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Dual Role of METCAM/MUC18 Expression in the Progression of Cancer Cells

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Abstract

The altered expression of cell adhesion molecules (CAMs) correlates with the malignant progression of many epithelial tumors. MUC18/CD146/A32/MelCAM/S-endo 1, a CAM in the immunoglobulin gene superfamily, is an integral membrane glycoprotein. MUC18 is not a mucin, resulting from its misleading nomenclature by the original discoverer. We re-named it as METCAM (metastasis-regulating CAM), based on its very interesting biological roles in tumor formation and metastasis of many epithelial tumors. Initial findings show that METCAM/MUC18 expression has a positive effect (as a tumor and metastasis promoter) on the progression of breast cancer, most melanoma cell lines, nasopharyngeal carcinoma (NPC) type II, and prostate cancer. Later research results show that METCAM/MUC18 expression has a negative effect (as a tumor suppressor and metastasis suppressor) on the progression of ovarian cancer, one mouse melanoma cell line, and nasopharyngeal carcinoma type I, and perhaps hemangioma. Since the above dual function of METCAM/MUC18 occurs only in different cell lines from the same cancer type or in those from different cancer types, we suggest that the different effect of METCAM/MUC18 on tumor formation and metastasis of different cancer cell line may be due to different intrinsic properties (co-factors) in each cancer cell line that modify the biological functions of METCAM/MUC18 in the intrinsic properties of tumor cells and their interactions with the tumor microenvironment. This chapter will review the published work and present some possible mechanisms for the METCAM/MUC18-mediated cancer progression for future studies.

Keywords: METCAM/MUC18, breast cancer, melanoma, nasopharyngeal carcinoma, ovarian cancer, prostate cancer, tumors

1. Introduction

Cancer progression is very complex because many genes are directly or indirectly involved in the process. The accumulation of the multiple intrinsic changes leading to aberrant alterations

of gene expression can contribute to tumorigenesis and its progression to the malignant phenotype. This is because the genome of malignant tumor cells has greater instability than non-malignant tumor cells and renders malignant tumor cells more prone to acquiring multiple mutations [1]. Tumorigenesis involves expression of many oncogenes and tumor suppressor genes [2, 3], which will not be elaborated here. Likewise, metastasis also involves many metastasis enhancer genes and metastasis suppressor genes [2, 3] ever since the successful conversion of a non-metastatic Ha-ras-transformed NIH 3 T3 fibroblast cells to metastatic tumor cells by transfecting the cells with the DNA fragments isolated from a human metastatic tumor [4]. This also suggests that many alternative pathways are possible for metastasis, similar to multiple alternative pathways leading to tumorigenesis [2, 3]. This includes the genes encoding cell adhesion molecules (CAMs), such as E-cadherin [5], integrins $\alpha 2\beta 1$ [6] and $\alpha V\beta 3$ [7], CD44 [8], EPCAM [9, 10], ALCAM [11], and METCAM/MUC18 [12, 13]. The list of these genes has been rapidly lengthened because of the advent of modern state-of-the-art technologies, such as SAGE analysis [14, 15], DNA chip microarray analysis [16–19], and proteomics [20–22]. Some of these genes may be commonly used by metastatic tumors derived from different tissues, if these genes render tumor cells with a metastatic advantage over other tumor cells, regardless of their origins. Some oncogenes or tumor suppressor genes may also play direct or indirect roles in tumor metastasis, if they directly or indirectly alter cytoskeleton structure, cellular motility, invasiveness, and render them having growth advantages in target organs.

Tumor metastases fortunately are a rare event due to metastatic inefficiency. It was originally thought that only a very small population of the metastatic cells could reach and establish the growth in the distant target organs after they successfully intravasate or extravasate the vasculatures or lymphatics, and then survive the assaults in the circulatory system, which includes the attacks from the immune system and the destructive hydrodynamic shearing [23]. However, recent results of observing the process with *in vivo* video microscopy appear to support the notion that metastatic inefficiency is more likely due to that only a small percentage of tumor cells are able to dock and establish secondary growths in distant organs after survival from the attacks from the immune system and the assault from the mechanical shearing, since both highly metastatic cells and non-metastatic cells have similar migratory and invasive abilities to intravasate or extravasate the circulatory systems [24]. The successful establishment of secondary growth by metastatic cells may result from a complex interaction of tumor cells with the extracellular matrix in the favorable microenvironment of the target organs. This interaction may also be due to the altered expressions of many cell adhesion molecules (CAMs) in metastatic cells that alter their ability to interact with the extracellular matrix.

CAMs govern the social behaviors of the cells. The altered expression of CAMs affects cell-cell interactions and cell-extracellular matrix interactions, which results in changing the cellular motility and invasiveness [25]. Altered expression of CAMs also can affect survival and growth of tumor cells and alter angiogenesis [26]. As such, CAMs may promote or suppress the metastatic potential of tumor cells [26]. The metastatic potential of a tumor cell could be the consequence of a complex participation of many over- or under-expressed CAMs, as documented in many carcinomas [27]. For example, integrins αV , $\alpha 4$, and $\beta 3$, I-CAM, METCAM/MUC18, and HLA-DR are over-expressed, whereas E-cadherin, α -catenin, and VCAM are under-expressed in metastatic melanomas [28]. On the other hand, the metastatic potential of a tumor cell could be due to the altered expression of a single CAM. For example, over-expression of integrin

$\alpha 2\beta 1$ decreases the metastasis of breast carcinoma cells [6], whereas over-expression of integrin $\alpha V\beta 3$ increases the metastatic potential of human prostate carcinoma cells [7].

Effects of altered expression of I-CAM, V-CAM, some integrins (αV , $\alpha 4$, and $\beta 3$), L1CAM, METCAM/MUC18 [28], and E-cadherin [29] on the metastasis of melanoma have been demonstrated. Studies of the altered expression of CAMs on the metastasis of prostate cancers are E-cadherin [5], CD44 [8, 30], CEA-CAM [31, 32], and some integrins [7, 33, 34]. Increased expression of E-cadherin [35] and the standard form of CD44 [8] suppresses metastasis of prostate carcinoma. On the contrary, increased expression of a splicing variant form of CD44, CD44v7-v10, correlates with the progression of prostate carcinoma and enhances *in vitro* invasiveness of human prostate cancer cell lines [30]. Increased expression of CEA-CAM1 suppresses tumorigenesis [32]; however, the effect on metastasis has not been tested. The effect of a single integrin mostly is not obvious except $\alpha V\beta 3$, $\alpha 6\beta 1$, and $\alpha 3\beta 1$ integrins in prostate cancer, perhaps many members of the integrin family are functionally compensatory to each other [33–36]. Aberrant expression of CAMs has been associated with nasopharyngeal carcinoma (NPC). For examples, up-regulation of ICAM [37] and down-regulation of E-cadherin [38, 39] and connexin 43 [40] correlate with the progression of NPC; however, the expression of CD44 does not [38]. Aberrant expression of various CAMs associated with the malignant progression of ovarian cancer are mucins [41], integrins [42], CD44 [43], L1CAM [44], E-cadherin [45], claudin-3 [46], EpCAM [9, 10], and METCAM/MUC18 [47, 48].

For the past two decades, we have focused our research on the role of METCAM/MUC18 in the progression of several epithelial tumors: first, we tried to correlate the expression level of METCAM/MUC18, which was determined by using the methods of immunohistochemistry and/or RT-PCR, with the pathological state of the tumor tissues and from the results to propose a hypothesis to predict the possible positive or negative role played by METCAM/MUC18 in the progression of each cancer. Then we tested the hypothesis by investigating effects of METCAM/MUC18 over-expression or under-expression on *in vitro* cellular behaviors and tumorigenesis and on *in vivo* tumorigenesis in athymic nude mice, or if possible, in syngeneic mice. We studied the effect of the expression of METCAM/MUC18 on the progression of melanoma cell lines [49, 50] and prostate cancer cell lines [51–55], and then extended our research to breast cancer cell lines [56–58], ovarian cancer cell lines [48, 59, 60], and nasopharyngeal carcinoma cell lines [61]. Possible mechanisms played by METCAM/MUC18 were preliminarily determined by analyzing the expression levels of several downstream effectors in the tumor tissues excised from these mice. In this chapter, I will summarize the findings of the above research activities and review the dual role of METCAM/MUC18 in the progression of breast cancer, melanoma, nasopharyngeal carcinoma, ovarian cancer, and prostate cancer [48–62]. I will also propose some possible mechanisms of METCAM/MUC18-mediated tumorigenesis and metastasis for future studies.

