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MOLECULAR MECHANISMS THAT MEDIATE METASTASIS SUPPRESSOR ACTIVITY OF NM23-H1

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ABSTRACT OF DISSERTATION

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MOLECULAR MECHANISMS THAT MEDIATE METASTASIS SUPPRESSOR ACTIVITY OF NM23-H1

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

By
Qingbei Zhang

Lexington, Kentucky

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Lexington, Kentucky

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ABSTRACT OF DISSERTATION

MOLECULAR MECHANISMS THAT MEDIATE METASTASIS SUPPRESSOR ACTIVITY OF NM23-H1

Metastasis is the spread of cancer cells from the primary tumor to distant sites. It is the most dangerous attribute of cancer, and also the principle cause of cancer-related morbidity and mortality. Metastasis suppressor genes are a group of genes that suppress tumor metastasis without significant effect on tumorigenicity. NM23 was the first identified metastasis suppressor gene, and loss of its expression is a frequent hallmark of metastatic growth in multiple cancers (e.g. melanoma, carcinomas of breast, stomach and liver). NM23-H1 possesses at least three enzymatic activities, including nucleoside diphosphate kinase (NDPK), histidine kinase (hisK), and a more recently described 3'-5' exonuclease (EXO). While the hisK has been shown to be linked to the suppression of cell motility, the NDPK has been reported to be unrelated to the suppression of metastatic potential indirectly. Relevance of EXO has not been addressed. Other known 3'-5' exonuclease are closely associated with DNA repair functions, suggesting NM23-H1 may suppress mutations required for metastasis.

As a transcription factor, NM23 has been shown to modestly downregulate the transcription on PDGF-A chain, a growth factor oncogene, either alone or in association with another transcriptional factor, Pur . At the same time, identification of NM23-H1 as a 3'-5'exonuclease suggests the role of NM23-H1 in DNA repair.

Etoposide and cisplatin elicited nuclear translocation of H1 within 4 h in HeLa and HepG2 cells, seen as accumulation of H1 in small intranuclear foci, strongly suggesting the DNA repair function of H1. To investigate the enzymatic function contributing to metastasis suppressor activity of H1, complementation system was used by transfecting NM23-H1 with individually disrupted enzymatic function into 2 melanoma cell lines, 1205LU and WM793. Overexpression of H1 in 1205LU suppressed lung metastasis *in vivo* without effect on indices of transformation (e.g. proliferation, soft agar colonization). EXO- deficient H1 and NDPK-deficient H1 lost suppression of lung metastasis, while hisK-deficient H1 maintained suppressor activity. Consistent with the results in 1205LU cells, EXO-deficient H1 and NDPK-deficient H1 lost suppression of the progression of WM793 cells in protein-free medium, while WT and hisK-deficient H1 prevented the progression. Taken together, these data suggest that the NDPK and/or 3'-5'EXO activity of H1 inhibits the progression of premetastatic cells to the metastatic phenotype, possibly via a DNA repair function or other structural transactions with DNA.

KEYWORDS: NM23, exonuclease, DNA repair, genomic integrity, metastasis

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This dissertation is dedicated to my husband Can, my daughter Helen, my parents Shuyuan Wang and Xuemin Zhang.

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always have my statistic problem resolved. Dr. Xin Wang is my favorite consultant by admiring her unique talents and bountiful experience. Cynthia Long assisted me with cryostat and histological staining. Dr. Steve Post taught me how to use their imaging device to scan fluorescence in the tissue samples. I'm so grateful for their backing me up.

