoma cells. It also offers many advantages over a xenograft mouse system for metastatic studies. (*a*) It has a complete wild-type immune system; thus, the model is more closely related to the real situation in clinical human melanomas. (*b*) The system is useful for testing biological responses of various preclinical immunotherapeutic trials before they are to be applied to human cases. (*c*) The significance of a gene in promoting or potentiating melanoma metastasis can be evaluated in genetically engineered mice. (*d*) The model may serve as a whole animal imaging system (luminescence, fluorescence, magnetic resonance imaging, micro–positron emission tomography) for a real-time visualization of the dynamics of melanoma metastasis.

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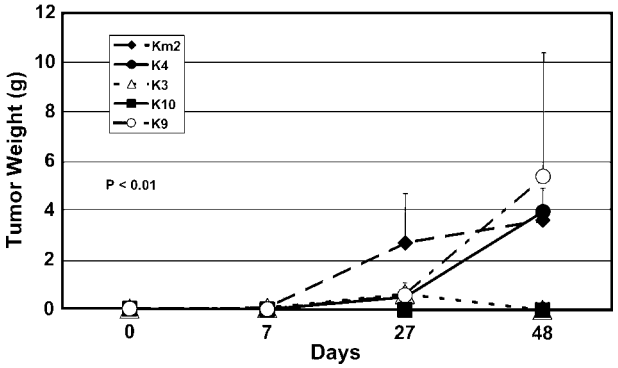

FIGURE 1. Tumor formation of five mouse melanoma K1735 sublines. Tumor formation was done by subcutaneous injection of 1.5×10^6 cells of each of the five K1735 sublines (Km2, K3, K4, K9, and K10) into the dorsal flank of C3H syngeneic mice. Tumors were palpable for Km2 cells after 7 d. Five mice were necropsized and then the tumor was excised after 27 d, and another five mice followed after 48 d. The tumor weight in each mouse was determined by a balance. The mean tumor weight from each group of mice was plotted against time. Data obtained from K10 cells were used as the reference for statistical analyses using the Student's *t* test.

To establish the MCAM/MUC18-mediated metastasis of melanoma cells in a syngeneic mouse model, we have isolated and determined the sequence of the mouse MCAM/MUC18 (moMCAM/MUC18) cDNA from several mouse melanoma cell lines. The longest open-reading frame of the cDNA gene encodes 648 amino acids, two amino acids longer than the huMCAM/MUC18 (5). Nevertheless, similar to huMCAM/MUC18, the moMCAM/MUC18 is also categorized as a cell adhesion molecule (CAM) in the immunoglobulin gene superfamily (5, 6) because the amino acid sequence of moMETCAM/MUC18 protein delineates five immunoglobulin-like domains and it is 76.2% identical to the amino acid sequence of huMCAM/MUC18 (5). We have also shown that the moMCAM/MUC18 expression level in nine mouse melanoma cell lines is directly proportional to their metastatic abilities (5). In this report, we further tested the hypothesis that overexpression of moMCAM/MUC18 should also promote the metastasis of mouse melanoma cells in a syngeneic mouse system and, perhaps, reveal new information (6).

To test the hypothesis, we introduced the moMCAM/MUC18 cDNA gene, which was driven by a HCMV-IE promoter cloned in a mammalian cell expression vector, into two mouse melanoma K1735 sublines, K1735-3 (K-3) and K1735-10 (K-10). The two K1735 sublines were chosen because of their negative expression of moMCAM/MUC18 (5) and low tumorigenicity and metastatic ability (7): K-10 has a phenotype of tumor$^-$/met^{low} and K-3 tumor$^+$/met^{low}. This different phenotype of the two sublines, but in an isogenic background (7), made them especially interesting for testing the effect of ectopically expressed moMCAM/MUC18 on their metastatic ability. We isolated the G418-resistant (G418^R) clones from these sublines and selected for clones that expressed high levels of moMCAM/MUC18 for testing the effect of moMCAM/MUC18 expression on their *in vitro* growth rate, motility, and invasiveness. We also determined their ability to form subcutaneous tumors and cause pulmonary metastases. The preliminary result has been presented in the annual meeting of the American Association for Cancer Research (8).

Results

No Correlation of moMCAM/MUC18 Expression with the Tumorigenicity of Five K1735 Sublines

In contrast to metastasis tests, the tumorigenicity of five K1735 sublines (clones Km2, K3, K4, K9, and K10) has not been determined (7). To study the effect of moMCAM/MUC18 overexpression in some of these sublines on their tumorigenicity, we determined the tumor formation by these sublines in syngeneic C3H mice after subcutaneous injection of these cells. The results are shown in Fig. 1 and Table 1. As shown in Fig. 1 and Table 1, K10 did not form subcutaneous tumor even 7 months after injection of the tumor cells, whereas the one positive control subline, K4 (tumor^{+++}/met^{+++}), formed tumors after 21 to 26 days. Tumor of K4 reached the maximal size after 48 days. The other positive control subline, Km2 (tumor^{+++}/met^{+++}), formed detectable tumors as early as 7 days; the tumors reached a substantial size of about five times larger than K4 at 27 days and slowly reached a maximal size at 48 days. K3 formed tumors of smaller size, reached the maximal size at 27 days, began to shrink after 27 days, and continue to shrink to an undetectable size at 48 days. K9 (tumor^{+++}/met^{low}) cells formed tumors, which reached a size similar to that of K3, at 27 days but then continued to grow to a very large size, some of which even surpassed K4 at 48 days. The result shows that each subline had a different capability to form subcutaneous tumor and temporally manifested different tumor growth rate. We concluded that the ability of tumor formation and tumor growth rate and tumor size did not correlate with the

Table 1. moMETCAM/MUC18 Expression, Tumor Growth, and Pulmonary Metastasis of Five K1735 Sublines in C3H/HEN/Hsd Syngeneic Mice

Cell lines/properties	K1735-m2	K1735-4	K1735-9	K1735-3	K1735-10	References
MCAM/MUC18 expression	+++	++++	None	None	None	7
Tumor size (g), after 7 d*	0.05	None	None	0.05	None	7
Tumor size (g), after 27 d*	2.7 ± 2.0	0.5 ± 0.22	0.58 ± 0.35	0.63 ± 0.46	0	7
Tumor size (g), after 48 d*	3.63 ± 0.4	3.97 ± 0.95	5.4 ± 5.0	0	0	7
Pulmonary metastasis (no. lung nodules), after 35 d †	Yes (>150)	Yes (156)	None (0)	Yes (1)	Yes (0.5)	8
Phenotype	tumor^{+++}/met^{+++}	tumor^{+++}/met^{+++}	tumor^{+++}/met$^-$	tumor$^+$/met^{low}	tumor$^-$/met^{low}	7

*1.5×10^6 cells were subcutaneously injected per mouse.
†1×10^5 cells were intravenously injected per mouse.