2. METCAM/MUC18

Human METCAM/MUC18 (huMETCAM/MUC18), a CAM in the immunoglobulin gene superfamily, is an integral membrane glycoprotein [63]. The name “MUC18”, which was originally coined by Judy Johnson [63], has often been mistaken as a new member in the mucin family. Other names, such as CD146, A32, and S-endo 1, were not used because they did not reflect its biological functions [63–68]. The names MCAM and MELCAM with an

over-emphasis on its role in melanoma [28] are discarded because MUC18 is involved in the metastasis of many cancers besides melanoma ([69] and this review). To eliminate confusion and to reflect its biochemical properties and key role in the progression of epithelial tumors, we have created a new name for MUC18: METCAM (metastasis CAM), an immunoglobulin-like CAM that regulates metastasis [69]. MUC18 is included in our nomenclature to commemorate its original discovery [63].

Judy Johnson's group was the first group to clone and characterize the sequence of a huMETCAM/MUC18 cDNA from human melanoma [63]. Later my group cloned the huMETCAM/MUC18 cDNA from several human melanoma cell lines and human prostate cancer cell lines [70]. The DNA sequences of our huMETCAM/MUC18 cDNA clones from three human melanoma cell lines, three human prostate cancer cell lines, and several human nasopharyngeal tissues are similar; therefore, we conclude that our huMETCAM/MUC18 cDNA gene is the major common form in comparison to that of Judy Johnson. The amino acid sequences deduced from the DNA sequence of our huMETCAM/MUC18 cDNA differ from that of Johnson's group in seven amino acids [63, 70]. Regardless of minor differences in amino acid sequences, all huMETCAM/MUC18 cDNAs encode 646 amino acids that include a N-terminal extracellular domain of 558 amino acids, of which at the N-terminus it has 28 amino acids characteristic of a signal peptide sequence, a transmembrane domain of 24 amino acids (amino acid #559–583), and an intracellular cytoplasmic domain of 64 amino acids at the C-terminus (**Figure 1**). The molecular weight of the un-glycosylated form of huMETCAM/MUC18 protein is estimated to be about 72 kDa [63, 69, 70]. Since huMETCAM/MUC18 has eight putative N-glycosylation sites (Asn-X-Ser/Thr), it is often heavily glycosylated and sialylated resulting with an apparent molecular weight between 113,000 and 150,000, dependent upon the tissue origin. The extracellular domain of the protein contains five immunoglobulin-like domains (V-V-C2-C2-C2) [63, 70] and an X domain [69, 70]. The cytoplasmic domain contains peptide sequences that are potentially be phosphorylated by protein kinase A (PKA), protein kinase C (PKC), and casein kinase 2 (CK 2) [63, 64, 69, 70]. My lab has also cloned the mouse METCAM/MUC18 (moMETCAM/MUC18) cDNA, which contains 648 amino acids with 76.2% identity with huMETCAM/MUC18 [71]. The structure of the huMETCAM/MUC18 protein is illustrated in **Figure 1**.

Similar to other CAMs, the functions of huMETCAM/MUC18 has been studied in relation to cell-cell and cell-extracellular matrix interactions, which trigger a cascade of signals that affect cytoskeleton structure and cellular motility and invasiveness. **Figure 1** shows the six conserved

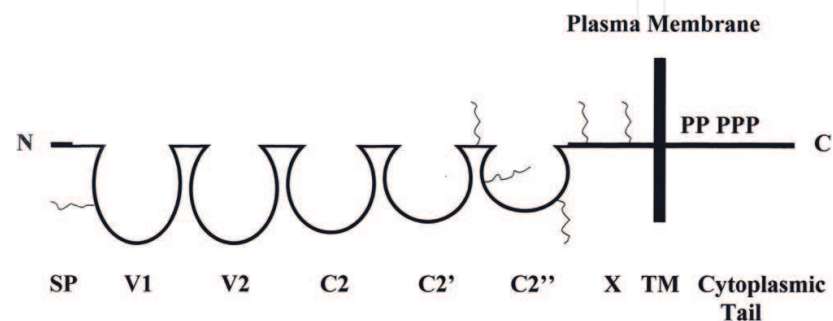


Figure 1. The protein structure of human METCAM/MUC18.

N-glycosylation sites, which are located in the V1, the region between the C2' and the C2'', the C2'', and the X domains in the extracellular domain, and five potential phosphorylation sites in the intracellular cytoplasmic tail. From the protein structure, we predicted that METCAM/MUC18 may have additional functions, which may include synergistic interactions with growth factor receptors (as a co-receptor) to modulate cell functions and to trigger on intracellular signaling pathways, activation of matrix metalloproteinases (MMPs), serving as a co-activator for other cell functions, and serving as a co-transporter for extracellular small molecules (for example, calcium ion influx) [69], as summarized in **Figure 2**.

HuMETCAM/MUC18 is expressed in several normal tissues/cells, such as endothelial cells, hair follicular cells, smooth muscle cells, normal breast epithelial cells, basal cells in bronchial epithelium, the cerebellum, intermediate trophoblasts, some activated T cells [66], ovarian epithelial cells [48], and normal nasopharynx epithelial cells [61]. In addition, huMETCAM/MUC18 is expressed in several cancers, such as melanoma, gestational trophoblastic tumors, leiomyosarcoma, angiosarcoma, hemangioma, Kaposi's sarcoma, schwannoma, some lung squamous and small cell carcinomas, some breast cancer, and some neuroblastoma [66]. For the past two decades, we have also found that huMETCAM/MUC18 is also expressed in prostate cancer [72], ovarian cancer [48], and nasopharyngeal carcinoma [61].

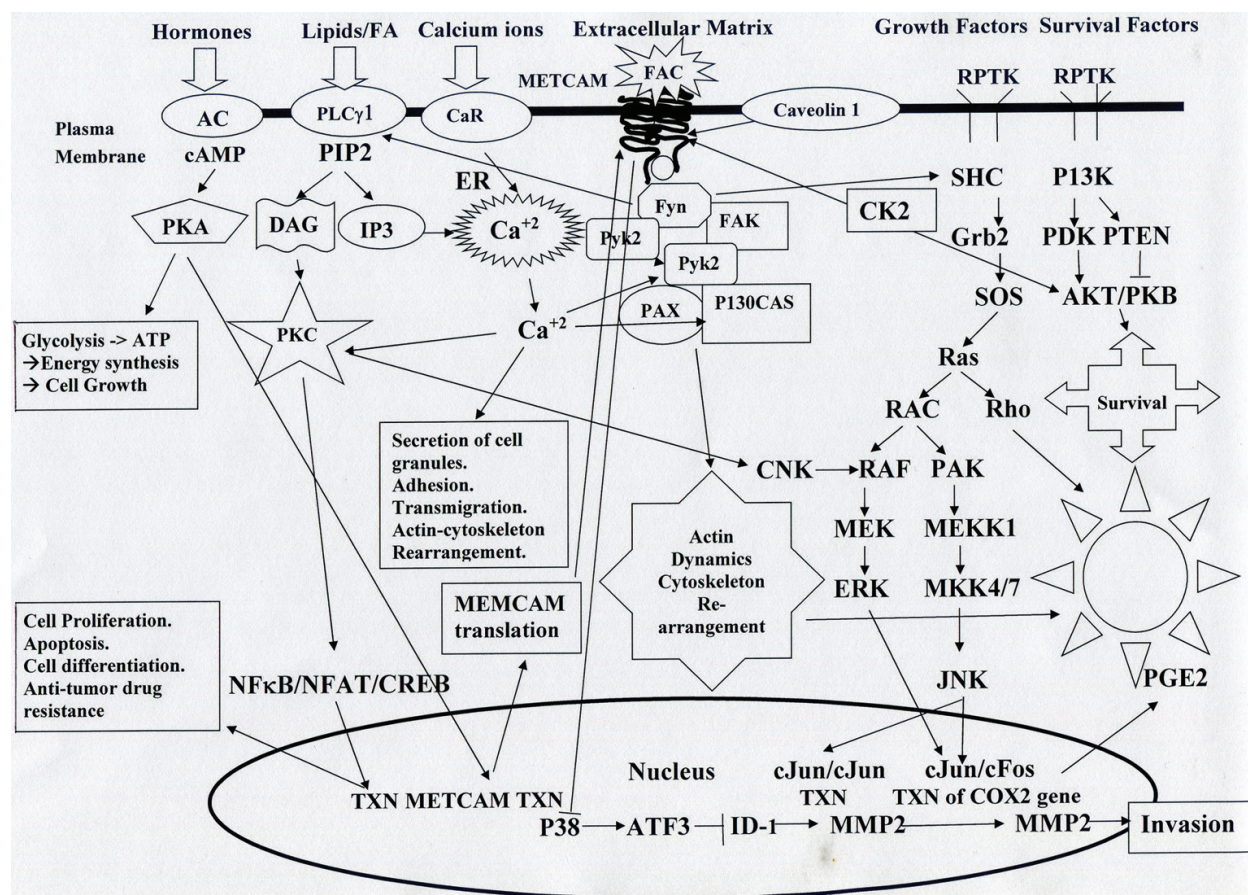


Figure 2. A proposed model for the outside-in and inside-out signaling of METCAM/MUC18 and its possible crosstalk with signal transduction pathways.

