Chapter 4
Metastasis Suppressor Genes

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Below is a list of the most extensively studied MSGs, describing their initial characterization as well as what possible mechanism has been characterized for each.

4.1 NM23

Steeg and coworkers discovered and validated the first MSG in 1988, which they named *NM23*. The results of the study showed that the RNA levels of *NM23* were highest in cell lines and tumors with relatively low metastatic potential in two different experimental systems; the first was in murine K-1735 melanoma cell lines and the second was in rat mammary carcinomas. Since this date, eight isotypes of human *NM23* have been described (Nm23-H1 through Nm23-H8) (Lacombe et al. 2000). Among these isotypes, it has been shown that only Nm23-H1 and Nm23-H2 possess anti metastatic abilities. These isotypes have been studied extensively in different types of tumors including melanomas (Hartsough and Steeg 2000). The metastasis-suppressive function of *NM23* was previously correlated with its histidine protein-kinase activity in site-directed mutagenesis experiments (Freije et al. 1997; Wagner et al. 1997). Recently, Steeg et al. reported that *NM23* co-immunoprecipitated with the kinase suppressor of RAS (KSR) protein, which is thought to be a scaffold protein for the extracellular signal regulated kinase–mitogen activated protein kinase (ERK–MAPK) pathway (Hartsough et al. 2002; Morrison 2001). *NM23* was shown to phosphorylate KSR serine (Ser) 392 (a 14-3-3 binding site) and Ser 434, which was phosphorylated *in vivo (*Cacace et al. 1999; Volle et al. 1999*).* Therefore, it was

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hypothesized that the phosphorylation of KSR by *NM23* altered its scaffold function, possibly by altering the docking of proteins or KSR intracellular localization, which lead to reduced ERK activation in response to signaling (Steeg 2003). In agreement with these findings, Steeg et al. showed that MDA-MB-435 breast carcinoma cells that over expressed *NM23* showed reduced ERK activation levels compared with vector-alone control transfectants. In addition, a histidine-kinase-deficient mutant of *NM23* (Nm23-H1P96S) showed high levels of activated ERK, comparable to those of the control transfectants. Combining all this research, the authors concluded that altered levels of *NM23* in metastatic versus non-metastatic tumor cells might impact ERK activation through a complex interaction with the KSR scaffold protein (Steeg 2003).

NM23 represents the most extensively studied and confirmed MSG. The afore-mentioned data was recently extended by the characterization of Nm23-M1 knockout mouse, where they induced hepatocellular carcinoma in mice that contained Nm23-M1 or lacked Nm23-M1. The results of the study showed that the size of the primary tumor of the knockout mice did not change significantly, but the incidence rate of metastases increased significantly in the knockout mice (Boissan 2005).

Different expression levels of *NM23* gene products have been widely reported in different human tumor cohorts. Reduced *NM23* gene products expression are correlated with aspects of high metastatic potential including reduced overall survival time, the presence of lymph node metastases, and poor tumor differentiation grade in a subset of breast, gastric, ovarian, cervical, hepatocellular carcinomas and melanoma cohorts.

A study performed by Heiman et al. showed that the fifteen-year disease-free survival rate was found to be significantly better in breast cancer patients with high Nm23 immunostaining compared to low Nm23 immunostaining. Moreover, Nm23 was associated with excellent survival, even when there were other unfavorable prognostic markers such as angiogenesis or nuclear grade (Heimann et al. 1998).

Differential colony hybridization between low and high metastatatic murine melanoma cell lines identified different levels of Nm23. The mRNA levels of the Nm23 were ten-fold higher in low metastatic clones compared to high metastatic clones.

Similarly, significantly lower levels of Nm23 mRNA were detected in cell lines that were derived from aggressive primary melanomas. These melanomas showed higher Clark's level and greater Breslow thickness, which are considered to be poor prognostic markers for cutaneous melanoma patients (Caligo et al. 1994). Nm23 protein expression in primary cutaneous melanomas was found to be significantly and inversely correlated with the dermatopathological predictors of poor prognosis in patients with localized melanoma, including thickness, ulceration, level of invasion, and mitotic figures (Ferrari et al. 2007). Using tissue microarray analysis in one hundred and twenty patients with primary cutaneous melanoma an important role for Nm23 assessment in these patients was suggested. The results of the study showed that Nm23 expression was strongly correlated with Clark's level ($P < 0.001$), Breslow depth ($P = 0.002$) and patient age ($P = 0.014$). In addition, Nm23 expression was significantly associated with poor patient outcome ($chi2 = 7.2219$, $P = 0.0072$).