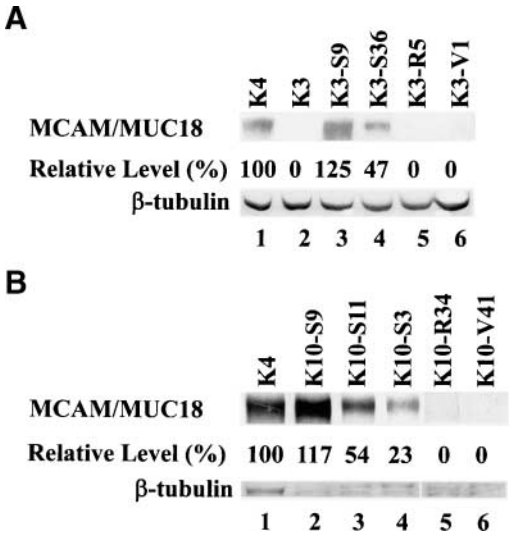

FIGURE 2. Expression of mMCAM/MUC18 protein in clones derived from K3 (**A**) and K10 (**B**), which were transfected with the expressible moMCAM/MUC18 cDNA. **A.** K4 (*lane 1*), a highly metastatic subline, was used as a positive control, and K3 (*lane 2*), a low metastatic subline, was used as a negative control. Two K3-derived clones, K3-S9 (*lane 3*) and K3-S36 (*lane 4*), which were transfected with the moMCAM/MUC18 cDNA in sense orientation, expressed high and low levels of moMCAM/MUC18, respectively. One K3-derived clone, K3-R5 (*lane 5*), which was transfected with the moMCAM/MUC18 cDNA in the antisense orientation, expressed no moMCAM/MUC18. One K3-derived clone, K3-V1 (*lane 6*), which was transfected with the empty vector, also did not express any moMCAM/MUC18. The relative level of moMCAM/MUC18 expression in each clone per cell line is indicated at the bottom as a number, assuming that the level of moMCAM/MUC18 expression in the K4 subline was 100%. β-Tubulin was used as the loading control. **B.** K4 (*lane 1*), a highly metastatic subline, was used as a positive control. Three K10-derived clones, K10-S9, K10-S11, and K10-S3 (*lanes 2-4*), which were transfected with moMCAM/MUC18 cDNA in the sense orientation, expressed different levels of moMCAM/MUC18. One K10-derived clone, K10-R34 (*lane 5*), which was transfected with moMCAM/MUC18 cDNA in the antisense orientation, and one K10-derived clone, K10-V41 (*lane 6*), which was transfected with the empty vector only, did not express any moMCAM/MUC18. The relative level of moMCAM/MUC18 expression for each clone per cell line is indicated at the bottom as a number, assuming that the level of moMCAM/MUC18 expression in the K4 subline was 100%. β-Tubulin was used as the loading control.

expression level of moMCAM/MUC18. In contrast, the metastatic ability of these sublines positively correlates with the expression of moMCAM/MUC18, as shown in Table 1. The phenotypes of these sublines are summarized in Table 1.

moMCAM/MUC18 Expression in G418^R Clones Derived from K3 and K10 Sublines

The two K1735 sublines, K3 and K10, are most suitable to be used for testing the effect of enforced expression of moMCAM/MUC18 on their *in vitro* motility and invasiveness and their ability of *in vivo* tumorigenesis and metastasis, because they have isogenic background, do not express any detectable level of moMCAM/MUC18 mRNA and protein, and have a very low ability to form tumor and metastasize (5). To obtain moMCAM/MUC18-expressing clones from these two K1735 sublines, we transfected these two sublines with an HCMV-IE promoter-driven moMCAM/MUC18 cDNA gene in a mammalian cell expression plasmid vector, pcDNA3.1+, which also contained the neomycin-resistant gene driven by SV40 promoter, to facilitate screening of expressing clones. Many G418^R clones, which expressed different levels of MCAM/MUC18, were identified by our anti-moMCAM/MUC18 antibody in the Western blot analysis.

Figure 2A shows different expression levels of moMCAM/MUC18 protein in four typical G418^R clones derived from the K3 subline. The moMCAM/MUC18 level expressed in the K3-S9 clone was higher than the positive control, the K4 subline; lower in the K3-S36 clone; and none in the parental K3 cells and two negative control clones, K3-R5 (moMCAM/MUC18 cDNA in antisense orientation) and K3-V1 (the vector control).

Figure 2B shows different expression levels of moMCAM/MUC18 protein in five typical G418^R clones derived from K10. The moMCAM/MUC18 level expressed in the clone K10-S9 was higher than the positive control, the K4 subline. The moMCAM/MUC18 level expressed in clones K10-S11 and K10-S3 was lower than K4 and the K10-S9 clone. In contrast, similar to the parental K10 subline, the moMCAM/MUC18 level was not detectable in both the two negative controls, the K10-R34 clone, in which the transfected moMCAM/MUC18 cDNA was in antisense orientation, and the K10-V41 clone, in which only the empty vector was transfected. These clones were used for the following tests.

Possible Effects of moMCAM/MUC18 Expression on the In vitro *Growth Rate, Motility, and Invasiveness of the Two K1735 Sublines*

The result of the effect of moMCAM/MUC18 expression on the growth rate of the above G418^R clones derived from K3 sublines is shown in Fig. 3. Expression of ectopically transfected moMCAM/MUC18 did not significantly alter the growth rate of the K3 cells (Fig. 3). Similar finding was also observed in the K10 cells (data not shown). We, thus, concluded that overexpression of moMCAM/MUC18 did not significantly affect the growth rate of these two K1735 sublines.

The results of the effect of moMCAM/MUC18 expression on the *in vitro* motility of the above G418^R clones derived from

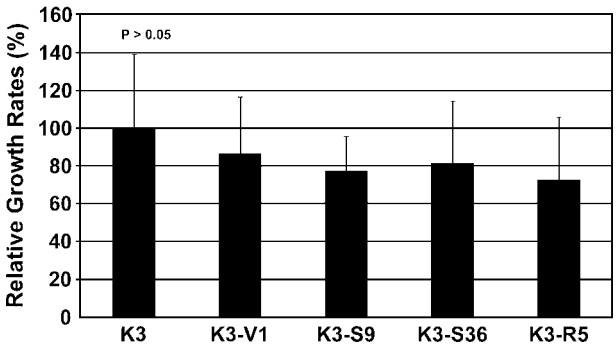

FIGURE 3. The growth rates of K1735-3 clones. Effect of moMCAM/MUC18 expression on the growth rate of G418^R clones derived from moMCAM/MUC18 cDNA-transfected mouse melanoma K1735-3 subline. Two clones, K3-S9 and K3-S36, were derived from K3 by transfection with the moMCAM/MUC18 cDNA in the sense orientation. One clone, K3-R5, was derived from K3 by transfection with the moMCAM/MUC18 cDNA in the antisense orientation. One clone, K3-V1, was derived from K3 by transfection only with the empty vector. The growth rates of the four G418^R clones and the parental K3 cells were determined as described in Materials and Methods and statistically analyzed by the Student's *t* test.