3. Role of METCAM/MUC18 in the tumorigenesis and metastasis of different human cancers

3.1. METCAM/MUC18 plays a positive role in the progression of breast cancer

METCAM/MUC18 was suggested by two groups to play a tumor suppressor role [73, 74], but by the two other groups as a tumor promoter in the progression of human breast cancer [75, 76]. To resolve the controversial role of METCAM/MUC18 in the progression of human breast cancer, we set out independent studies to investigate the actual role played by METCAM/MUC18 in the progression of human breast cancer. We found that enforced expression of METCAM/MUC18 in both MCF-7 and SK-BR-3 cell lines increased their *in vitro* motility, invasiveness, and colony formation in soft agar (*in vitro* tumorigenesis). Furthermore, enforced expression of METCAM/MUC18 in both cell lines increased tumor-take and tumorigenesis in athymic nude mice [56–58].

Moreover, anti-METCAM/MUC18 antibody decreased the motility and invasiveness of the two basal-like cell lines, MDA-MB-231 and MDA-MB-468 [57]. Enforced expression of METCAM/MUC18 increases the metastasis of both basal-like cell lines in athymic nude mice [77]. Taken together, METCAM/MUC18 plays a positive role in the progression of four human breast cancer cell lines. Therefore, METCAM/MUC18 is a novel oncogene for mammary carcinoma cells and may be useful as a therapeutic target for the treatment of breast cancer. From further preliminary mechanical studies we suggest that METCAM/MUC18 promotes the progression of human breast cancer cells by increasing proliferation, angiogenesis, switching to aerobic glycolysis, and epithelial-to-mesenchymal transition (EMT) [56–58], thus its downstream signaling molecules may also be used as therapeutic targets for the treatment of breast cancer.

3.2. METCAM/MUC18 plays a dual role in the progression of melanoma

HuMETCAM/MUC18 was highly expressed on the cellular surface of most malignant human melanomas and has been suggested to play a positive role in the progression of human melanoma [63, 64]. Three groups demonstrate that the stably ectopic expression of the huMETCAM/MUC18 cDNA gene in three non-metastatic human cutaneous melanoma cell lines increases the metastatic ability of these cell lines in immune-deficient mouse models [12, 67]. Our group focused our studies on mouse melanomas, we show that stable, ectopic expression of moMETCAM/MUC18 in two low-metastatic mouse melanoma cell lines, K1735-3 and K1735-10, increases their metastatic abilities in immune-competent syngeneic mice [49]. METCAM/MUC18 enables melanoma cells to establish pulmonary metastasis only when the cells are injected into the tail vein (experimental metastasis) [12, 13, 49, 50], thus bypassing the initial stages of metastasis. In contrast, no metastasis was found when METCAM/MUC18-expressing melanoma cells were injected subcutaneously (spontaneous metastasis) either in immune-deficient mouse models [12, 67] or in immune-competent syngeneic mouse models [13, 49]. We concluded that moMETCAM/MUC18 may promote melanoma metastasis only in the later stages of metastasis. This result is consistent with the observation of one of the three original groups that huMETCAM/MUC18 does not initiate the conversion (transformation) of melanocytes into melanoma [78].

In contrast to the role of moMETCAM/MUC18 in promoting metastasis in the two mouse melanoma cell lines K1735 clone 3 and clone 10, surprisingly we found that over-expression of

moMETCAM/MUC18 in one mouse melanoma cell line K1735 clone 9 decreased subcutaneous tumorigenesis and decreased pulmonary lung nodule formation when cells were injected into the tail vein in an isogenic mouse model [50]. Thus MCAM/MUC18 acts as a tumor and metastasis suppressor for the K1735-9 subline, different from its role in other K1735 sublines, K1735-3 and K1735-10. We suggest that ectopic expression of MCAM/MUC18 in different sublines may interact with different intrinsic co-factors/ligands, which may contribute to these intrinsic properties, such as adhesion-associated signaling cascades and cytoskeleton rearrangement, leading to different epithelial-to-mesenchymal transition of these cells and hence the intrinsic tumorigenic and metastatic potential of these cells. Different intrinsic co-factors in different K1735 sublines, which may modulate the functions of MCAM/MUC18 in the cells, leading to interact differently with the tumor microenvironment, may render sublines manifest differently in tumorigenicity and metastasis *in vivo*. Moreover, interactions of METCAM/MUC18 with these co-factors/ligands may render different sublines/cell lines being regulated by other physiological factors *in vivo*, which may enhance or inhibit *in vivo* growth of the tumor cells by altering metabolic switch, by altering apoptosis, or by up-regulating or down-regulating angiogenesis, as well as by boosting up or suppressing immune system in the tumor microenvironment and in the lung [50].

These syngeneic mouse systems are more useful models than the immune-deficient mouse systems for better understanding mechanisms of the complex role played by MCAM/MUC18 in the progression of melanoma cells. Furthermore, since these syngeneic mouse models more closely mimic the clinical melanoma cases in comparison to xenograft models, the knowledge gained from using these systems may also be useful for designing efficacious clinical therapies. Therefore, when therapeutic means are developed, we should keep in mind the dual role played by MCAM/MUC18. We should also be aware of the response of immunotherapy by using anti-METCAM/MUC18 monoclonal antibodies [79] and therapy by using MCAM/MUC18-specific siRNAs [80] may be different in different patients.

3.3. METCAM/MUC18 plays a dual role in the progression of nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a malignant head and neck cancer; 90% of that develops in the non-lymphomatous, squamous epithelial lining of posterior nasopharynx [81]. NPC is heterogeneous: it manifests one of the three subtypes (or three patterns): keratinizing squamous cell carcinomas (WHO type I), non-keratinizing squamous cell carcinomas (WHO type II), and undifferentiated carcinomas (WHO type III) [61, 81]. Epidemiological studies suggest that three major etiological factors, such as genetic susceptibility, environmental factors, and infection with Epstein Barr virus (EBV), contribute to the extraordinary incidence in endemic areas [61]. However, how these major etiological factors contribute to the initiation and development and final progression is not known. Nevertheless, these etiological factors may induce aberrant expression of cell adhesion molecules (CAMs) in NPC and leading to tumorigenesis and malignant progression. Aberrant expression of CAMs has been associated with the progression of NPC [37–40]. However, the possible aberrant expression of METCAM/MUC18 in nasopharyngeal carcinoma has not been studied.

We initiated the study of the possible roles of METCAM/MUC18 in the malignant progression of NPC by using immunohistochemistry to determine the expression of the protein in the tissues of

normal nasopharynx and NPC and two established NPC cell lines [61]. We found that METCAM/MUC18 was expressed in all of the normal nasopharynx, but weakly expressed in only 27% of the NPC tissues, suggesting that METCAM/MUC18 may function as a tumor suppressor in the development of NPC during the progression of the disease [61]. To test the hypothesis, we investigated the effect of METCAM/MUC18 over-expression on *in vitro* cellular behavior and *in vivo* tumorigenesis of two NPC cell lines in athymic nude mice. Indeed, METCAM/MUC18 over-expression suppressed the tumor growth of NPC-TW01 cells, which were established from type I NPC [81], as shown in [82, 83]. We suggested that METCAM/MUC18 plays a tumor suppressor role in the type I NPC [82, 83]. On the contrary, over-expression promoted the tumor growth of NPC-TW04 cells, which were established from type II NPC [81], as shown in [82, 84]. We suggest that METCAM/MUC18 plays a tumor promoter role in the type II NPC [82, 84].