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CHAPTER ONE

INTRODUCTION

1.1. Metastasis and genomic instability

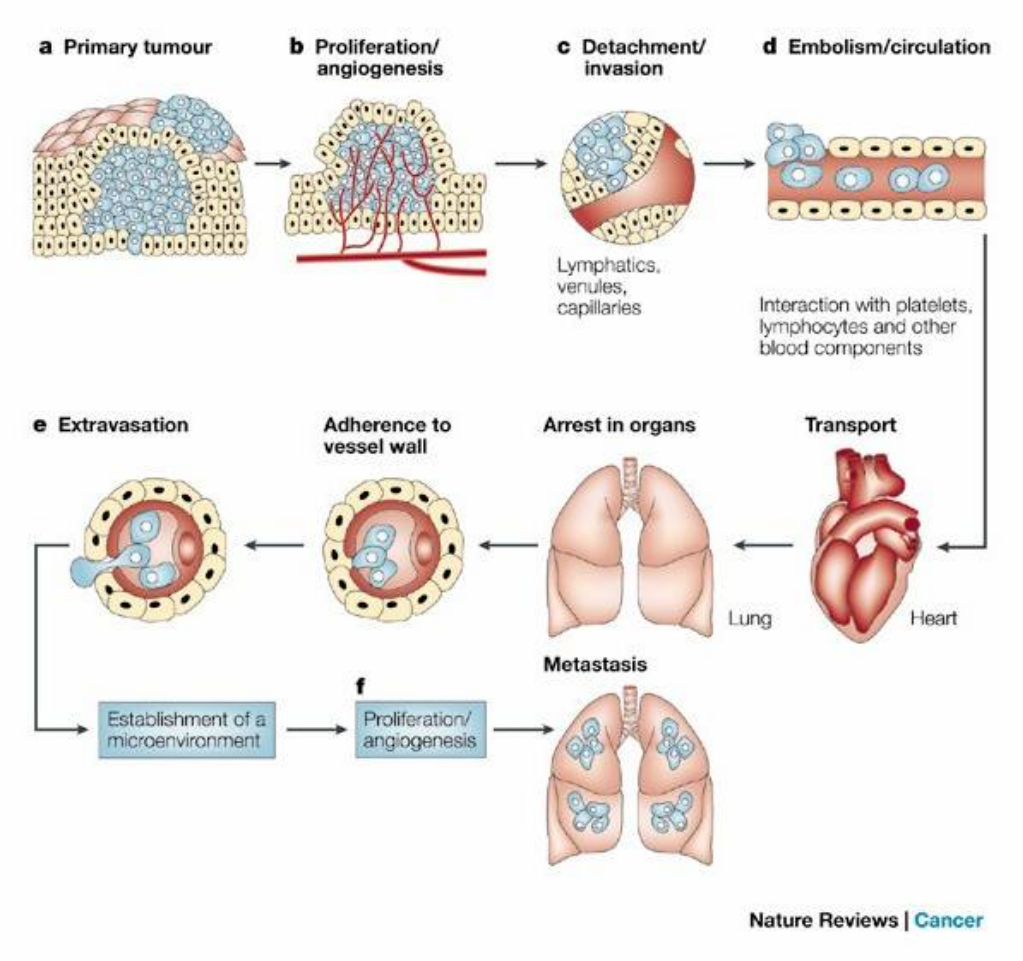
Metastasis is the spreading of tumor cells from their primary sites to distant locations. It may occur via the blood, a lymphatics route, or through the body cavities. Because of the limited treatment options for metastatic disease, metastasis is the principle cause of morbidity and mortality of cancer patients (reviewed in Steeg, 2003).

Metastasis is the end-result of a complicated, multistage process. Each of these sequential steps is rate-limiting (reviewed in Shevde and Welch, 2003; reviewed in Fidler, 2003). First, tumor cells detach from the primary tumor, invade the host stroma and penetrate blood vessels (Intravasation). Secondly, after tumor emboli enter the circulation, cells must survive shear forces, immune surveillance, or maybe nitric oxide produced by cytokine-activated endothelial cells (Dong *et al*, 1994). Finally, surviving tumor cells penetrate blood vessels (Extravasation) and colonize in the secondary site. Metastases form with proliferation of tumor cells and angiogenesis (Figure 1 from Fidler, 2003).

The majority of data show that metastasis is a highly selective process (reviewed in Talmadge and Fidler, 1982; Talmadge *et al*., 1982) and is quite inefficient (reviewed in Weiss, 1990). Progressing from premetastatic to metastatic state, a tumor cell must overcome a series of barrier to finish all steps of metastasis. During the process of evolution, tumor cells may have undergone many coordinated changes and acquired genetic variability to become more malignant. Nowell (reviewed in Nowell, 1976) suggested that increasing genetic instability of the evolving cells and associated selection process result in accelerating tumor progression toward malignancy. This

hypothesis was tested by Fidler group, and they found that high metastatic clone of fibrosarcoma cells were phenotypically less stable than low metastatic clone. Furthermore, the spontaneous mutation rate of high metastatic cells were determined to have 3- to 7- fold increase as compared to their low metastatic counterparts. These data support the hypothesis that tumor evolution could be the consequence of acquired genetic alterations (Cifone and Fidler, 1981).