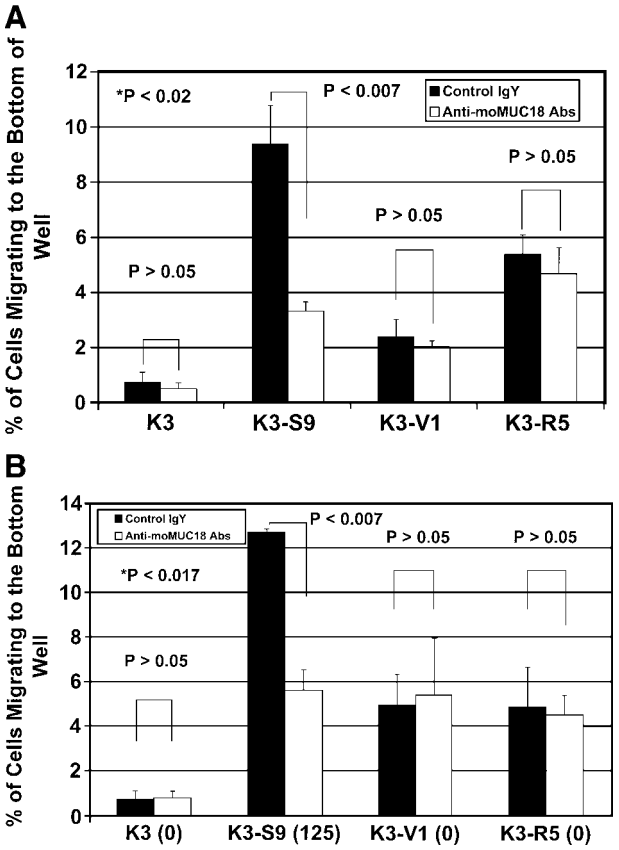

FIGURE 4. Motility and invasiveness of mouse melanoma K1735-3 clones. Effect of moMCAM/MUC18 expression on the *in vitro* motility (**A**) and invasiveness (**B**) of the mouse melanoma K3 subline. The motilities (**A**) and the invasiveness (**B**) of the parental K3 cells, one G418^R clone (K3-S9), in which K3 cells were transfected with the moMCAM/MUC18 cDNA in sense orientation, one G418^R clone (K3-R5), in which K3 cells were transfected with the moMCAM/MUC18 cDNA in antisense orientation, and one G418^R clone (K3-V1), in which K3 cells were transfected with the empty vector, which were determined as described in Materials and Methods. The numbers in the brackets adjacent to each clone name in the *x* axis represent the level of MCAM/MUC18 in each clone, respectively. Anti-moMCAM/MUC18 antibody (*open columns*) or control chicken IgY (*filled columns*) was added to block the motility and invasiveness of these clones. Means and SDs of triplicate values of motility tests were indicated, except in K3 and K3-V1, in which means and SDs of 10 and 12 repeated values of motility tests were indicated. Means and SDs of six repeated values of invasiveness tests were indicated. The **P* value (the values of different control clones versus those of the K3-S9 cells) and *P* values (the values between the anti-moMCAM/MUC18 antibody and the control IgY in each clone) were obtained by analyzing the experimental data with the Student's *t* tests, as described in Materials and Methods.

K10 and K3 sublines are shown in Figs. 4A and 5A. Figure 4A shows that a high moMCAM/MUC18-expressing clone, K3-S9, had ~2-fold to 3-fold higher motility than that of the control clones, K3-V1 and K3-R5. Figure 5A shows that a high moMCAM/MUC18-expressing clone, K10-S9, had ~2-fold higher motility than that of the control clone, K10-V41, whereas the lower-expressing clones (K10-S11 and K10-S3) did not increase the motility.

The results of the effect of moMCAM/MUC18 expression on the *in vitro* invasiveness of the above G418^R clones derived from K3 and K10 sublines are shown in Figs. 4B and 5B. Figure 4B shows that expression of moMCAM/MUC18 increased the invasiveness ~2-fold of the clones derived from K3 subline. Figure 5B shows that high expression of moMCAM/MUC18 in the clone K10-S9 increased the invasiveness ~4-fold of the control clone K10-V41, derived from the K10 subline, and the lower-expressing clones (K10-S11 and K10-S3) also increased the invasiveness, albeit to a less extent, ~2-fold to 3-fold of the control clone K10-V41.

As also shown in Figs. 4 and 5, the increased motility and invasiveness of the moMCAM/MUC18-expressing clones were significantly decreased by the presence of anti-moMCAM/MUC18 antibodies, whereas those of the control clones, which did not express moMCAM/MUC18, were not affected by the antibodies.

Taken together, we concluded that the increased motility and invasiveness in these clones were due to the presence of the moMCAM/MUC18 protein. It seemed that a low expression

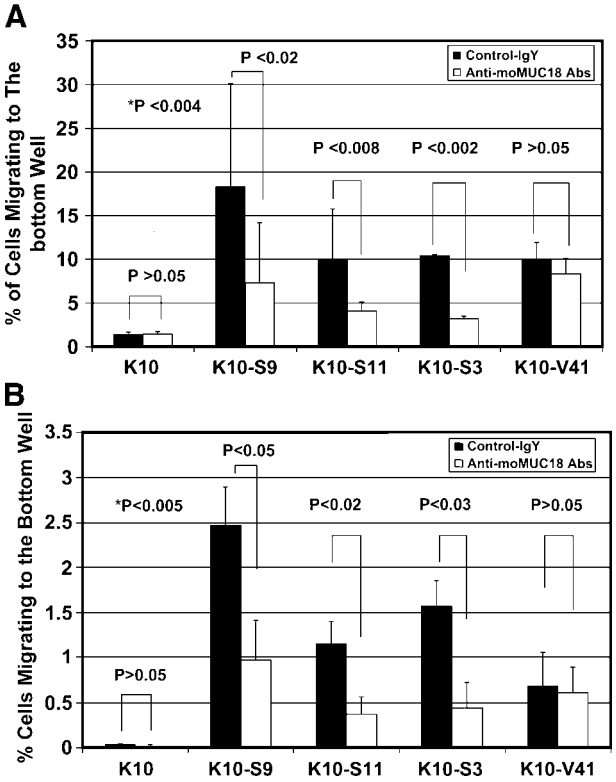

FIGURE 5. Motility and invasiveness of mouse melanoma K1735-10 clones. Effect of moMCAM/MUC18 expression on the *in vitro* motility (**A**) and invasiveness (**B**) of the mouse melanoma K10 subline. The motilities (**A**) and the invasiveness (**B**) of the parental K10 cells, three G418^R clones (K10-S9, K10-S11, and K10-S3), in which K10 cells were transfected with the moMCAM/MUC18 cDNA, and one G418^R clone (K10-V41), in which K10 cells were transfected with the empty vector, which were determined as described in Materials and Methods. Anti-moMCAM/MUC18 antibody (*open columns*) or control chicken IgY (*filled columns*) was added to block the motility and invasiveness of these clones. Means and SDs of triplicate values of motility tests were indicated. Means and SDs of six repeated values of invasiveness tests were indicated. The **P* value (the values of different clones versus those of the K10 cells) and *P* values (the values between the anti-moMCAM/MUC18 antibody and the control IgY in each clone per cell) were determined by analyzing the data with the Student's *t* tests, as described in Materials and Methods.

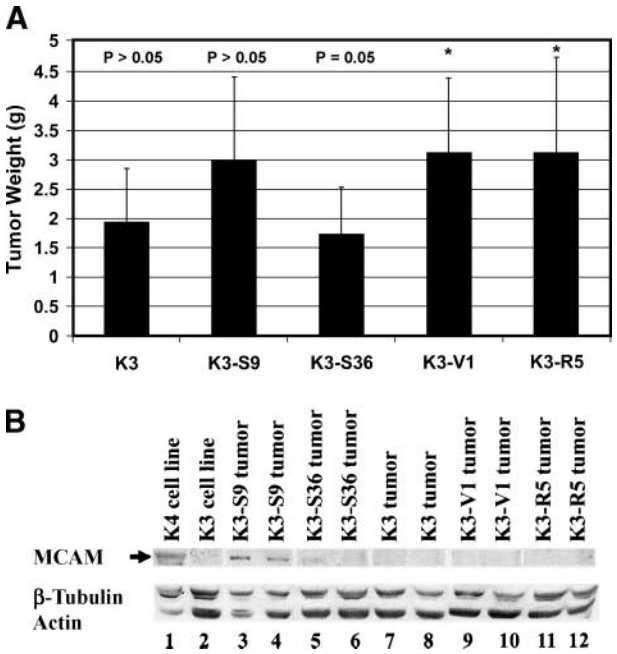

FIGURE 6. Tumor formation of K3 clones. Cells (5×10^6) from various clones of the K3 subline were injected subcutaneously into the syngeneic C3H brown mice. The mice were euthanasized, tumors were excised, and tumor weight was determined after 35 to 39 d. **A.** Tumor formation of K1735-3 clones. The quantitative result of the tumor formation in five mice per clone. *P* values of the data from parental K3 subline, and K3-S9 and K3-S36 clones were compared with either those of K3-V1 or K3-R5 clone (*). **B.** The expression of moMCAM/MUC18 in the tumors formed from K3-S9 (*lanes 3 and 4*), K3-S36 (*lanes 5 and 6*), the parental K3 subline (*lanes 7 and 8*), K3-V1 (*lanes 9 and 10*), and K3-R5 (*lanes 11 and 12*) clones. The expression of moMCAM/MUC18 in the two K1735 sublines (K4 and K3) is shown as controls (*lanes 1 and 2*, respectively).

level of moMCAM/MCU18 was sufficient to increase the invasiveness, but a high expression level was required to increase the motility of these two mouse melanoma sublines. Furthermore, the significant decrease in motility and invasiveness upon the addition of anti-moMCAM/MUC18 antibodies reveals that the moMCAM/MUC18 expression mediated these cellular behaviors.