We suggest that the dual role played by METCAM/MUC18 in the progression of two different types of NPC's may be modulated by different intrinsic factors and also in different stromal microenvironment. These two NPC cell lines may serve as models for understanding the contribution of three etiological factors to trigger the malignant progression of NPC and for translational applications. Radiotherapy has been used for the treatment of NPC; however, NPC has been notoriously resistant to radiotherapy. Thus, we sought the possibility of altering the radio-sensitivity of NPC by ectopically increased expression of METCAM/MUC18 in NPC cell lines. Our preliminary studies show that radio-sensitivity of the tumors induced from both cell lines in athymic nude mice was increased by increased expression of METCAM/MUC18. Thus, ectopically increased expression of this protein may be used for clinical treatment [data not shown].

3.4. METCAM/MUC18 plays a negative role in the progression of ovarian cancer

METCAM/MUC18 expression has been recently to correlate with the progression of ovarian cancer [47, 48], and perhaps affect the *in vitro* behaviors of ovarian cancer cells [85]; however, the role of METCAM/MUC18 in the progression of epithelial ovarian cancer has not been directly studied in animal models. For this purpose, we initiated the studies by directly testing the effect of over-expression of METCAM/MUC18 on the ability of SK-OV-3 cells in *in vitro* motility and invasiveness, and *in vivo* tumor formation in nude mice after subcutaneous (SC) injection and *in vivo* progression in nude mice after intraperitoneal (IP) injection. Over-expression of METCAM/MUC18 inhibited *in vitro* motility and invasiveness [59] and suppressed *in vivo* tumorigenesis and malignant progression of the human ovarian cancer cell line SK-OV-3 [59]. Similar results were shown in another human ovarian cancer cell line, BG-1 (data not shown).

Taken together, we provided *in vitro* and *in vivo* evidence to support the notion that METCAM/MUC18 plays a suppressing role in tumorigenesis and malignant progression of two human ovarian cancer cell lines [59, 60]. We strongly suggested that METCAM/MUC18 is a novel tumor and metastasis suppressor for the progression of human ovarian cancer cells.

3.5. METCAM/MUC18 plays a positive role in the progression of prostate cancer

Over-expression of METCAM/MUC18 is not limited to melanoma as previously thought and also later research carried out by one of the original three groups proved that MelCAM/MCAM/MUC18 did not play an important role in converting normal melanocyte into melanoma [78]. With this in mind, we have initiated the study of trying to correlate the huMETCAM/MUC18

expression with prostate cancer at different pathological stages. Molecular biological and immunological methods were used to study the expression of huMETCAM/MUC18 in two prostate cancer cell lines (DU145 and PC-3) and in human tissues of normal prostates, BPH, PIN, and prostate cancer, and immunohistochemistry was used for its expression in tissue sections of paraffin-embedded human prostate cancer [70, 72]. From the results, we suggested a possibility that huMETCAM/MUC18 may be used as a novel early diagnostic marker for the metastatic potential of human prostate cancer. These notions are further strengthened by the results of our studies in a transgenic mouse model, *transgenic adenocarcinoma mouse prostate* (TRAMP) [52]. Furthermore, we have suggested a hypothesis that huMETCAM/MUC18 very likely plays an important role in tumorigenesis and metastasis of human prostate cancer cells [72]. Then we carried out systematic studies of huMETCAM/MUC18-mediated prostate cancer metastasis in animal models to test the above hypothesis. We have tested the effect of ectopic expression of huMETCAM/MUC18 in human prostate LNCaP cells on their ability to form tumor in the non-orthotopic subcutaneous sites [53] and in the orthotopic prostate glands and to initiate metastasis in nude mice [51]. In contrast to melanoma cells, we have obtained evidence to prove that huMETCAM/MUC18 is a key determinant in initiating the metastasis of prostate cancer [51]. The detailed evidence is briefly described as follows:

3.5.1. Over-expression of huMETCAM/MUC18 correlates with the development and malignant progression of human prostate cancer

Two groups initiated the studies by testing possible huMETCAM/MUC18 expression in prostate cancer cell lines and prostate cancer tissues; however, they were unable to obtain positive results because the monoclonal antibodies used were incapable of recognizing the huMETCAM/MUC18 epitopes in prostate cancer cell lines and tissues [65, 86]. But we were able to use our chicken polyclonal antibodies for Western blot analysis and immunohistochemistry to detect the expression of huMETCAM/MUC18 antigens in prostate cancer cell lines and human prostate cancer tissues. We confirmed these results by using the RT-PCR analysis to show the presence of huMETCAM/MUC18 mRNA [70, 72]. We found that huMETCAM/MUC18 was neither expressed in most (90%) of the normal epithelial cells in the prostatic ducts/acini nor in any (100%) of these cells in BPH, but it was detectable in the majority (greater than 80%) of the neoplastic prostate epithelial cells (high-grade PIN), high-grade prostate adenocarcinomas, and metastatic lesions. HuMETCAM/MUC18 was expressed in two metastatic human prostate cancer cell lines, DU145 and PC-3, and one bladder cancer cell line, Tsu-Pr1, but not in one non-metastatic prostate cancer cell line, LNCaP [70, 72]. Thus, we conclude that huMETCAM/MUC18 is not expressed in normal and benign hyperplastic human prostate tissues, but its expression increases during prostate cancer initiation (high-grade PIN), progression to carcinoma, and in metastatic cell lines and metastatic lesions. Taken together, over-expression of METCAM/MUC18 correlates with the initiation of malignant progression of human prostate cancer [70, 72], suggesting that huMETCAM/MUC18 may be a useful marker for monitoring the metastatic potential of prostate cancer cells [70, 72]. Furthermore, the extent of *in vitro* motility and invasiveness is directly proportional to the extent of huMETCAM/MUC18 expression in four human cancer cell lines [70]. Our anti-huMETCAM/MUC18 antibody was able to significantly block the *in vitro* motility and invasiveness of various human prostate cancer cell lines [70]. Therefore, we propose the hypothesis that huMETCAM/MUC18 may directly mediate the increased epithelial-to-mesenchymal transition and initiate the progression of prostate cancers.

3.5.2. Correlation of over-expression of mouse METCAM/MUC18 with the malignant progression of prostate cancer in a transgenic mouse model (TRAMP)

If the above hypothesis is correct, we should be able to correlate moMETCAM/MUC18 expression with the development and progression of prostate cancer in a transgenic mouse model. To test this possibility, we have used the autochthonous TRAMP (*transgenic adenocarcinoma mouse prostate*) model for the experiment by collaborating with Dr Norman Greenberg's group. The TRAMP model established by Dr Norman Greenberg [87] is one of two transgenic mouse models that have been established for studying the tumorigenesis and metastasis of prostate cancer [87, 88]. This model was created by transfecting the germ line of the C57BL/6 inbred strain of mice with fusion gene of the rat probasin (PB) gene promoter and the SV40 T antigen (Tag) gene. The expression of the PB-Tag transgene is regulated by androgens and only localized to the prostatic epithelial cells in the dorsolateral and ventral lobes. When the mice reach an age of 12–20 weeks, TRAMP mice histologically show mild to severe hyperplasia with cribriform structures. By an age of 26 weeks, severe hyperplasia and adenocarcinoma is manifested. By an age of 26–33 weeks, all TRAMP males show primary tumors and metastasis in the lymph nodes and lungs and less frequently in the bones, kidney, and adrenal glands. In this transgenic model, the epithelial origin of the tumors and metastatic deposits has been successfully shown [87] in prostates.

MoMETCAM/MUC18 expression was determined by Western blot analysis and/or immunohistochemistry by using our chicken anti-moMETCAM/MUC18 antibodies [71] during the progression of mouse prostate adenocarcinoma in this transgenic mouse model. When these mice reached 12–20 weeks of age, they began to show PIN in the prostate glands. When they reached 178–181 days of age (25.7–25.9 weeks), they had primary tumors in the prostate glands and the expression of moMETCAM/MUC18 mRNA and protein was detectable. Tumors continued to grow beyond an age of 32.4 weeks, when some mice were found dead. Interestingly, metastasis was found even when tumors were small (less than 0.5 g) and the level of moMETCAM/MUC18 expression was much lower. MoMETCAM/MUC18 was not detectable in the prostates of the control group (presumably having the normal organ). The tumor metastasizes to peri-aortic lymph nodes in all the mice that had primary tumors. Metastatic lesions were also observed in seminal vesicles, abdomen cavity, livers, and lungs in some mice. The expression of MoMETCAM/MUC18 was detectable in all PINs, prostate adenocarcinomas, and metastatic lesions. We concluded that the moMETCAM/MUC18 expression was increased during the progression of the mouse prostate cancer in this transgenic mouse model [52]. Bone metastasis has been observed, though we have not analyzed the bone samples of these transgenic mice [89].