Further analysis revealed that the intensity of Nm23 expression was also correlated with patient outcome (chi2 = 11.3281, P = 0.0035) (Pacifico et al. 2005).

A study performed by Bakalian et al. showed that the invasive abilities of different human uveal melanoma cell lines with different metastatic potentials increased after silencing the expression of Nm23-H1 in these cell lines with small interference RNA. Furthermore, uveal melanoma patients with high immunostaining intensity for Nm23-H1 survived longer as opposed to patients with low immunostaining intensity (Kaplan-Meier test P = 00097). They concluded that Nm23-H1 could be a prognostic marker to predict the survival rate of uveal melanoma patients and could be a potential marker to identify high-risk patients (Bakalian et al. 2007).

4.2   RHOGDI2

Theodorescu and coworkers recently has identified the Rho family GDP dissociation inhibitor 2 (RhoGDI2) protein as a functional metastasis suppressor gene in bladder cancer. RhoGDI2 was originally identified during differential studies of invasive and metastatic properties of isogenic human bladder carcinoma cell lines, where T24 (non-metastatic), and T24T (highly invasive and metastatic) cell lines, using experimental metastasis models and comparative genomic studies (Seraj et al. 2000a; Titus et al. 2005). The results of the studies demonstrated that reduced expression of RhoGDI2 correlated with increasing invasive and metastatic activities in T24T highly invasive and metastatic cells. In human bladder tumors, the RhoGDI2 level inversely correlated with the development of metastatic disease, and multivariate analysis identified RhoGDI2 as an independent prognostic marker of tumor recurrence following radical cystectomy (Theodorescu et al. 2004). Therefore, RhoGDI2 is considered a prognostic marker in bladder cancer patients after cystectomy, where diminished expression is associated with decreased patient survival (Theodorescu et al. 2004). Using DNA microarrays to monitor the changes in gene expression following restoration of RhoGDI2 expression, Titus and coworkers identified several potentially targetable proteins, including the endothelin-1 ligand (ET-1), that were suppressed in the presence of RhoGDI2 protein. The results of the study revealed that loss of RhoGDI2 during the clinical progression of bladder carcinoma might lead to up-regulation of the endothelin axis. The later was confirmed by examining the relationship between RhoGDI2 expression levels and those of ET-1 in human tumor samples and cell lines. These findings suggested that adjuvant trials with endothelin antagonists might be contemplated for patients with advanced bladder carcinoma following the initial therapy (Titus et al. 2005). Lately, RhoGDI2 has been found to suppress the expression of neuromidinU, a molecule that mediates both increased growth of metastases and increased tumor cachexia in animal models (Wu et al. 2007). All these experiments showed a novel approach of identifying downstream therapeutic targets of metastasis suppressor genes. This new therapeutic approach warrants further clinical evaluation.

4.3   Ndrg1

N-myc downstream regulated gene 1(NDRG1) was originally identified by differential displays as being significantly up regulated by induction of *in vitro* differentiation of colon carcinoma cells (van Belzen et al. 1997). NDRG1 has been shown to act as a tumor suppressor as well as a metastasis suppressor depending on cell context (Kovacevic and Richardson 2006). The level of the NDRG1 expression was inversely related with the status of metastasis in breast and prostate carcinoma patients, supporting the notion that NDRG1 is a tumor metastasis suppressor gene (Bandyopadhyay et al. 2003, 2004). Ectopic expression of the NDRG1 gene in a highly metastatic prostate cancer cell line significantly reduced the incidence of lung metastases, suggesting that NDRG1 was able to block the metastatic process without affecting the primary tumor growth (Bandyopadhyay et al. 2003, 2004). NDRG1 significantly suppressed the invasive potential of prostate and breast cancer cells as tested by *in vitro* invasion chamber assay (Bandyopadhyay et al. 2003, 2004). Similar metastasis suppressor effect of NDRG1 was also observed in colon carcinoma cells (Guan et al. 2000).