Effect of moMCAM/MUC18 Expression in K3 and K10 Clones on Their Tumor Formation in Syngeneic C3H Mice

To further determine the effect of moMCAM/MUC18 expression on tumor formation of the two K1735 sublines with an isogenic background, the tumor cells from various $G418^R$ clones of K3 and K10 sublines were subcutaneously injected to C3H syngeneic mice. The number of cells injected was increased to 5×10^6 per mouse to improve the previous behavior of temporal decreasing tumor formation by the K3 subline, as observed in Fig. 1 and Table 1. When the tumor cell loadings were increased to 3.3 times of that in the previous experiment, the tumor formed by K3 cells did not shrink after 5 weeks when mice were necropsized. As shown in Fig. 6A, the tumor size of a moMCAM/MUC18-expressing clone, K3-S9, was not statistically different from that of the two control clones, which were transfected with the vector alone (K3-V1) and with the moMCAM/MUC18 cDNA in antisense orientation (K3-R5), respectively. As shown in Fig. 6B, moMCAM/MUC18 expression in tumors reflected the injected clones, indicating that moMCAM/MUC18 expression was not altered during the *in vivo* tumor growth. We, thus, concluded that moMCAM/MUC18 expression did not have a significant effect on the tumor formation ability of K3 cells. A similar experiment was done for the $G418^R$ clones derived from the K10 subline; however, similar to the parental K10 cells, no tumor was formed from all the clones tested even after 7 months (data not shown). From the above result, we concluded that ectopic moMCAM/MUC18 expression did not significantly affect the tumor formation of these two mouse melanoma K1735 sublines.

Effect of moMCAM/MUC18 Expression in K3 and K10 Clones on Their Pulmonary Nodule Formation in Syngeneic C3H Mice by Using Experimental Metastatic Assays (via Intravenous Injection)

Figure 7 shows the effect of moMCAM/MUC18 expression on the formation of lung nodules by the $G418^R$ clones of the K10 subline after intravenous injection. The lung nodules formed by these clones were only detectable by microscope, as shown in Fig. 7, and in 86% of mice. The quantitative results in Fig. 8 show that the formation of pulmonary nodules by the high moMCAM/MUC18-expressing clone, K10-S9, was significantly increased.

Figure 9 shows the effect of moMCAM/MUC18 expression on the formation of lung nodules by the $G418^R$ clones of the K3 subline after intravenous injection. As shown in the middle column of Fig. 9A, both the number and the size of lung nodules were dramatically increased by the high moMCAM/MUC18-expressing clone, K3-S9; the effect even exceeded

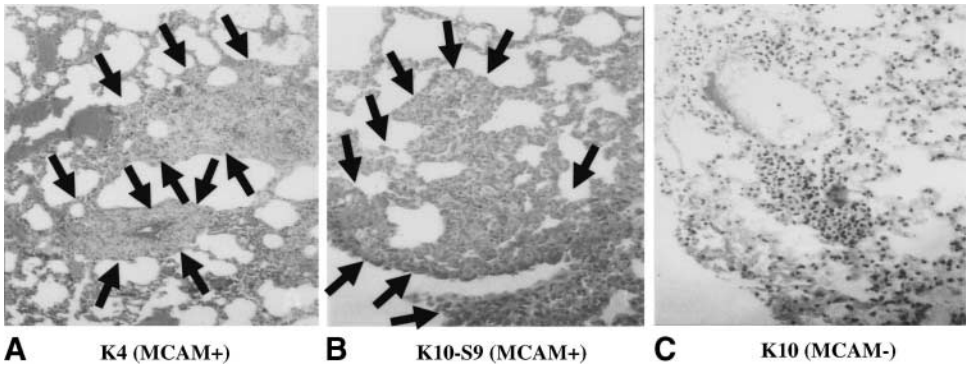

FIGURE 7. Lung nodule formation by various clones derived from the K10 subline. The histology of microscopic size of lung nodules formed by intravenous injection of various K10 clones. The lung nodules formed in the lung by injection of the K4 cells (**A**; as a positive control), high-level moMCAM/MUC18-expressing $G418^R$ clone K10-S9 (**B**), and the parental K10 cells or a vector-transfected K10 clone (**C**; as a negative control). Arrows, lung nodules.

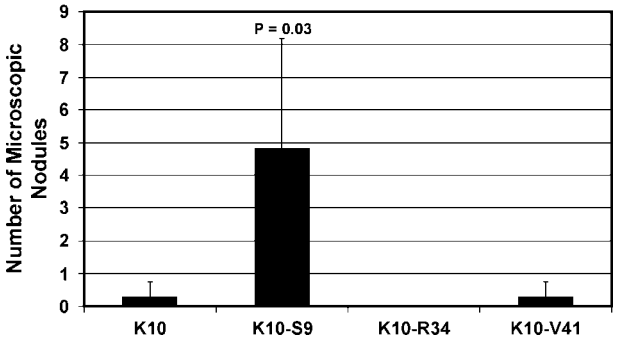

FIGURE 8. Pulmonary nodule formation of K1735-10 clones. The quantitative result of the effect of MCAM/MUC18 expression on the lung nodule formation. Five mice were used for the injection of each clone. *P* value was determined with the Student's *t* test (as described in Materials and Methods) by comparing the data of the K10-S9 clone with the data of the parental K10 subline and the K10-R34 and K10-V41 clones.

those formed by the positive control K4 cells (Fig. 9A, *right column*). The quantitative results in Fig. 9B show that the number of lung nodules formed by the K3-S9 clone was significantly increased in 100% of mice over the control clones, K3-V1 and K3-R5.

Taken together, we concluded that overexpression of the moMCAM/MUC18 gene increased the pulmonary metastasis lesion formation in these two K1735 sublines.

Effect of moMCAM/MUC18 Expression in K3 and K10 Clones on Their Pulmonary Nodule Formation in Syngeneic C3H Mice by Using Spontaneous Metastatic Assays (via Subcutaneous Injection)

None of the $G418^R$ clones, either moMCAM/MUC18-expressing clones or nonexpressing controls derived from both K3 and K10 sublines, produced any lung nodules via subcutaneous injection (data not shown), whereas the positive control of the K-4 subline was able to form lung nodules by subcutaneous injection (data not shown).

Immunohistochemical Detection of moMCAM/MUC18 Antigen in Metastatic Lesions Formed by K3 and K10 Clones

Figure 10 shows the immunohistochemistry of the metastatic pulmonary nodules formed by the clones of the K10 subline. Anti-moMCAM/MUC18 antibody stained only the sections of lung nodules induced by the K10-S9 clone, but not by the control clones (K10 and K10-V41). We concluded that these lung nodules were induced by the expression of the moMCAM/MUC18 in the K10 clone, but not by the control clones that did not express the protein.