3.5.3. Over-expression of huMETCAM/MUC18 increases the tumor-take and metastasis of human prostate cancer cells

To test the hypothesis that huMETCAM/MUC18 may increase the metastatic potential of human prostate cancer cells, we successfully obtained G418-resistant clones that express a high level of huMETCAM/MUC18 after transfecting the huMETCAM/MUC18 cDNA gene into a human prostate cancer LNCaP cell line that did not previously express huMETCAM/MUC18 and had a minimal ability to metastasize. We then injected these clones orthotopically into one of the dorsolateral lobes of the prostate. We found that ectopically enforced huMETCAM/MUC18 expression increases the tumor-take and initiates the metastasis of LNCaP cells to various organs, such

as the seminal vesicles, the ureter, the kidney, and the peri-aortic lymph nodes, in athymic nude mice [51]. Since metastatic lesions were only observed in the mice with tumors, we also concluded that metastasis is closely associated with the tumorigenesis, as suggested by Weiss [23], but tumor formation without the expression of huMETCAM/MUC18 did not lead to metastasis.

Alternatively, we have also established a xenograft mouse model to further study how the expression of huMETCAM/MUC18 mediates tumorigenesis of LNCaP cells. We subcutaneously injected the huMETCAM/MUC18-expressing LNCaP cells together with Matrigel and observed the appearance of tumors at different times in a nude mouse model [53]. We found that ectopic (or enforced) expression of huMETCAM/MUC18 increased the early on-set of tumorigenesis of LNCaP cells in this mouse model [53]. Ectopic (or enforced) expression of huMETCAM/MUC18 increases the tumor formation of LNCaP cells [53]. We concluded that the enforced expression of huMETCAM/MUC18 in human prostate cancer LNCaP cells increased the tumor growth more than the control cells.

These results confirming that huMETCAM/MUC18 plays an important role in increasing tumorigenesis and initiating metastasis of LNCaP cells, consistent with our earlier findings that huMETCAM/MUC18 is frequently expressed in the pre-malignant high-grade PIN and in human prostate cancer tissues [72]. Taken together, the hypothesis that METCAM/MUC18 plays an important role in initiating prostate cancer progression is well-supported by evidence.

Recently, we further shown that huMETCAM/MUC18 also played a positive role in the progression of another prostate cancer cell line, DU145, by showing that the tumorigenesis of DU145 in an athymic nude mouse model was decreased when the endogenously expressed METCAM/MUC18 was decreased by knock-down with SiRNAs [54, 55].

3.6. METCAM/MUC18 plays a dual role in other tumors

In addition to melanoma, prostate cancer, breast cancer, ovarian cancer, and NPC, METCAM/MUC18 is also expressed in other cancers, such as gestational trophoblastic tumors, leiomyosarcoma, angiosarcoma, Kaposi's sarcoma, some lung squamous and small cell carcinomas, and some neuroblastoma; however, the role of METCAM/MUC18 in the development of most of these cancers has not been investigated [65, 66]. In our preliminary tests, we observed that moMETCAM/MUC18 was expressed at a higher level in one angiosarcoma clone, SVR, which was transfected with H-Ras, than in an immortalized normal endothelial cell line control, MS-1 [62]. The higher expression level of moMETCAM/MUC18 was correlated with the higher tumorigenicity of the SVR cell line [69, 90], suggesting that METCAM/MUC18 promoted the development of angiosarcoma [62, 69, 90]. Recent findings from other groups also suggest that METCAM/MUC18 also plays a positive role in the progression of osteosarcoma [91], hepatocellular carcinoma [92, 93], gastric cancer [94], non-small cell lung adenocarcinoma [95], small cell lung cancer [96], and pancreatic cancer [98].

On the other hand, the possible tumor and metastasis suppressor role of METCAM/MUC18 has also been extended from melanoma, ovarian cancer, and NPC, to colorectal cancer [97], pancreatic cancer [99], and perhaps, hemangioma [100]. **Table 1** summarizes the role of METCAM/MUC18 in the tumor formation and/or cancer metastasis of various tumors/cancers.

Tumor/cancers	Tumorigenesis	Metastasis	References
Clinical prostate cancer and human prostate cancer cell lines	Increasing	Increasing and affecting initiation in the early stage (PIN)	[51, 53–55, 70, 72]
Prostate adenocarcinoma in TRAMP mice	Increasing	Increasing and affecting initiation in the early stage	[52]
Clinical melanoma and human melanoma cell lines	No effect	Increasing and affecting at the late stage	[12, 67]
Mouse melanoma cells (K1735-3 and 10)	No effect or suppression	Increasing and affecting at the late stage	[13, 49, 71]
Mouse melanoma cells (K1735-9)	Suppression	Suppression	[13, 50, 71]
Angiosarcoma	Increasing	Possible promotion, by not determined	[62, 69, 90]
Human breast cancer cell line MCF-7	Promotion	Not determined	[56]
Human breast cancer cell line SK-BR-3	Promotion	Not determined	[57–58]
Human breast cancer cell lines MDA-MB-231 and 468	Promotion	Promotion	[57, 77]
Hemangioma	Possible suppression, but bot determined	Not determined	[100]
Nasopharyngeal carcinoma type I	Suppression	Possible suppression, but not determined	[82, 83]
Nasopharyngeal carcinoma type II	Promotion	Possible augmentation, but not determined	[82, 84]
Ovarian cancer	Suppression	Suppression	[48, 59, 60]
Colorectal cancer	Suppression	Suppression	[97]
Gastric cancer	Promotion	Not determined	[94]
Non-small cell lung carcinoma	Promotion	Not determined	[95]
Small cell lung carcinoma	Promotion	Not determined	[96]
Hepatocellular carcinoma	Promotion	Promotion	[92, 93]
Osteosarcoma	Promotion	Not determined	[91]
Pancreatic cancer	Promotion or suppression	Not determined	[98, 99]

Table 1. The possible role of METCAM/MUC18 in tumor formation and/or cancer metastasis of various tumors/cancers.

Taken together, the dual role of METCAM/MUC18 may be explained by that the intrinsic properties of each cancer cell line may provide co-factors that either positively or negatively regulate METCAM/MUC18-mediated tumorigenesis and metastasis. To understand further the role of METCAM/MUC18 in these processes, it is essential to identify these intrinsic co-factors in the future studies.

4. Possible molecular mechanisms of the huMETCAM/MUC18-mediated progression of cancer

Since the discovery of huMETCAM/MUC18 in the 1980s, about three groups have worked on the role of huMETCAM/MUC18 in melanoma metastasis [12, 63, 64, 67], another group on the role of huMETCAM/MUC18 in the biology of endothelial cells [68], and our group joined in the effort to study the role of huMETCAM/MUC18 in the progression of melanoma and prostate cancer, and later breast cancer, ovarian cancer, and NPC, as described above. Though we are beginning to understand the biology of METCAM/MUC18-mediated cancer progression, however, many questions of linking the regulation of the expression of this gene to its role in cancer progression are remained to be answered. For examples, the contribution of the protein structure and the glycosylation of the protein to the function of huMETCAM/MUC18 in the progression of cancer have not been systematically studied. How the protein mediates the interaction of tumor cells with the tumor microenvironment is not well studied. Though only limited information is available for huMETCAM/MUC18's outside-in and inside-out signaling in endothelial cells, and the signaling information for the METCAM/MUC18-mediated progression of various cancer cells are not much known. How the expression of METCAM/MUC18 is regulated at the level of transcription is minimally studied. We will try to address these questions by taking advantage of the currently known information from various sources to propose possibilities for much needed studies in the future.

4.1. The presentation of huMETCAM/MUC18 on the surface of cancer tissues may be different from cancer to cancer

Our unique contribution to the biology of METCAM/MUC18 was mainly attributed to the high specificity of our chicken antibodies [70, 71], to reconcile the different, sometimes controversial, findings [73, 86], we suggest that the presentation of huMETCAM/MUC18 on the cellular surface in normal and carcinoma tissues may be different from cancer to cancer [70, 72]. HuMETCAM/MUC18 may be presented differently in various cancer cell lines because of different carbohydrate composition, differential distribution of huMETCAM/MUC18 in the cholesterol-enriched lipid raft membrane fraction of the plasma membrane, different interactions of huMETCAM/MUC18 with other membrane proteins, and different lipid modification of the protein. There appears to have some correlation of the predominant cytoplasmic expression of huMETCAM/MUC18 antigens with the malignant progression of human prostate carcinomas, but not in human melanoma. To test the above possibilities, it is necessary to express the whole or a fragment of the protein in a large scale in human cancer cells lines in order to purify the protein for biochemical characterization and for crystallization and physical structure studies. Alternatively, to probe the functions of different parts of the molecule and for translational research, a complete set of monoclonal antibodies can be induced from the purified whole protein or its fragments. In line with these, a repertoire of mouse monoclonal antibodies has been successfully constructed by Yen's group [101]. We also intend to make a repertoire of rabbit monoclonal antibodies for the above purpose.