Studies in which mice injected with SW620 colon cancer cells over expressing Ndrg-1 resulted in only 23 % of these developing liver metastases,compared to 75 % in the control group (Guan et al. 2000). To date, there has been little assessment of the molecular targets of Ndrg-1 that mediate its anti-metastatic activity. The adhesion molecule and metastasis suppressor, E-cadherin was found to be up regulated by Ndrg-1 (Guan et al. 2000). Increased expression of E-cadherin has been shown to reduce the motility of metastatic breast cancer cells *in vitro* (Liu et al. 2005). However, it is widely believed that E-cadherin is not the only molecular target of Ndrg-1 that contributes to metastasis suppression. Recently, it has been shown that Ndrg-1 expression was regulated by cellular iron levels and induced by iron chelators (Kovacevic and Richardson 2006). These latter compounds were identified as potential anticancer agents as they selectively prevent cancer cell proliferation and lead to apoptosis. The discovery that iron chelators increase Ndrg-1 expression further augments their antitumor and anti metastatic activity and offers a potential new strategy for the treatment of cancer and metastases.

4.4   RKIP

It has been shown that RKIP negatively regulates the Raf/MEK/ERK pathway by interfering with the activity of Raf-1. In its phosphorylated state, RKIP dissociates from Raf-1 and inhibits GRK-2, a negative regulator of G-protein coupled receptors (GPCRs). In addition, it has been demonstrated that RKIP is a negative regulator of the NF-kappaB pathway. Recent studies have also shown that phosphorylated RKIP binds to the centrosomal and kinetochore regions of metaphase chromosomes, where it may be involved in regulating the partitioning of chromosomes and the progression through mitosis. Therefore, evidence based research indicates that RKIP regulates

the activity and mediates the cross talk between several important cellular signaling pathways of metastasis, angiogenesis, resistance to apoptosis, and genome integrity (Klysik et al. 2008).

The first evidence about RKIP came from cell lines derived from metastatic prostate cancers, which displayed decreased levels of RKIP mRNA and protein as compared with primary tumor cell lines (Fu et al. 2003). Furthermore, studies showed that over-expression of RKIP in metastatic cancer cells decreased their invasive capabilities. Consistent with the notion that RKIP is a potent suppressor of metastases, experiments from several laboratories have demonstrated that malignant melanomas, breast cancer lymph node metastases, colorectal cancer, and hepatocarcinoma cells frequently display a marked decrease in RKIP expression (Klysik et al. 2008).

Recently, measuring the levels of RKIP in the blood has been proposed as a prognostic marker for prostate cancer patients, where RKIP plays a major role (Fu et al. 2006). A study performed by Zhu et al. showed that a small molecule, called locostatin, has the ability to abrogate RKIP's ability to inhibit Raf (Zhu et al. 2005). Interventions capable of enhancing RKIP-1 activity would be particularly useful for the control of metastatic cells that display attenuated steady-state levels of RKIP-1. Therefore, future studies evaluating the drug-induced modulation of RKIP expression may provide a potent means of controlling metastases.

4.5 KISS1

KISS1 is one of the metastasis suppressor genes that appears to function in the dormancy phase of the metastatic cascade. In addition, the gene encodes a protein that is further cleaved (called metastin) that likely exerts its function through the binding of metastin to a G-coupled-receptor. This event makes *KISS1* a possible candidate for therapy.

KISS1 was identified as a metastasis suppressor gene in melanoma cells in 1996 by Lee et al., when transfection of a full-length *KISS1* cDNA into C8161 melanoma cells suppressed metastasis in an expression-dependent way (Lee et al. 1996).

Nash et al. were the first to show that the introduction of *KISS1* into highly metastatic human melanoma cell lines C8161 and MelJuSo suppressed *in vivo* metastases to the lung by more than 95 % (Miele et al. 1996). Interestingly, introduction of *KISS1* into a metastatic breast cancer cell line MDA-MB-435 also showed a > 95 % suppression of metastases to the lung (Lee and Welch 1997).

It was also demonstrated that loss of KISS1 mRNA expression correlated with the conversion from benign to malignant phenotype in human melanoma (Shirasaki et al. 2001). This data strongly suggested *KISS1* metastasis suppression being pertinent in tumors of broadly different origins, a conclusion that was confirmed by later studies (Ikeguchi et al. 2004). Furthermore, reduced KISS1 expression has been shown to be a strong prognostic marker in patients with urinary bladder cancer (Sanchez-Carbayo et al. 2003) and gastric carcinoma (Dhar et al. 2004).