Figure 1. The steps of the process of metastasis



1.2. Metastasis suppressor genes and NM23

By definition, metastasis suppressor genes suppress the ability of tumor cells to metastasize, without affecting their primary tumor growth characteristics. These genes are identified by their decreased expression in metastatic tumor cells, compared with their expression in non-metastatic cells (reviewed in Steeg, 2003). Since the identification of the first metastasis suppressor gene, *nm23*, in 1988 by P.S. Steeg, more than a dozen of metastasis suppressor genes have been identified, and their functional data have been completed or not characterized yet (reviewed in Shevde and Welch, 2003). Many high-throughput techniques have been used during the discovery of these genes, such as differential display, microarrays, and serial analysis of gene expression (SAGE). A very instrumental method employed in identifying several metastasis suppressor genes (e.g., BRMS1, KISS-1, MKK4, and CD44-s) is microcell-mediated chromosome transfer (MMCT), a method to identify a chromosomal region that has metastasis suppression function *in vivo*. In the case of genetic loss, transfer of a single human tagged chromosome into metastatic cells, would suppress metastasis. Identified metastasis suppressor genes can be validated by re-expression in metastatic cell lines. Its re-expression should significantly inhibit metastasis, but have no effect on primary tumor growth. Even though the number of metastasis suppressor genes is continuing to grow, the roles they play in predicting the prognosis of metastatic diseases as well as therapeutic strategies is not yet known.

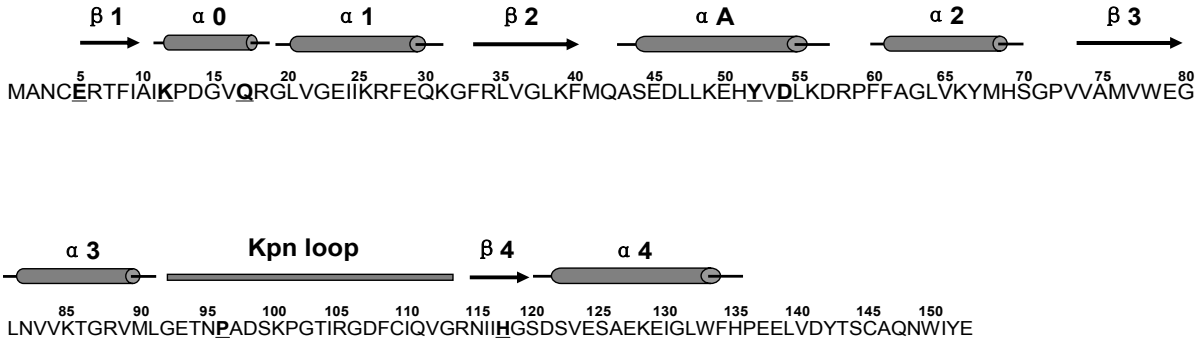
NM23 was the first identified metastasis suppressor gene by screening cDNA libraries of matched metastatic/non-metastatic murine melanoma cell lines by substraction cloning (Steeg *et al.*, 1988). NM23 stands for ‘non-metastatic clone 23 gene’. Loss of NM23 expression is linked to metastasis in multiple cancers (e.g. melanoma, breast cancer, and stomach and liver carcinomas). The human NM23

protein has sequence homology over the entire translated region with developmentally regulated protein in Drosophila, encoded by the abnormal wing discs (*awd*) gene. Mutations in *awd* cause abnormal tissue morphology and necrosis and widespread aberrant differentiation in Drosophila, analogous to changes in malignant progression (Rosengard *et al.*, 1989). To date, eight NM23 family members have been identified, designated NM23-H1 through NM23-H8 (Lacombe *et al*., 2000). Of these, only H1 and H2 have been reported metastasis suppressor activity (Shevde and Welch, 2003).