4.2. Which domains of huMETCAM/MUC18 are required for tumorigenesis and metastasis?

The relation of the protein structure of huMETCAM/MUC18 to its functions in tumorigenesis and metastasis has not been systematically defined. For this purpose, the partial known information of the functional domain(s) of the two huMETCAM/MUC18-related cell adhesion molecules in the Ig-like gene superfamily, such as CEA-CAM [31, 32] and ALCAM [102–104], might be used as a guide for designing tests to probe the functional domains in the external domain of huMETCAM/MUC18. The known functions of the cytoplasmic tail of several cell adhesion molecules may also provide clues to understand the function of the cytoplasmic tail of huMETCAM/MUC18 in crosstalk with signal pathways leading to tumorigenesis and metastasis [105–107]. For example, the domain of CEACAM1 required for tumorigenesis seems to reside in the intracellular cytoplasmic tail, but not in the extracellular domain [31, 32]. The N-terminal most Ig-like domain of the extracellular portion of ALCAM seems to be responsible for homophilic and heterophilic interactions [103, 104]. However, the domain(s) of these two molecules required for metastasis have not yet been studied. Since the information from these related proteins is very limited, we cannot logically predict the role of both the extracellular domain and the intracellular tail of the Ig-like CAMs in tumorigenesis and metastasis. To begin addressing this question, in the past, we have generated mutants deleted different domains of huMETCAM/MUC18 by using a special PCR method [108] and used them to determine their contribution to tumorigenesis. The ecto-domain of huMETCAM/MUC18 was similar to the whole wild type cDNA to be able to induce tumorigenesis of LNCaP cells in nude mice, suggesting that the ecto-domain alone was sufficient to induce tumor of human prostate cancer cells *in vivo*, implying that the cytoplasmic domain was not essential for this process [data not shown].

However, similar tests of using only the cytoplasmic domain have not been performed for LNCaP cells. Rather, the function of the cytoplasmic domain of huMETCAM/MUC18 was tested for the human ovarian cancer cell line BG-1 that the cytoplasmic domain alone was able to induce tumors in nude mice [data not shown].

In conclusion, we are not clear if this is true only for BG-1 cells or this is generally true for all cancer cell lines, which requires further studies.

4.3. The possible role of glycosylation in the huMETCAM/MUC18-mediated cancer metastasis

Glycosylation of a protein has been shown to affect the proper folding, stability, and/or activity of a protein [109]. Furthermore, the glycosylation of a cell adhesion molecule may affect its ability to induce metastasis of cancer cells [110–113].

Both huMETCAM/MUC18 and moMETCAM/MUC18 have an apparent molecular weight of about 150 kDa [114], because they are heavily glycosylated, sialylated, and post-translationally modified. We have shown that the apparent molecular weight of huMETCAM/MUC18 expressed in different human cancer cell lines was decreased after digestion with N-glycosidase F and neuraminidase (sialidase), but not with O-glycosidase or endoglycosidase H [62, 69], suggesting that huMETCAM/MUC18 has both sialic acid and N-glycans as carbohydrate side chains. Glycosylation in different cancer may be different that has been shown by using the anti-HNK-1 monoclonal antibody Leu 7 to probe the expression of HNK-1 epitope,

a sulfated-glycan with the structure of sulfo $\rightarrow 3\text{GlcA}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNA-c}\beta 1 \rightarrow \text{R}$, in melanomas and prostate cancer tissues [115–120]. The expression level of the HNK-1 epitope is proportional to that of human METCAM/MUC18 in human melanoma, and both are predominantly expressed on the plasma membrane of melanoma cells. In contrast, the HNK-1 epitope is found to be predominantly present in the cytoplasm of human prostate epithelial cells. In addition, different from the increased expression level of huMETCAM/MUC18 in prostate cancer, the expression of the HNK-1 epitope is decreased in the more advanced grades of human prostate cancer [120]. We suggest that the sugar moieties in the N-glycans of huMETCAM/MUC18 in human prostate cancer cells are different from that in melanoma cells; this may be related to its more profound effect on promoting the tumorigenesis and metastasis of human prostate cancer LNCaP cells than that of melanoma cells [12, 51, 69, 70]. Thus, we hypothesize that the N-glycans at the N-glycosylation sites of huMETCAM/MUC18 should have a significant effect on their *in vitro* motility and invasiveness and other *in vitro* cellular behaviors (for example, cell-cell adhesion and cell-extracellular matrix interaction) as well as tumorigenesis and metastasis of human prostate cancer and melanoma cells and very likely also in other human cancer cells that express METCAM/MUC18, since glycosylation of huMETCAM/MUC18 may affect its ability to mediate cell-cell adhesion and cell-extracellular matrix interaction. This notion is supported by a recent publication that GCNT3 is an upstream regulator of METCAM and it glycosylates METCAM/MUC18 and extends its half-life, leading to further elevation of S100A8/A9-mediated cellular motility in melanoma cells [121].

The human huMETCAM/MUC18 protein has nine potential N-glycosylation sites (Asn-X-Ser/Thr or N-X-S/T sites) [63, 64, 70] and the mouse METCAM/MUC18 has seven [71]. Their locations on huMETCAM/MUC18 are depicted in **Figure 1**. Six N-glycosylation sites are conserved between the two proteins: 56/58 NL/FS, 418/420NRT, 449/451NLS, 467NGT/469NGS, 507NTS/509NTT, and 544/546NST [69]. Since the apparent molecular weight of huMETCAM/MUC18 and moMETCAM/MUC18 in the SDS gel are similar, we suggest that only these six conserved N-glycosylation sites are actually glycosylated. All these N-glycosylation sites are located in the external domains of V1, C', C'', and X. The effect of N-glycosylation on the function of huMETCAM/MUC18 can be tested by using genetic means to alter the N-glycosylation sites. First, we should test the effect of mutations in the six conserved sites of the huMETCAM/MUC18 on *in vitro* cell-cell aggregation and cell-extracellular matrix adhesion and on *in vivo* tumorigenesis and metastasis of human cancer cells. The N-glycosylation site can be point mutated from Asn to Ala or Gln [122, 123] or may be linker-scanning mutated by replacing the three codons (the nine nucleotide sequence) with a nine bp oligonucleotide containing a unique restriction site sequence [124]. Both kinds of mutation are better than deletion mutations, since they do not change the relative physical location of the mutated sequences and thus the phenotype of the mutant is directly related to the substituted sequence without the complicated influence of the added sequences from the surrounding region of interest. The linker-scanning mutations from our experience are superior to the point mutations because they usually manifest a more dramatic phenotype [124].

4.4. The heterophilic ligands of METCAM/MUC18 may play an important role in the cell-cell and cell-extracellular matrix interactions and cancer metastasis

To further understand how huMETCAM/MUC18 mediates metastasis of cancer cells to certain target organs, it is important to identify the heterophilic ligand(s) of huMETCAM/MUC18 and to know how it regulates cellular behaviors, and how it interacts with members of the signal

transduction pathways. Previous studies suggest that huMETCAM/MUC18 prefers to interact with heterophilic ligand(s) [125, 126], which, however, has not been identified. But at least we know that some components of proteoglycans such as, glycosaminoglycan, hyaluronic acid, dermatan sulfate, keratan sulfate, heparin, heparan sulfate, chondroitin-6-sulfate, and heparan sulfate proteoglycan, have been excluded [125, 126]. The heterophilic ligands of huMETCAM/MUC18 are highly likely to be proteins. The proteins possessing SH3 domain [127] may be the possible candidates; however, the proteins containing the SH3 domain may not be the real ligands since they interact with MUC18 with a low affinity. The neurite outgrowth factor (a member of the laminin family) [128] may be another possible candidate; however, the cDNA gene of the neurite outgrowth factor has not been cloned for further characterization. To identify the authentic heterophilic ligands of huMETCAM/MUC18, stringent biochemical criteria should be employed.