Figure 11 shows the histology of the metastatic pulmonary nodules formed by the clones of K3 subline. In addition to the formation of the lung metastasis, the K3-S9 clone also induced the formation of heart metastatic lesions, as respectively shown in Fig. 11B and C. Figure 12 shows the immunohistochemistry of the metastatic pulmonary nodules formed by the K4 (as a positive control) and the metastatic pulmonary nodules and the metastatic heart lesions formed by the K3-S9 clone. Similar to the metastatic pulmonary nodules formed by K4 cells, anti-moMCAM/MUC18 antibody stained numerous patches of micrometastases only in the lung nodules and heart lesions induced by the K3-S9 clone, as shown in Fig. 12C and E, but not by a negative control clone, K3-V1, as shown in Fig. 12G. From the results, we concluded that these lung nodules were induced by the expression of moMCAM/MUC18 in the K3 clones, but not by the negative control clones that did not express the protein.

Taken together, we concluded that moMCAM/MUC18 expression significantly increased the abilities of the two K1735 sublines in the formation of pulmonary metastasis lesions.

Discussion

MoMCAM/MUC18 has ~76.2% amino acid sequence identical to huMCAM/MUC18, suggesting that moMCAM/MUC18 may have properties and biological functions similar to huMCAM/MUC18 (5, 6). Consistent with this notion, the expression of moMCAM/MUC18 mRNA and protein correlates directly with the metastatic abilities of the nine mouse melanoma cell lines (5). Fidler's group had isolated >40 clones from the original mouse melanoma K1735, and each clone presumably had the isogenic background (7), which served as an excellent denominator for testing the effect of moMCAM/MUC18 expression on metastasis of melanoma cells in a syngeneic mouse model. In this report, we have first shown that the phenotypes of these sublines are different in that each had a different capability to form subcutaneous tumor (Table 1) and behaved differently in reference to both the tumor growth rate and the final tumor size (Fig. 1). However, the ability of tumor formation, the tumor growth rate, and the tumor size did not correlate with the expression level of moMCAM/MUC18.

To further support the notion with a direct evidence, we transfected the two mouse melanoma sublines K10 and K3 with moMCAM/MUC18 cDNA and selected $G418^R$ clones, which expressed high levels of moMCAM/MUC18, to test the effect of the moMCAM/MUC18 expression in the clones derived from these two sublines on tumorigenicity. The high moMCAM/MUC18-expressing K10 (a nontumorigenic subline) clones and K3 (a tumorigenic subline) clones did not significantly change the tumor-take nor the tumor size compared with the vector-transfected K1735 clones, suggesting that expression of a high level of moMCAM/MUC18 may have little effect on tumorigenesis. Furthermore, overexpression of moMCAM/MUC18 in the two isogenic K1735 sublines did not confer that these cells increased proliferative benefit in both *in vitro* and *in vivo* tumor growth. The former was reflected in a similar growth rate of the moMCAM/MUC18-expressing clones and the control clones *in vitro*. The latter is consistent with the observation that the ability of tumor formation, the tumor growth rate, and the tumor size did not correlate with the expression level of moMCAM/MUC18 in the five mouse melanoma K1735 sublines, as shown in Fig. 1 and Table 1, and is also reflected in the observation that ectopic moMCAM/MUC18 expression did not significantly affect the tumor formation of these mouse melanoma K1735 sublines. From the

results, we also deduced a notion that the tumor size of mouse melanoma cells does not seem to be directly linked to metastasis, similar to some of the carcinomas (reviewed in ref. 9).

We have presented evidence to directly prove that enforced expression of moMCAM/MUC18 increased *in vitro* motility and invasiveness and *in vivo* lung nodule formation (lung metastasis) of the two K1735 sublines. From the result of the

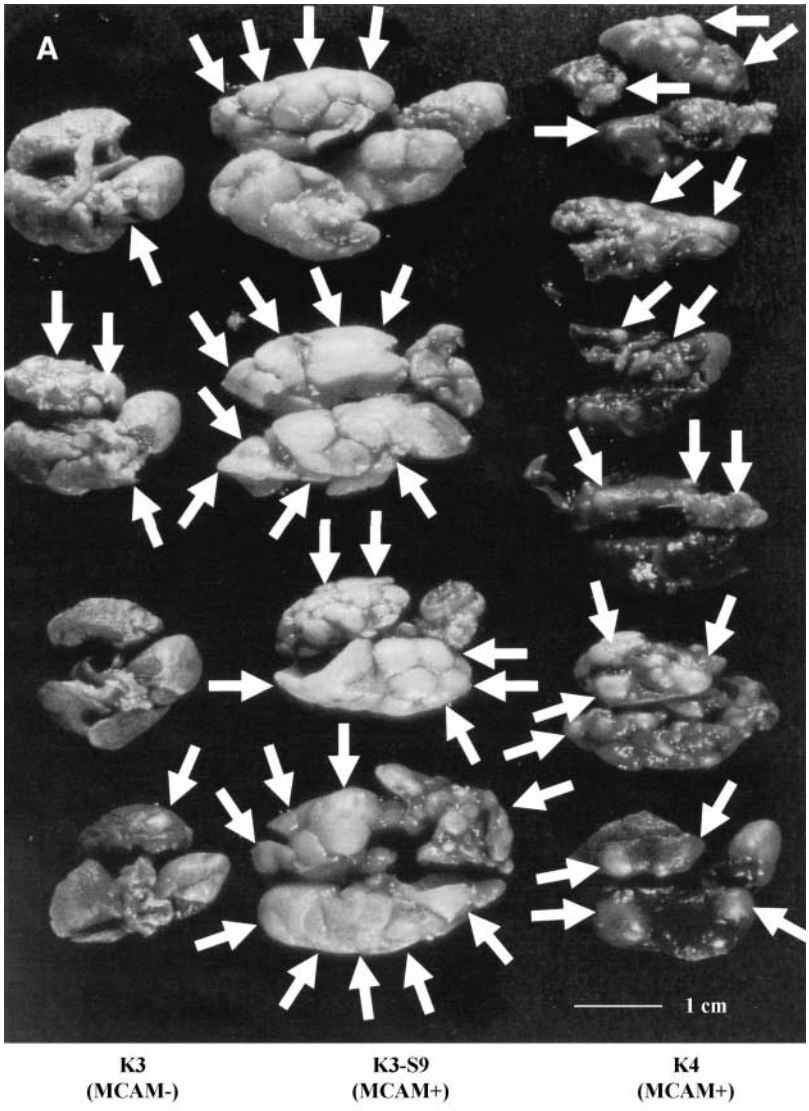

FIGURE 9. Lung nodule formation by various clones derived from K3 subline (**A**) and the quantitative results of these lung nodules (**B**). The cells of different K3 clones were injected intravenously, as described in Materials and Methods. The lungs were excised and fixed in Bouin's solution, which made lung nodules light yellow color, indicated by the arrows, and normal lungs have a dark brown background. **A.** The lung nodules formed by the K4 subline (as a positive control) in the right column, the parental K3 cells (as a negative control) in the left column, and K3-S9, a high moMCAM/MUC18-expressing $G418^R$ clone, in the middle column. Arrows, lung nodules. **B.** Pulmonary nodule formation of K1735-3 clones. The quantitative results of the effect of overexpression of moMCAM/MUC18 on the lung nodule formation by the K3 clones. Five mice were used for the injection of each clone. *P* values of the data from the K4 subline, the parental K3 subline, and the K3-S9 clone were determined with the Student's *t* test (as described in Materials and Methods) by comparing to either the data of K3-V1 or K3-R5 clone (*).