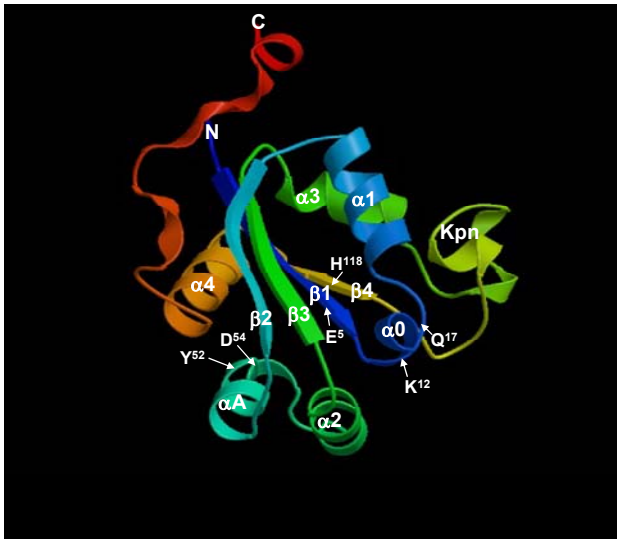
NM23-H1 and NM23-H2 are 88% identical in sequence and map 4 kb apart on chromosome 17q21-22 near the BRCA1 locus (De la Rosa *et al*., 1995). High resolution X-ray structures indicate that NM23-NDPK enzymes are hexameric consisting of subunits of 152 amino acids. These amino acids fold into four-stranded antiparallel β sheets and surrounding α helices (reviewed in Postel, 1998) (Figure 2). The Killer of prune (K-pn) mutation is located in awd (Dearolf et al., 1988) and substitutes a serine for a proline in Drosophila NDP kinase (A.Shearn, personal communication; Lascu et al., 1992). K-pn is a dominant lethal mutation (Orevi and Falk, 1975) in flies carrying the prune eye colour mutation, which suggests that the product of awd interacts with that of prune.

Figure 2. The structure representations of NM23-H1 (A) Amino acid sequence and secondary structure assignment of NM23-H1 (B) Ribbon diagram of the NDPK/NM23-H1 monomer. Helices, b-sheets, and K-pn loop are all labeled as indicated (C) Space filling representation of the hexamer of NM23-H1. The key residues studied in this project are demonstrated in both monomer and hexamer stuctures.

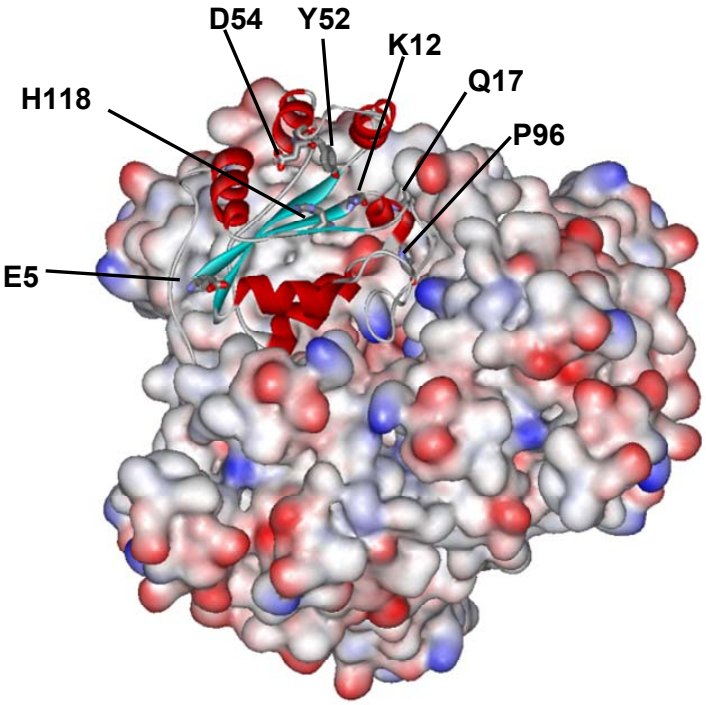
A.



B.


C
N
α3
α1
Kpn
H118
α4
β1
β4
β2
β3
E5
α0
Q17
D54
Y52
K12
αA
α2

C.


D54
Y52
K12
Q17
H118
P96
E5

NM23-H1 possesses at least three enzymatic activities, including nucleoside diphosphate kinase (NDPK), histidine kinase (hisK), and a 3'-5' exonuclease (EXO) more recently described by our lab (Ma *et al*., 2002, 2004). Mutations of P96 or S120 of H1 lacking motility suppressive capacity upon transfection are deficient in histidine-dependent protein phosphotransferase pathways *in vitro*, suggesting the hisK is linked to the suppression of metastasis (Freije *et al*., 1997). Some indirect evidence has demonstrated that the NDPK appears to be unrelated to metastasis suppression (MacDonald *et al*., 1993), but this has not been tested directly. Relevance of EXO has not been addressed. Other known 3'-5' exonucleases are closely associated with DNA repair functions and the maintenance of genomic integrity, and their loss would be expected to cause increased mutation rates, suggesting NM23-H1 may have the potential to suppress mutations required for metastasis.