To search for the ligand(s), many methods may be employed, such as immunoaffinity pull-down method [129], METCAM/MUC18-GST fusion protein pull-down method [69], METCAM/MUC18-AP fusion method to screen an expression library [130, 131], or METCAM/MUC18 used for screening counter-acting peptides in a phage library expressing random peptides [132, 133]. So far, we have attempted to use an immunoaffinity pull-down method to identify the heterophilic ligands of huMETCAM/MUC18. From our preliminary results, we found that a protein of 72 kDa may be a potential ligand, which is expressed on the cellular surface of two human prostate cancer cell lines and one human melanoma cell line [69]. This putative ligand protein appeared to be present in the extract of the human prostate cancer cell line, PC-3, more than that in DU145. We excluded the possibility that this protein may be the breakdown product of huMETCAM/MUC18 because of its discrete size. We are in the process of preparing a sufficient quantity of the protein for further characterization with mass spectrometry. In addition, we have successfully expressed a huMETCAM/MUC18-ectodomain-GST fusion protein in LNCaP cells. The fusion protein will be purified and also used for pulling down ligands. Alternatively, we have used a huMETCAM/MUC18-AP fusion protein to screen a human brain cDNA expression library; preliminary trials indicated that this methodology appears to be promising.

4.5. METCAM/MUC18-mediated signal transduction and cancer metastasis

The intracellular cytoplasmic tail of huMETCAM/MUC18 contains three consensus sequences to be recognized by PKC, one by PKA, and one by CK2 [63, 69, 70]. Though not biochemically proven, the cytoplasmic tail presumably is phosphorylated by these kinases. It probably has a capacity to crosstalk and network different signal pathways, similar to the cytoplasmic tails of other CAMs [106, 107, 134, 135]. Thus METCAM/MUC18, as an integral membrane protein and a cell adhesion molecule, should mediate inside-out and outside-in signals, which may be participating in cell-cell communication, cell-extracellular matrix interaction, and affecting the cellular motility and invasiveness [134, 135]. Furthermore, its interaction with cognate heterophilic ligand(s) may affect how it promotes angiogenesis and how it mediates targeting to specific organs and facilitates metastasis. Moreover, it may interact with androgen/androgen receptor, growth factors/receptors, chemokines/receptors, and Ca^{2+} -mediated signaling members. **Figure 2** summarizes the possible crosstalk of huMETCAM/MUC18 with many members of signal transduction pathways, which are supported by the following:

The downstream signal transduction of this protein has been studied in endothelial cells. Anfosso et al. [68] showed that antibody cross-linked huMETCAM/MUC18 (which mimics cell

adhesion on the cell surface) induces phosphorylation of both FAK and PyK2 (a member of the FAK family) and association of FAK with paxillin in the endothelial cells, which leads to the activation of focal adhesion complexes, similar to the outside-in signaling triggered by the engagement of integrins with the extracellular matrix. In addition, the engagement of huMETCAM/MUC18 also triggers direct association with Fyn, a member of the c-Src family, which activates adhesion-associated signaling cascades and cytoskeleton rearrangement, leading to increasing motility, and invasiveness. Since FAK and PyK2 do not directly associate with huMETCAM/MUC18 and the molecules that link the huMETCAM/MUC18 with FAK or PYK2 have not been identified, but huMETCAM/MUC18, similar to integrins, may use Fyn (and talin) for this purpose. We suggest that it is likely that focal adhesion complexes and signaling cascades may also be present in the prostate cancer and melanoma cells, since the over-expression of huMETCAM/MUC18 increases motility and invasiveness of human prostate cancer cells and melanoma cells.

Anfosso et al. further found that huMETCAM/MUC18 engagement also induces a Ca^{2+} influx, indicating that it is also able to initiate a store-operated calcium mobilization [136]. Ca^{2+} inside the cell may play a very important role—more so than other secondary messengers—in focal adhesion-induced actin cytoskeleton rearrangement and cellular motility, which is supported by the fact that locally elevated Ca^{2+} inside the cell triggers focal adhesion disassembly and enhances residency of focal adhesion kinase at focal adhesions [137, 138]. The link between the cell adhesion role of huMETCAM/MUC18 with Ca^{2+} influx is supported by the evidence that human METCAM/MUC18 is coupled to a Fyn-dependent pathway that triggers activation of phospholipase C- γ 1 via tyrosine phosphorylation, which leads to increased Ca^{2+} influx that is in turn required for the tyrosine phosphorylation of PyK2 and p130^{Cas} and formation of a complex between PyK2, p130^{Cas}, and paxillin, which in turn leads to cell adhesion and cell motility. Taken together, huMETCAM/MUC18 is a signaling molecule involved in the dynamics of actin cytoskeleton rearrangement. The elevation of Ca^{2+} influx also has other effects: it is linked to Ras-Raf1-MAP kinase via PyK2 and involved in cell proliferation by activating phospholipase C- γ 1, which in turn activates PKCs that affects cell proliferation, differentiation, and transcriptional control of other genes (for example, c-Myc target genes); and it may directly activate transcription factors, such as NF- κ B, NF-AT, and CREB, which regulate transcription of genes in proliferation, survival, and/or apoptosis [139].

AKT (or PKB), the cellular homolog of the retroviral oncogene v-AKT, is a serine/threonine kinase. AKT is a key member in the AKT/PI3K/PTEN signaling pathway [140]. AKT is activated by phosphorylation at Thr 308 and Ser473 by its upstream factor, PI3 kinase, and is inactivated by de-phosphorylation by PTEN, a tumor suppressor. Upon activation, AKT promotes cell survival by interfering with the cell apoptosis (when cells are exposed to pro-apoptotic signals, such as growth factor withdraw, irradiation, DNA damage, detachment, and the administration of apoptosis-inducing reagents) [140] and also promotes motility, proliferation, growth, angiogenesis, and the activation of mTOR (mammalian target of rapamycin) [141]. AKT also can be activated by Ras, which in turn is activated by a growth factor receptor or by RTK and survival factors, IGF1. In melanoma cells, when AKT activation is inhibited, huMETCAM/MUC18 expression is reduced. When AKT is super-activated, huMETCAM/MUC18 expression is increased. On the other hand, the over-expression of huMETCAM/MUC18 in melanoma cells led to further activation of AKT, resulting in cell survival under stress conditions [142]. It is not clear how the expression of AKT is directly or indirectly affecting the expression of huMETCAM/MUC18 and which isoforms is involved, since there are

three isoforms of AKT: AKT-1, AKT-2, and AKT-3. One possible crosstalk between METCAM/MUC18 and AKT may be linked by casein kinase 2, as described further.

Casein kinase 2 (CK2) was discovered in the 1950s. The history of CK2 is full of paradoxes and unexpected findings [143]. The α -subunits and the β -subunits of CK2 are found to be un-coordinately distributed in different cellular compartments, suggesting that the interaction partners of the α -subunits and the β -subunits may be different and much more complex than we have previously thought. CK2 is constitutively active, and no oncogenic CK2 mutant is known; but high CK2 activity correlates to neoplasia. It was thought to be one of the most pleiotropic protein kinases. However, a phospho-proteomics analysis of these CK2 null cells suggests that CK2 pleiotropy may be less pronounced than expected, supporting the idea that the phospho-proteome generated by this kinase is flexible and not rigidly pre-determined. Furthermore, CK2 is dispensable, since it can be replaced by other kinases to perform the phosphorylation of critical sites whenever CK2 activity is nullified. CK2 may also be involved in cell survival by directly affecting AKT activity or indirectly interacting with members of the AKT survival pathway. We suggest that a potential sequence present in the huMETCAM/MUC18 cytoplasmic tail may be recognized by CK2 as its substrate and may link the reciprocal mutual effect between huMETCAM/MUC18 and AKT.

PKC is the most extensively studied among all protein kinases. A huge number of members are involved in the family of PKC. PKCs are encoded by nine different genes. It has a large family including three major families of isozymes with distinct regulation: cPKC (PKC α , PKC β I, PKC β II, and PKC γ), nPKC (PKC δ , PKC ϵ , PKC η , and PKC θ), and aPKC (PKC λ , PKC ζ , and PKC ι) [144]. In addition, there are the PKC-related kinases, PRK $\frac{1}{2}$ [145] and PKC-binding partners [146]. The classical PKCs (cPKCs) are activated by PS, calcium, and DAG or PMA; the novel PKCs (nPKCs) are activated by PS and DAG or PMA; the atypical PKCs (aPKCs) are independent of PS, calcium, and DAG. PKC isozymes are key regulators of cellular function, such as growth, differentiation, cell survival, neurotransmission, carcinogenesis, and cancer progression [144]. PKCs control multiple functions associated with cancer progression, in many cases in opposite manners. Depending on the context, PKCs can act either as promoters or suppressors of the cancer phenotype [147]. The PKC-related kinases, PRK $\frac{1}{2}$ and PKC-binding partners may regulate and expand the functions of PKCs by positioning individual PKCs in the appropriate location to respond to specific receptor-mediated activating signals, bringing them in close contact with substrate proteins, directing them in vesicle trafficking between compartments, or integrating PKC-mediated signaling with other signaling pathways [145, 146]. Increased regulation of PKC α has been associated with the malignant progression of melanoma and that of PKC β II with the progression of colon/rectal cancer [144]. Up-regulation of PKC α , PKC ϵ , and PKC ζ , and down-regulation of PKC β was associated with the progression of prostate cancer [144]. Over-expression of PKC ϵ was sufficient to transform androgen-sensitive LNCaP cells into an androgen-independent variant [144]. The contribution of PKCs to METCAM/MUC18-mediated tumor growth and metastasis has not been studied. At least, it is highly possible that PKCs may phosphorylate the cytoplasmic tail of METCAM/MUC18 at the three potential PKC phosphorylation sites; however, it is not clear which isoform of PKC is responsible for it. Furthermore, some inside-out signals may turn on some of the PKC members, which in turn may interact with the cytoplasmic tail of huMETCAM/MUC18, leading to tumor cell survival, tumor vascularization, cytoskeleton reorganization, focal adhesion, migration, and invasiveness [147].