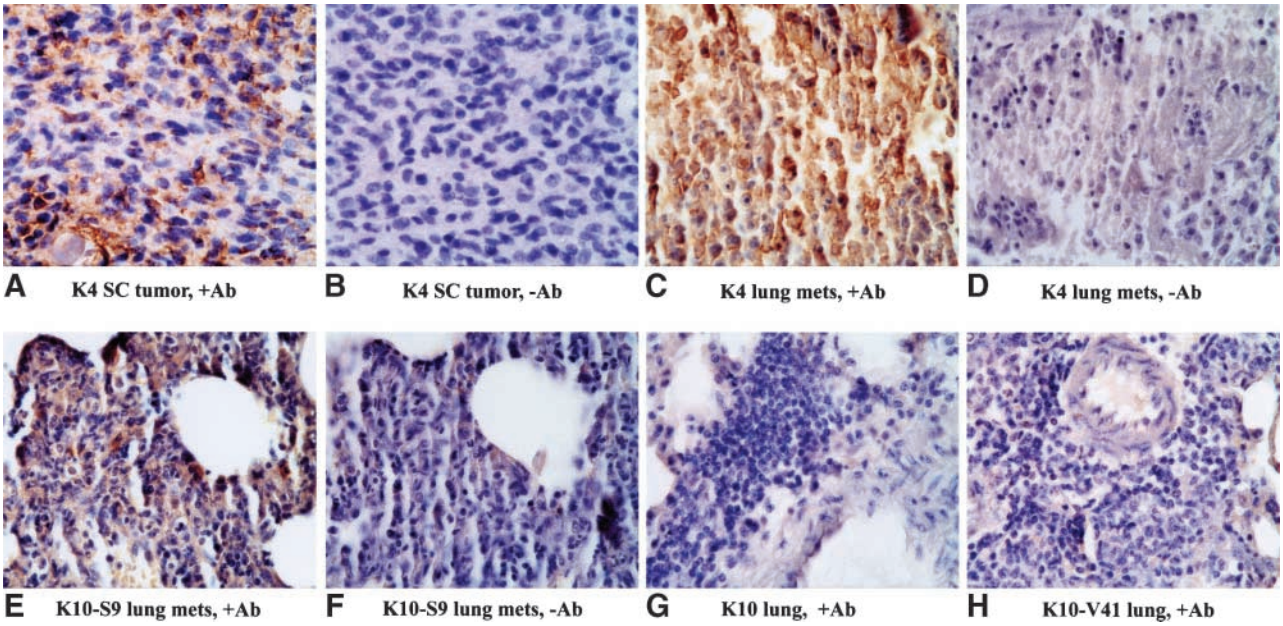

FIGURE 10. Immunohistochemistry of lung lesions formed by injection of K4 cells, K10 cells, and G418^R clones of K10. The anti-moMCAM/MUC18 antibody–stained subcutaneous tumor formed by subcutaneous injection of K4 cells (**A**; *K4 SC tumor*) and stained lung metastatic lesions (*K4 lung mets*) formed by intravenous injection of K4 cells (**C**; as positive controls) and the corresponding no antibody controls (**B** and **D**). The anti-moMCAM/MUC18 antibody–stained lung metastatic lesions formed by intravenous injection of K10-S9 clone (**E**) and the corresponding no antibody control (**F**). **G** and **H.** No staining by anti-moMCAM/MUC18 antibody in the lung sections of the parental K10 cells and the K10-V41 clone after intravenous injection. The magnification was 600× in all frames.

experimental metastasis test, increased expression of moMCAM/MUC18 in the two mouse melanoma K1735 sublines increased their ability to metastasize to lungs in syngeneic mice. The effect of moMCAM/MUC18 expression on pulmonary metastasis was more dramatic for the K3-derived clones, which induced more numerous and larger lung nodules in all mice than the K10-derived clones, which only induced less numerous and microscopic lung nodules in 86% of mice. We conclude that moMCAM/MUC18 plays an important role in increasing the metastasis of mouse melanoma cells in a syngeneic C3H mouse model. We also suggest that metastasis induced by the expression of moMCAM/MUC18 may be affected by the different intrinsic properties of different sublines because the lung nodule formation by the mouse melanoma cells that express high levels of moMCAM/MUC18 varies among these two K1735 sublines. It is very likely that distinct intrinsic factors, absent or present in different cell lines, may modulate the role of MCAM/MUC18 in the MCAM/MUC18-mediated metastasis. This may also explain the variable results of the effect of the overexpression of huMCAM/MUC18 on the

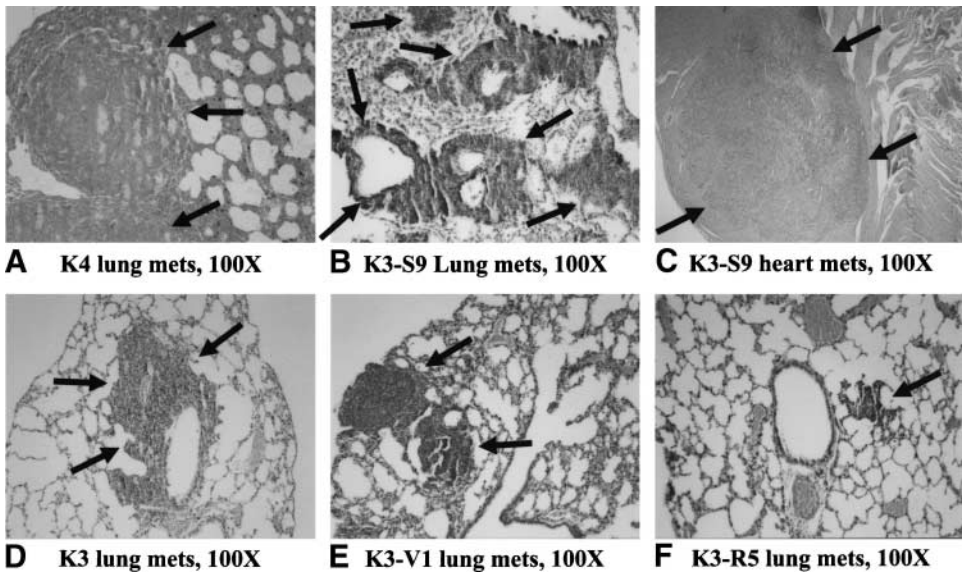

FIGURE 11. The histology of metastatic lesions formed by G418^R clones of K3 cells. Lung nodules formed by K4 subline (**A**; as a the positive control), many metastasis nodules in lungs (**B**), and the metastatic lesions in the heart formed by the K3-S9 clone (**C**). The minimal lung nodule formation by the three negative controls, parental K3 (**D**), the empty vector-transfected (K3-V1; **E**), and the antisense-oriented moMCAM/MUC18 cDNA-transfected (K3-R5; **F**) clones per cells. Arrows, lung or heart nodules.