1.3. 3'-5' exonucleases and DNA repair

DNA replication is a highly organized process. DNA polymerases play an essential role to maintain the integrity of the genome during this whole process. Besides their 5'-3' DNA synthesis function, many DNA polymerases (e.g. pol γ, pol δ and pol ε) contain intrinsic proofreading 3'-5' exonuclease activity. The role of 3'-5' exonuclease is to proofread for the polymerase. Thus, this enhances the accuracy of DNA synthesis and ensures the high-fidelity of DNA replication.

However, some 3'-5' exonucleases are not "polymerase-associated", and are, thus, termed "autonomous 3'-5' exonucleases" (reviewed in Shevelev and Hübscher, 2002). At least eight human autonomous 3'-5' exonucleases have been identified in mammals, including the protein TREX1, TREX2, p53, MRE11, RAD1, RAD9, APE1

and VDJP. These exonucleases are thought to maintain the accuracy of DNA replication and improve the relatively inefficient proofreading of polymerase-associated exonuclease (the error rate of DNA polymerases in DNA synthesis is 10^{-4}-10^{-5}). They may also assist exo-deficient polymerases under different cases of genotoxic stress (such as, imbalance of dNTPs, action of DNA-damaging agents). However, none of these functions has been demonstrated to date.

The spontaneous mutation rate in eukaryotic cells is low (estimated 10^{-10}-10^{-12} per cell division) because of the base pairing rules recognized by the 5'-3' catalytic function as well as the 3'-5' exonuclease proofreading function. Inactivation of any of the gene products responsible for mechanisms that limit errors following DNA replication results in an increased spontaneous mutation rate, termed the "mutator" phenotype. The *E.coli* chromosome is replicated by the pol III holoenzyme. The catalytic core of the enzyme contains the α subunit (the polymerase), the ε subunit (3'-5' exonuclease, product of the *dnaQ* gene), and the θ subunit (exonuclease stabilizer) (reviewed in Kelman and O'Donnell, 1995). The enhanced expression of *dnaQ* in wild-type *E.coli* results in about 100-fold decrease in frequency of spontaneous mutations and 10-fold the frequency of UV-induced mutations (Ciesla *et al*., 1990). These results suggest that increasing the cellular level of an autonomous 3'-5' exonuclease has an antimutator effect. Furthermore, if *dnaQ* was mutated and transferred to the chromosome, replacing the wild-type gene, the cells became inviable (Fijalkowska and Schaaper, 1996). These results demonstrated that loss of proofreading exonuclease activity in *dnaQ* is lethal due to excessive error rates (error catastrophe). Error catastrophe can occur when mutations accumulate to such an extent that the entire system breaks down, which leads to strong mutator phenotypes, cancer susceptibility and progression, and even inviability.

The 3’-5’ exonuclease activity of NM23-H1 is dependent upon the presence of Mg^{2+}, is most pronounced with single-stranded substrates or mismatched bases at the 3' terminus of double-stranded substrates, and is inhibited by both ATP and the incorporation of cordycepin, a 2'-deoxyadenosine analogue, into the 3'-terminal position (Ma, *et al*., 2004). Each of these is a common feature of other 3’-5’ EXOs described previously, suggesting the possibility of NM23-H1 functioning as an autonomous EXO. NM23-H1 possesses a Yx_1D motif in the primary sequence, revealing a strong homology to the EXO III motif found in most 3’-5’ EXOs, which is Yx_3D. Interestingly, *E.coli* NDPK retains the conventional Yx_3D form. However, the purpose of EXO activity of NM23-H1 in the cell and antimutator function remain to be established. If NM23 is identified as both a 3’-5’ exonuclease and suppressor of the mutator phenotype, that may provide the strong evidence of anti-metastasis and anti-progression of NM23 by decreasing mutation rates and thus maintaining genomic stability.

1.4. Nucleoside diphosphate kinase and histidine kinase

Until recently, nucleoside diphosphate kinase (NDPK) was the only known activity of NM23. NDPKs are ubiquitous enzymes catalyzing the transfer of phosphate groups from triphosphate to diphsophate nucleotides, thereby maintaining an appropriate balance of cellular NTP and dNTP levels.