In general, loss or reduction of KISS1 expression in different tumor types negatively affected tumor progression, metastatic potential, and survival (Nash and Welch 2006).

Goldberg et al. showed that when chromosome 6 hybrid cells were injected intravenously into athymic mice, grossly detectable metastases did not form. Despite arriving in the lungs at frequencies comparable to the controls, the *Mkk4* and *KISS1* metastasis suppressor transfectants failed grow (Chekmareva et al. 1998; Goldberg et al. 1999). The results of these studies are responsible for identifying the role of these genes in the dormancy phase of the metastatic disease.

KISS1 encodes a 145-amino acid residue peptide that is further processed posttranslationaly. One of the products, a 54-amino acid peptide is called Metastin or Kisspeptin-54 and is a natural ligand to a G-coupled receptor known as HOT7T175/AXOR12/GPR54 (Ohtaki et al. 2001).

Evidence suggests that KISS1/metastin promotes dormancy of solitary cells (Nash et al. 2007) and acts in the final stage of tumor cell colonization at the metastatic site (Steeg 2004). A potential therapeutic approach involves administering exogenous KISS1 which has been shown to suppress cutaneous melanoma metastasis to multiple organs and enhanced median survival almost three-fold (Steeg and Theodorescu 2008). This has become a possibility due to the advent of small molecule mimetics.

Orsini et al in 2007 reported a molecule in which structure-activity approach may yield pharmacologically useful compounds relevant in defining and modulating metastin receptor function (Orsini et al. 2007).

4.6   KAI1

KAI1 is also known as CD82, R2, C33, IA4, and 4F9. It structurally belongs to tetraspanin family while categorized as metastasis suppressor gene (Malik et al. 2009). Tetraspanins are large group of cell surface transmembrane proteins with four transmembrane structures, which can form complexes with integrins.

KAI1/CD82 was initially identified as a metastasis suppressor of prostate cancer. However, evidence supports KAI1/CD82 as a invasion- and metastasis-suppressor during the progression of a variety of solid tumors (Malik et al. 2009).

The role of KAI1/CD82 in cancer progression was discovered by a genetic screen attempting to identify metastasis suppressor genes. Using microcell-mediated chromosome transfer, human gene(s) responsible for suppressing metastasis of the highly metastatic rat AT6.1 prostate cancer cells was mapped to the short arm of human chromosome 11. Later on, an important progress was made, by cloning the metastasis suppressor gene located on human chromosome 11 p11.2–13, which was named KAI1 (Ichikawa 1992).

KAI1/CD82 expression leads to a marked suppression of lung metastases of AT6.1 prostate cancer cells, with no effect on the growth rate of the primary tumor (Dong et al. 1995).

KAI1 appears to prompt dormancy in solitary tumor cells by binding DARC on the surface of vascular endothelial cells and inducing tumor cell growth arrest.

An inverse correlation between KAI1/CD82 expression and the invasive and metastatic potentials of cancer has been frequently observed in a wide range of malignancies such as prostate, gastric, colon, cervix, breast, skin, bladder, lung, pancreas, liver, and thyroid cancers (Liu and Zhang 2006).

4.7   MKK4 and MKK7

Mitogen-activated protein (MAP) kinase kinase 4 (MKK4) is a component of stress activated MAP kinase signaling modules. It directly phosphorylates and activates the c-Jun N-terminal kinase (JNK) and p38 families of MAP kinases in response to environmental stress, pro-inflammatory cytokines, and developmental cues (Whitmarsh and Davis 2007). The human MKK4 gene is located on chromosome 17 and encodes a protein of 399 amino acids (Yoshida et al. 1999).

MKK7 (also known as JNKK2) and MKK6 are also mitogen-activated protein kinase kinase that specifically phosphorylate JNK and p38, respectively (Vander Griend 2005)

Yoshida et al. first characterized MKK4 as an MSG in 1999 when they reported a reduction of the metastatic potential of prostate cancer cells by 80 % in a spontaneous metastasis assay (Yoshida et al. 1999). These experiments using the highly metastatic rat prostate cancer cell line AT6.1 (which lacks MKK4 expression) as a model system have demonstrated that the overexpression of MKK4 significantly reduces their metastatic ability (Whitmarsh and Davis 2007).