PKA is involved in cAMP-mediated signal transduction. The huMETCAM/MUC18 promoter may be regulated by PKA [148], since it contains a consensus CREB-binding site, as shown in **Figure 3**. Thus, the expression of huMETCAM/MUC18 may be regulated by PKA. Furthermore, the activity/function of huMETCAM/MUC18 may be regulated by PKA-mediated signals, perhaps after the phosphorylation of the cytoplasmic tail. Since PKA has been shown to regulate the Ca^{2+} channels and pumps, the Ca^{2+} influx induced by the huMETCAM/MUC18 engagement may also be regulated by PKA [139]. The effect of PKA on *in vitro* tumor growth and *in vitro* motility and invasiveness may be investigated after augmentation of the PKA activity by treatment of cells with forskolin. The effect of PKA on these processes may be reversed by treatment with specific inhibitors to reduce the PKA activity.

4.6. Transcriptional regulation of huMETCAM/MUC18 gene

Only a few studies have been done to understand the mechanism of transcriptional control of METCAM/MUC18 gene. The sequence of 900 bp in the core promoter region of huMETCAM/MUC18 has been characterized [64]. This promoter does not have a TATA box, but is GC-rich, which contains putative binding sites for SP-1, CREB [148], AP-2 [149, 150], c-Myb [151], N-Oct2 (Brn2) [152], Ets [153], CArG [154], and Egr-1 [155] and three insulin responsive elements (one Ets and two E-box motifs) [156], suggesting that huMETCAM/MUC18 expression may be regulated by growth-related signals [62, 69] (**Figure 3**).

AP-2 is a transcription repressor for the huMETCAM/MUC18 gene and it is also a suppressor for the huMETCAM/MUC18-mediated metastasis [149, 150]. This is supported by the evidence that transfection of highly metastatic melanoma cells ($\text{AP-2}^-/\text{MUC18}^+$) with the AP-2 gene resulted in a down-regulation of huMETCAM/MUC18 gene and inhibited their tumor growth and metastasis in nude mice [149]. Similar down-regulation of huMETCAM/MUC18 probably occurs in human prostate cancer cells [150]. Since the loss of AP-2 expression is at the early stage of prostate cancer development, this is consistent with our notion that MEMCAM/MUC18 plays an important role in initiating the development of prostate cancer. The loss of AP-2 expression at the late stage of melanoma is also consistent with our hypothesis that MEM/MUC18 plays a key role only in the late stage of the development of melanoma. ZBTB7A has

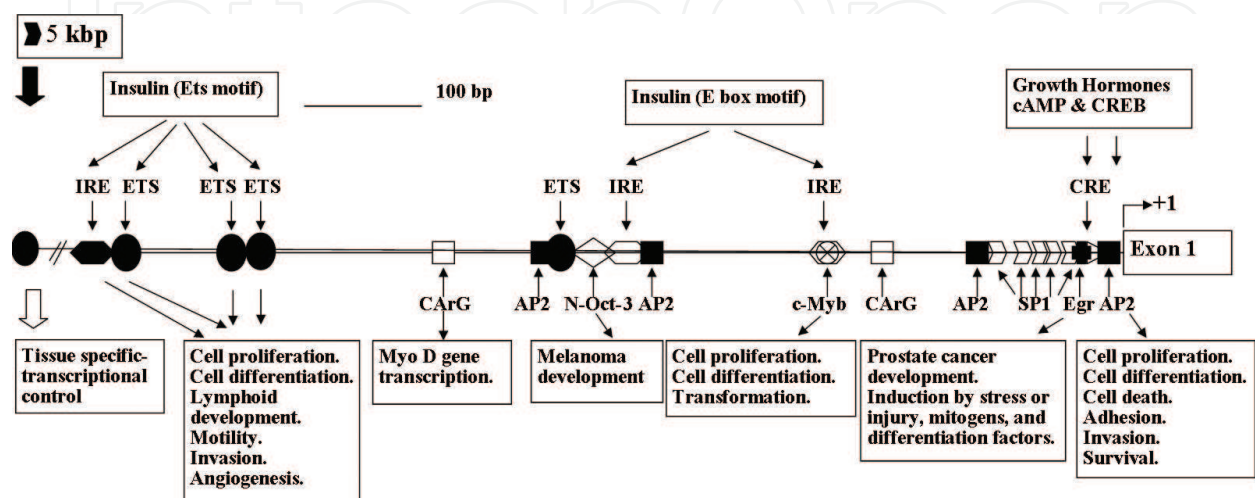


Figure 3. Putative transcription factor-recognized motifs in the 900 bp core promoter and 5–10 kbp upstream region of the huMETCAM/MUC18 gene.

been shown to repress transcription of METCAM/MUC18 gene in melanoma [157]. METCAM/MUC18 gene in osteosarcoma has been partly controlled by the transcription factor YY1 [158].

In addition to the sequence in the core promoter region, some upstream sequences should be required for the tissue-specific regulation of huMETCAM/MUC18 gene, since this 900 bp promoter region did not contain the necessary sequences to confer tissue specificity of the huMETCAM/MUC18 expression [159]. A recent finding appears to support this notion that Ets sequence in the 10 kbp upstream region is involved in the regulation of the expression of huMETCAM/MUC18 gene [160]. The sequence of the upstream region of the huMETCAM/MUC18 promoter may be obtained by searching in the Celera or other web sites. We have obtained several clones that contain at least 4 kbp of the gene for future studies.

Furthermore, epigenetic control of the huMETCAM/MUC18 gene has not been extensively studied. Nevertheless, the epigenetic control of the expression of huMETCAM/MUC18 gene has been demonstrated in NPC [161] and prostate cancer [162]. HuMETCAM/MUC18 gene is located on human chromosome 11q23.3 [127] which has been shown to be methylated in NPC, suggesting that the expression of this gene may be regulated by epigenetic controls. METCAM/MUC18 has been shown to be methylated in most of the early stage of prostate cancer [162].

5. Conclusions and clinical applications

METCAM/MUC18 may play a key positive function in the progression of prostate cancer, melanoma, breast cancer, gastric cancer, hepatocellular carcinoma, lung cancer, pancreatic cancer, and NPC type II. On the other hand, it may also have a key function in suppressing the progression of one mouse melanoma cell line, ovarian cancer, NPC type I, colorectal cancer, hemangioma, and perhaps other cancers. To further understand its role in these processes, it is essential to further define its functional domains, identify its cognate ligands and regulators, and study its crosstalk with members of various signal transduction pathways and the regulation of its expression at the level of transcription. The knowledge obtained from our studies should be useful for designing effective means to arrest, or even better, to block the metastatic potential of these cancers. For example, a preclinical trial of using doxazosin, a α 1-adrenergic antagonist, which has been used to treat the BPH patients, has been shown to reduce prostate cancer metastasis in the TRAMP mouse model [163]. Furthermore, the success of preclinical trials has been demonstrated in using a fully humanized anti-METCAM/MUC18 antibody against melanoma growth and metastasis [164] and in using a mouse anti-METCAM/MUC18 monoclonal antibody against angiogenesis and tumor growth (hepatocellular carcinoma, leiomyosarcoma, and pancreatic cancer) [165]. However, the dual role of METCAM/MUC18 in cancer progression warns us an important point in clinical applications that we should not be hasty in using monoclonal antibodies or siRNA for clinical trials, rather we should spend more effort on tailoring a personalized treatment in the future.

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