NDP + ATP <=> NTP + ADP;

dNDP + ATP <=> dNTP + ADP,

NDPK uses energy of ATP to make triphosphates of ribonucleoside diphosphates and deoxyribonucleoside diphosphates in both *de novo* and salvage biosynthesis.

Fidelity in DNA synthesis and repair is largely dependent on a balanced supply of deoxynucleotide triphosphate (dNTP) pools. It is therefore not surprising that (d)NTP/(d)NDP pool imbalances are associated with mis-incorporation of DNA synthesis, impairment of DNA repair, and thus compromised genomic integrity and malignant growth (Ji and Mathews, 1991). Evidence shows that the mutator phenotype of *ndk* (the structural gene for NDP kinase in *E.coli*) mutant cells is a result of the dNTP imbalance (Zhang *et al*., 1996). Furthermore, ATP/ADP and GTP/GDP distribution across the cytosol is important for the control of signaling pathways and actin-based mechanistic events involved in (tumor) cell motility, growth and invasion. The ability of NDPK to supply GTP implies a role in G-protein-mediated signaling. It was reported that NDPK could serve as a guanine nucleotide exchange factor as well as a GTPase-activating protein (Zhu *et al*., 1999). Rad is the prototypic member of a new class of Ras-related GTPases. Purification of the GTPase-activating protein (GAP) for Rad revealed NM23. In the presence of ATP, GDP-Rad was reconverted to GTP-Rad by the nucleoside diphosphate (NDP) kinase activity of NM23. Simultaneously, Rad regulated NM23 by enhancing its NDP kinase activity and decreasing its autophosphorylation. The interaction of NM23 and Rad provides a potential novel mechanism for bidirectional, bimolecular regulation in which NM23 stimulates both GTP hydrolysis and GTP loading of Rad whereas Rad regulates activity of NM23.

Besides NDPK activity, histidine kinase activity is another enzymatic function of NM23-H1. Histidine protein kinases are well described in prokaryotes and lower eukaryotes, where they form the ‘two-component’ signal transduction system. The two-component regulator system is comprised of two proteins, a histidine protein kinase (sensor protein), which is usually cell membrane-bound, and a partner

response regulator (effector protein), which is in the cytoplasm and associated with an internal response. The sensor kinase, when activated by a signal, autophosphorylates a histidine residue using ATP as a phosphodonor. Subsequently, the phosphorylated sensor kinase serves as a phosphodonor to a conserved aspartate residue in the response regulator. The response regulator protein in turn transmits the signal to the target protein to elicit an adaptive response to the stimulus (Calera *et al*., 1998). The signaling pathway also includes a phosphatase that dephosphorylates the response regulator, returning it to the nonstimulated state, where it once again can respond to the signal. The phosphatase may be the histidine kinase itself, the response regulator, or a separate protein.

NM23-H1 has been reported to exhibit a histidine protein kinase activity, and this activity correlated with motility suppression (Salerno *et al*., 2003). $P_{96}S$ mutant NM23-H1-transfected MDA-MB-435 human breast carcinoma lines exhibited motility levels at or above the control transfectants, indicating that these mutations can abrogate the motility-suppressive phenotype of NM23-H1 (MacDonald *et al*., 1996). This mutation exhibited normal autophosphorylation and nucleoside-diphosphate kinase (NDPK) characteristics but deficient histidine kinase activity, suggesting that histidine-dependent protein phosphotransfer activity of NM23-H1 may be responsible for metastasis suppression effects (Freije *et al*., 1997).

Even though NM23-H1 is “sticky” and has been shown to associate with numerous proteins (Salerno *et al*., 2003), the physiological substrates of NM23 are still unknown. Kinase suppressor of Ras (KSR) was shown to interact with NM23-H1 in a manner consistent with a “two-component” signal transduction system (Hartsough *et al*., 2002). NM23-H1 co-immunoprecipitated with KSR from lysates of

transiently transfected 293T cells and in MDA-MB-435 breast carcinoma cells. Autophosphorylated recombinent NM23-H1 was also shown to phosphorylate KSR *in vitro*, with phosphoamino acid analysis identified serine as the major target residue. Phosphorylated MAPK but not total MAPK levels were reduced in an *nm23-H1* transfectant of MDA-MB-435 cells. The $P_{96}S$ kinase-deficient NM23-H1 transfectant exhibited relatively high levels of activated MAP kinase, suggesting that the histidine protein kinase activity of NM23-H1 is needed for suppression of MAP kinase activation. Taken together, one hypothesis proposes that NM23-H1 promotes a histidine protein kinase signaling cascade, results in a novel pattern of KSR phosphorylation and diminishes its ability to facilitate MAP kinase activation (Salerno *et al*., 2003).