There is strong evidence that MKK4 play a role in dormancy of metastatic cells. In a model of spontaneous metastasis, MKK4 was shown to be required for suppression of overt metastases by inhibiting the ability of disseminated cells to colonize the lung (secondary site) (Vander Griend 2005). Ectopic expression of MKK4 also prolonged survival after surgical resection of the primary tumor from 7–20 days. Metastatic lung cells from mice were then cultured again in plaques and showed viability, stressing the dormant behavior of tumor cells expressing the gene (Vander Griend 2005). MKK-7 also plays an important role in dormancy, since it showed the same results as MKK-4 in these experiments. It was shown that ectopic expression MKK-7 suppresses the formation of overt metastases, whereas MKK6 had no effect (Vander Griend 2005).

A tissue-specific role for MKK7 was indicated when a difference between MKK4 and MKK7's regulation in dormancy was traced to the JNK arm of the MAPK pathway. In prostate cancer, MKK4 functions through the JNK pathway, which is also regulated by MKK7. NK activation leads to inhibition of the pro-survival gene Bcl2 and activation of the apoptotic genes BAX, Cytochrome C, Bim/Bmf, and c-Jun. The kinase activities of MKK4 and MKK7 were functional only in the metastatic site and not the primary tumor as identified using immunoprecipitation from primary and lung metastases.

The impaired expression of MKK4 in prostate and ovarian tumors appears to promote their metastasis (Yoshida et al. 1999; Yoshida et al. 2001), while reduced

MKK4 mRNA levels have been reported in breast cancer to brain metastases (Stark et al. 2005)

In normal prostate tissue there are high levels of MKK4 protein expression in the epithelial compartment but not in the stromal compartment, whereas in neoplastic prostate tissues the levels of MKK4 were reduced and there was in inverse relationship between the reduction of MKK4 expression and metastatic potential (Kim et al. 2001). Also, MKK4 protein expression is also reduced in ovarian metastatic tissues compared to normal ovarian epithelial cells (Yamada et al. 2002).

All these studies suggest that MKK4 functions as a metastasis suppressor that works in the dormancy phase of the metastatic cascade. It is probable that different tissues and organs dictate which MAP kinase pathway is targeted by MKK4 depending on specific stimuli.

4.8   BRMS1

BRMS1 (Breast Cancer Metastasis Suppressor) was identified using a combination of clinical observation and molecular biology. Seraj et al. identified BRMS1 by differential display comparing metastasis-suppressed chromosome 11 hybrids with metastatic, parental MDA-MB-435 human breast carcinoma cells. BRMS1 has subsequently been shown to suppress metastasis, but not tumorigenicity of human melanoma cells (Seraj et al. 2000b). A spontaneous metastasis assay was performed and originally showed to functionally suppress the metastatic capacity of breast cancer cells following injection into immunocompromised, athymic mice (Seraj et al. 2000b). BRMS1-transfected MDA-MB-435 cells demonstrated a decreased incidence and number of metastases to lung and regional lymph nodes when cells were injected orthotopically. These results demonstrated that BRMS1 suppressed metastasis without significantly affecting tumorigenicity, indicating that BRMS1 is a metastasis suppressor gene (Meehan and Welch 2003).

Further studies have proven that BRMS1 is not only a metastasis suppressor gene in breast cancer models but also in various other cancers such as melanoma and ovarian cancer (Meehan and Welch 2003). Recent studies have also demonstrated that low levels of BRMS1 protein correlated with poor prognosis in cancer patients with advanced metastatic disease. In addition, reduced BRMS1 mRNA levels have been shown to correlate with reduced disease-free survival in breast cancer patients.

BRMS1 is thought to possibly regulate metastasis through multiple mechanisms; such as the restoration of gap junctions, influencing phosphoinositide signaling, regulating genes through histone deacetylase (HDAC) interaction, and complex formation and inhibiting NFκB signaling in breast cancer. In particular, BRMS1 has been shown to downregulate osteopontin (OPN) expression by modulating the activity of NFκB signaling in breast cancer. Hedley et al. reported that decreased OPN associated with BRMS1 expression contributes to its metastasis suppression activity (Metge et al. 2010).

BRMS1 suppresses metastasis by inhibiting multiple steps in the metastatic cascade through different regulation mechanisms of many protein-encoding and metastasis-associated genes.

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