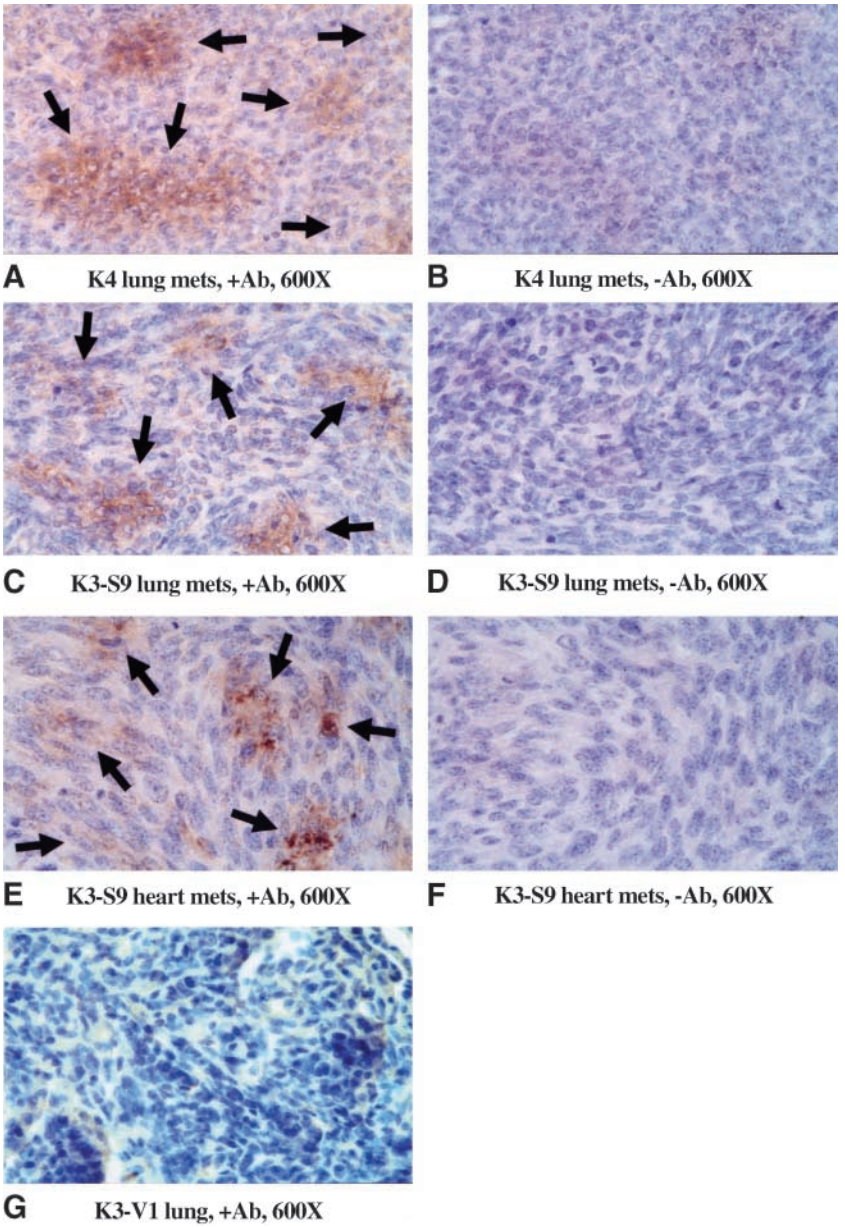

FIGURE 12. The immunohistochemistry of metastatic lesions formed by K4 and G418^R clones of K3 cells. The anti-moMCAM/MUC18 antibody–stained lung metastatic lesions formed by injection of K4 cells (**A**) and the corresponding no antibody control (**B**). The anti-moMCAM/MUC18 antibody–stained lung metastatic lesions formed by injection of K3-S9 clone (**C**) and the corresponding no antibody control (**D**). The anti-moMCAM/MUC18 antibody–stained heart metastatic lesions formed by injection of K3-S9 clone (**E**) and the corresponding no antibody control (**F**). **G.** No staining by the anti-moMCAM/MUC18 antibody in the lung from the injection of K3-V1 clone. Arrows, the MCAM/MUC18-positive patches of micrometastases in the lung or in the heart lesions.

metastasis of the two different low-tumorigenic, nonmetastatic human cutaneous melanoma cell lines, Xp-44 and SK-2, in SCID mice (4). We are in the process of using proteomic methods and microarray techniques to identify these factors.

All the clones from the two sublines showed negative results for the spontaneous metastasis test (subcutaneous injection), similar to human melanoma cells in xenograft immunodeficient mice, suggesting that moMCAM/MUC18 may not affect the metastasis of melanoma cells at the earlier steps but rather at the later steps of metastasis. This observation is consistent with the results observed in huMCAM/MUC18-transfected human melanoma xenografts in nude mice and SCID mice (3, 4). Because the subline K4, which expressed a high level of MCAM/MUC18, was able to cause spontaneous metastasis and also experimental metastasis in this syngeneic mouse model, we suggest that positive factors, present or negative factors, absent in the K4 may be required for efficient spontaneous metastasis for these two sublines.

Taken all these results together, we concluded that, in addition to the positive result similar to the effect of huMCAM/MUC18 expression on pulmonary metastasis of human melanoma cells, the effect of moMCAM/MUC18 expression on tumorigenesis and metastasis in our syngeneic mouse model revealed several significant new findings that were not shown in the human melanoma cell xenografts in immunodeficient mouse models (3, 4). (*a*) There is no statistically significant effect of increased moMCAM/MUC18 expression on increased tumorigenicity, which was not statistically clearly shown in the human xenografts. (*b*) There is an excellent correlation of overexpression of moMCAM/MUC18 with the increasing

in vitro motility and invasiveness and *in vivo* lung nodule formation (experimental metastasis), which was not shown in human xenografts, suggesting a mechanical significant contribution of moMCAM/MUC18 in increasing the intrinsic motility and invasiveness of melanoma cells during the process of metastasis. (*c*) The metastatic ability of different moMCAM/MUC18-expressing sublines/cell lines is different, perhaps due to the different intrinsic factors that may modulate the function of moMCAM/MUC18. (*d*) Overall, both human and moMCAM/MUC18 expression might affect tumorigenesis and metastasis of melanoma cells in a very complex way. Cofactors that are present in different variants may modulate the effect of MCAM/MUC18 on these processes.

In summary, we have successfully established a syngeneic mouse model system for further mechanical studies on the MCAM/MUC18-mediated metastasis in melanoma (10). This model should also serve as an excellent system for further studies of the mechanism of overexpression of an immunoglobulin-like CAM on the tumorigenesis and metastasis of melanoma cells (11). This is especially intriguing: that MCAM/MUC18 may be one of those genes that specify the final step (metastasis) in tumor progression would not confer increased proliferative advantage at the primary site (as reviewed in ref. 12). This system should also serve as an excellent preclinical model for designing immunotherapeutic means to arrest this dreadful skin cancer, as evident in a recent trial (13).

Materials and Methods

Cell lines and Cell Culture

Five mouse melanoma K1735 cell sublines, Km2, K3, K4, K9, and K10, from Dr. Isaiah J. Fidler (M. D. Anderson Medical Center/University of Texas; ref. 7) were maintained in Eagle's MEM supplemented with sodium pyruvate and 10% fetal bovine serum (Life Technologies/Bethesda Research Laboratories).

Lipofection of K1735 Variant Cells and Selection for the G418^R Clones

Cells (8.6×10^5 of K10 or 6.5×10^5 of K3) were seeded on 60-mm tissue culture Petri dish plates to give ~50% confluence. After 1-d growth, the monolayer cells were transfected with a mixture of 30 μg of Lipofectamine (14) or DMRIE-C (Life Technologies; ref. 15) and 5 μg each of the plasmid pcDNA3.1+, which contained a HCMV-IE promoter-driven moMCAM/MUC18 cDNA gene, as well as the neomycin-resistant gene driven by SV40 promoter. G418 (0.8 mg/mL; active component of 0.6 mg/mL) was added to the growth medium for the selection of G418^R clones derived from K10. G418 (0.3-0.5 mg/mL) was used for the selection of G418^R clones derived from K3. Clones were transferred and expanded sequentially from 24-well to 12-well plates and then to six-well culture plates. Cell lysate of each clone was made from the culture in each of the six-well plate by the addition of 100 μL of Western blot lysis buffer (16), which contained an antiproteolytic cocktail (1 mmol/L benzamidine, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL each of antipain, leupeptin, chymostatin, and pepstatin; Sigma Chemical Co.; ref. 5), boiled, and kept frozen at −20°C until Western blot analysis. The moMCAM/MUC18-positive clones were further expanded from six-well plates to T-25 flasks and subsequently to T-75 flasks and frozen in liquid nitrogen for preservation as stocks.