1.5. DNA binding and transcriptional regulation

By screening of a cervical carcinoma cell complementary DNA library with a DNA fragment containing PuF binding sites, human *c-myc* transcription factor PuF was identified as NM23-H2 nucleoside diphosphate kinase (Postel *et al*., 1993). The link between NM23 and the *c-myc* oncogene suggests that the NM23 protein can function *in vitro* in the transcriptional regulation of *c-myc* expression, their alteration or removal from the promoter may be necessary for activation of the *c-myc* gene (Postel, 1998). Unlike typical DNA binding proteins that are involved in transcriptional regulation, NM23-H2 has shown a preference for single-stranded polypyrimidine-rich sequences (Hilderbrandt *et al*., 1995). *In vivo*, NM23-H2 binds to and activates the translocated *c-myc* allele in Burkitt's lymphoma cells (Ji *et al*., 1995).

Our laboratory first demonstrated that NM23-H1 binds to a silencer element in the platelet-derived growth factor (PDGF) A-chain promoter and downregulates the transcription of the PDGF A-chain gene (Ma *et al*., 2002). As a growth factor, PDGF has been implicated in many forms of human cancer (reviewed in Silver, 1992) and may play an active role in the progression of some cancers to the metastatic phenotype. For example, overexpression of PDGF B-chain confers a tumorigenic and metastatic phenotype to human T98G glioblastoma cells (Potapova *et al*., 1996), while elevated levels of PDGF A-chain are correlated with angiogenic and metastatic potential in human breast cancer cells and human melanoma cell lines (Westermark *et al*., 1986; Anan *et al*., 1996).

Our lab has demonstrated that the A-chain gene is under the strong repressive influence of multiple silencer elements, located both in the 5'-flanking sequence and in the first intron (Liu *et al*., 1996). Further study revealed that a significant component of the silencer activity could be attributed to a 31bp sequence, termed the 5'-S1 nuclease-hypersensitive, or 5'SHS element (-1418 to -1388), and another silencer to a 24 bp sequence in the first intron, designated as the intSHS. Such nuclease-sensitive structures appeared in the promoter regions of many eukaryotic genes are often correlated with the induction of cell specific transcription. Many of these nuclease-hypersensitive transcriptional elements are characterized by homopurine/homopyrimidine tracts, and the overrepresentation of these motifs in eukaryotic genomes further suggests global involvement in transcription. Screening of a HeLa cDNA expression library with the C-rich strand of a PDGF-A silencer sequence (5'SHS) yielded three cDNA clones encoding NM23-H1. Transient transfection analyses in HepG2 cells revealed that both NM23-H1 and -H2 repressed transcriptional activity driven by the PDGF-A basal promoter (-82 to +8), and another

negative regulatory region (-1853 to -883), which contains the 5'-SHS (Ma *et al*., 2002). These studies demonstrate for the first time that NM23-H1 interacts both structurally and functionally with DNA. They also indicate a role for NM23 proteins in repressing transcription of a growth factor oncogene, providing a possible molecular mechanism to explain their metastasis-suppressing effects.

Recently, NM23/NDPK was shown to bind to *p53, WT1,* and *ING1* genes, suggesting that this protein may act as antimetastatic factor by favoring their transcription. Also, it was demonstrated that NM23 binds to the promoter of *NM23-H1*, suggesting the presence of a feedback regulation system for the protein level in the cells (Cervoni *et al*., 2006). All these results suggest that NM23/NDPK DNA binding may be involved in the transcription regulation of these genes.

1.6. Project objectives

Despite extensive study, the molecular mechanisms underlying the metastasis suppressor activity of NM23 are still not clear. The overall goal of this project has been to elucidate those mechanisms with a particular focus on its H1 isoform (NM23-H1).

Our first working model proposed that NM23s facilitate assembly of functional silencer complexes (FSCs) at the 5'SHS and related intSHS elements by binding to poly-purine/poly-pyrimidine motifs within their non-B-form DNA structures, and inducing silencer-competent DNA topology. Loss of NM23 proteins during tumor progression results in derepression of PDGF A-chain gene transcription, increased

A-chain expression, and ultimately, enhanced tumor growth and metastatic potential in at least some cancers.