Western Blot Analysis

Cell lysates from various cultured cell lines were prepared as described above (5, 16). The extract from a freshly dissected subcutaneous tumor or organs was prepared by first dicing with a sharp razor blade, adding 4 mL of cold PBS containing the antiproteolytic cocktail, and finally homogenizing in a polytron homogenizer at top speed for 10 to 15 s. One volume of the homogenate was mixed with one-third volumes of the 4× Western blot lysis buffer, added with antiproteolytic cocktails, and further diluted with about an equal volume of 1× Western blot lysis buffer, boiled, and kept frozen until use. The protein contents of all lysates were determined by the Bradford method (Bio-Rad Laboratories) and confirmed by SDS-PAGE after staining with Buffalo Black. Proteins in the lysates were electrophoretically separated by a SDS-PAGE (8-10% polyacrylamide slab gel) and blotted to a nitrocellulose membrane (Hybond-ECL, RPN3032D, Amersham; ref. 5). The standard procedure of Western blot analysis using a chicken anti-moMCAM/MUC18 antibody was used to determine the moMCAM/MUC18 expression in various mouse melanoma cell lines (5).

Determination of the Cellular Growth Rate by Direct Counting or the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

The growth rate of each G418^R clone parental K1735 variants and vector control clones was determined. Cells from monolayer culture were trypsinized, and the number of cells was counted with a hemacytometer. Five thousand cells in 0.2 mL of the growth medium with 10% fetal bovine serum were seeded to each well of a round-bottomed 96-well plate (Corning/Costar). Alternatively, 2×10^4 cells per well of each clone were initially seeded to 3 of 24 wells (triplicate). After 24, 48, 72, and 96 h, the cells from each well were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (17, 18) or trypsinized, and the number of cells was directly counted by a hemacytometer under a microscope. For MTT assay, 20 μL of MTT (5 mg/mL in PBS) were added to each well 4 h before each time point. After addition of MTT, the incubation was carried out at 37°C for 4 h. At the end of incubation, the MTT-growth medium mixture was removed by suction and 0.15 mL of DMSO was added into each well and mixed well for 5 min by pipeting up and down (or by rotational shaking). Absorbance difference of $A_{570\text{nm}}$ minus $A_{630\text{nm}}$ was directly read by using a microtiter plate reader autoreader (Bio-Tek Instruments). Means of hexaplicate readings (SDs mostly <20%) for each time point were used for calculating the growth rate for each clone. The relative growth rate was calculated by assuming the highest reading as 100%. The mean and SD of three relative growth rates for each clone were plotted.

Cell Motility Assay

The *in vitro* cell motility assay was carried out according to a published method (19) with minor modifications (17). Cells (2×10^5) of each clone in 0.4 mL of the growth medium containing 0.1% bovine serum albumin and 150 to 300 μg of

anti-moMCAM/MUC18 antibodies or control chicken IgY were initially seeded to each of the top wells (12 wells) of the Costar transwell Boyden system [the 12-well top inserts with 8.0-μm pore size of the polycarbonate membrane (Falcon 35-3182) that fits into the bottom wells of the companion 12-well plates (Fisher 08-771-22 or Falcon 35-3503)] and the regular growth medium with 10% fetal bovine serum was added to the bottom well. The growth medium (1.1 mL) containing 10% FCS was added to each bottom well. After 16 h, the cells migrating to the bottom well were trypsinized, concentrated by centrifugation, and counted with a hemacytometer. The experiments were repeated at least thrice, and means and SDs of at least triplicate values were indicated.

Cell Invasiveness Assay

The *in vitro* cell invasiveness assay was carried out according to a published method (19) with minor modifications (17). Cells (2×10^5) of each clone in 0.4 mL of the growth medium containing 0.1% BSA and 150 to 300 μg of anti-moMCAM/MUC18 antibodies or control chicken IgY were initially seeded to each of the top wells of the Costar 12-well Transwell Boyden system, of which the polycarbonate membrane of 12-μm pore size (Fisher and Costar) was coated with 150 to 300 μg of diluted Matrigel (Becton Dickinson MATRIGEL Basement Membrane Matrix, phenol red–free from Collaborative Research). The growth medium (1.1 mL) containing 10% fetal bovine serum was added to the bottom well. After 16 to 24 h, the cells migrating to the bottom well were trypsinized, concentrated by centrifugation, and counted with a hemacytometer. The experiments were repeated six times, and means and SDs of hexaplicate values were indicated.

In vivo *Tumor Formation Assay by Subcutaneous Injection and Pulmonary Metastasis Assay by Intravenous Injection of Cells*

For subcutaneous injection, 1.5×10^6 cells (in 0.2 mL PBS) of five K1735 sublines per C3H female mouse were subcutaneously injected at the right flank region. Ten 6-wk-old C3H female mice were used for injection of each clone per subline. After 7 d, some tumors were palpable. Palpable tumors were measured by a caliper after 3 wk, and the tumor volume was calculated by using the formula of $V = \pi / 6 (d1 \times d2)^{3/2}$ $(mm)^3$. Five mice were necropsied after 27 or 48 d. Cells (1×10^6; in 0.2 mL PBS) of various clones derived from K10 were subcutaneously injected per C3H mouse, and five mice were necropsied after 7 mo. Cells (5×10^6; in 0.2 mL PBS) of various clones derived from K3 were subcutaneously injected per C3H mouse, and five mice were necropsied after 35 to 39 d. When mice were euthanasized, the tumors were excised and weighed by a balance. For intravenous injection, 1 or 2.5×10^5 cells (in 0.1 mL PBS) were injected at the tail vein. After 5 to 6 wk, when lung nodules were formed, five mice per clone were euthanasized and lungs were dissected and fixed in 10% HCHO. All the lungs were stained in Bouin's solution overnight (7) and then washed several times in formaldehyde solution. When lung nodules became clearly distinguished from the lung, all of them in the lungs were counted under a dissecting microscope (7). The guidelines of Institutional Animal Care and Use Committee were followed for all the animal studies.

Statistical Analysis

To reveal the statistical significance of the results, we have used the Student's *t* test in the Microsoft Excel program to analyze the data generated from the tests of the effect of overexpression of moMCAM/MUC18 on *in vitro* growth rate, motility, invasiveness, and tumor weights and numbers of metastatic lesions formed *in vivo*. *P* values were obtained from the Student's *t* test by using the two-tailed distribution and type 2 (two-sample equal variance or homoscedastic) or type 3 (two-sample unequal variance) method to compare the data from the experimental groups with the data from the control groups.

Histology staining (H&E)

Mouse tissues were fixed in 10% phosphate–buffered formaldehyde (20) and embedded in paraffin, and 5-μm sections were made. A standard H&E staining was carried out (21).

Immunohistochemistry

Paraffin-embedded, formaldehyde-fixed tissue sections (5 μm) from the cutaneous melanoma and lung nodules were used. The mouse subcutaneous melanoma formed from the K4 melanoma cells was used as a positive external control for immunohistochemical staining. The mouse tissue section of the subcutaneous melanoma and lung nodules were deparaffinized and rehydrated (17, 20). Antigen retrieval was carried out by gentle boiling of citrate buffer (pH 6.0) in a boiling water bath (maintained at 95°C) for 10 min and cooled for 60 min. The endogenous peroxidase was quenched with 3% hydrogen peroxide. All tissue sections were blocked with 5% nonfat milk for 2 h and reacted sequentially with the primary antibodies (1:200 dilution of the chicken anti-moMCAM/MUC18 IgY) for 20 min, a secondary antibody (1:1,000 dilution of the biotinylated rabbit or goat anti-chicken IgY antibodies; G2891, Promega) for 20 min, a streptavidin-conjugated horseradish peroxidase complex (LSAAB-2 system, DAKO Co.) for 20 min, and diaminobenzidine as the chromogen for 5 min. The tissue sections were then counter-stained with hematoxylin for 1 min. All these incubations were done at room temperature; between incubations, sections were washed with TBS buffer. Negative controls had primary antibody replaced by the TBS buffer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Research

Enforced Expression of MCAM/MUC18 Increases *In vitro* Motility and Invasiveness and *In vivo* Metastasis of Two Mouse Melanoma K1735 Sublines in a Syngeneic Mouse Model

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