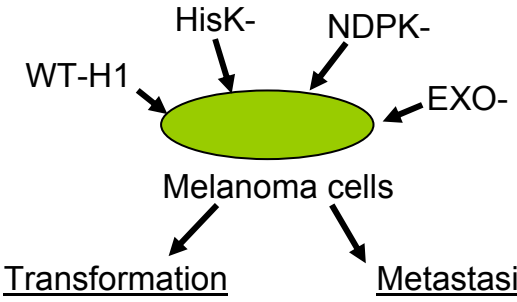
In order to test this hypothesis, two single-stranded DNA binding proteins, Purα and YB-1, were purified and EMSA was performed to identify that whether they could bind to 5'SHS silencer element and NHE (nuclease hypersensitive element) basal promoter region of PDGF A-chain as elements of FSCs. Transient transfection was conducted to demonstrate transcription regulation of each single protein and their combinations with NM23. Finally, ethylation interference footprinting was employed to localize the binding site of Purα on NHE element.

NM23 H1 was shown to possess a modest downregulation function on the PDGF A-chain gene, either on itself or with other single-stranded DNA binding proteins, suggesting this transcriptional inhibition of PDGF is very modest. At the same time, NM23-H1 was identified as a 3'-5' exonuclease (3'-5' EXO) in our lab. 3'-5' EXOs are well-accepted to play an essential role in maintaining genome integrity, with their loss resulting in an increased mutation rate. Accumulated genetic alterations are known to underlie tumorigenesis and metastasis, suggesting the novel hypothesis that the 3'-5' EXO of NM23-H1 may have potentially important implications for metastasis suppression as well. So, the alternative hypothesis is that loss of the 3'-5' EXO activity of NM23-H1 compromises genomic stability, which in turn, facilitates progression of the primary tumor cell to a metastasis-competent state and continuation of the metastatic phenotype.

To test the second hypothesis, a panel of melanoma cells was screened and a premetastatic cell line WM793 and a metastatic cell line 1205LU were chosen due to their low expression of NM23-H1 and –H2. Both cell lines were used for our

complementation system by stably transfecting wild type NM23-H1 and mutant variants, with disrupted 3'-5' EXO, NDPK, and HisK activities, back into cells respectively. Then, their metastasis suppressor activities were compared using *in vitro* models of motility and invasiveness, and *in vivo* models of metastasis, to determine which enzymatic activity contributing to the function of metastasis suppression (Figure 3). Besides, NM23 nuclear translocation and nuclear foci formation in response to DNA damage were investigated in both HepG2 and HeLa cells.

Figure 3. Schematic illustration of experimental design. In this complementation system, melanoma cell lines were stably transfected with wild type NM23-H1 and mutant variants, with disrupted 3'-5' EXO, NDPK, and HisK activities, respevtively. Metastasis suppressor activities of different transfected cells were compared using *in vitro* models of motility and invasiveness, and *in vivo* models of metastasis, to determine which enzymatic activity contributing to the function of metastasis suppression. Transformation phenotype of all the transfected cells has also been tested.



In vitro	Proliferation assay	Migration assay
	Soft agar assay	Matrigel invasion assay
		Progression assay
In vivo	Tumor explantation	Experimental metastasis
		Spontaneous metastasis

CHAPTER TWO

NM23-H1 HAS ONLY A MODEST EFFECT ON TRANSCRIPTIONAL ACTIVITY OF THE PDGF-A PROMOTER: IDENTIFICATION OF PURα AS A TRANSCRIPTIONAL ACTIVATOR VIA INTERACTION WITH SINGLE-STRANDED MOTIFS

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2.1. Introduction

Platelet-derived growth factors (PDGFs) are a family of glycoprotein dimers comprised of four constituent polypeptides, designated PDGF-A, PDGF-B, PDGF-C and PDGF-D (reviewed in Pietras *et al*., 2003). PDGF-A and PDGF-B exhibit considerable amino acid sequence homology and are encoded by distinct genes found on separate chromosomes (Bartram *et al*., 1984; Bonthron *et al*., 1988). The PDGFs possess potent mitogenic, chemotactic and angiogenic properties that contribute to normal embryonic development, cellular differentiation and wound

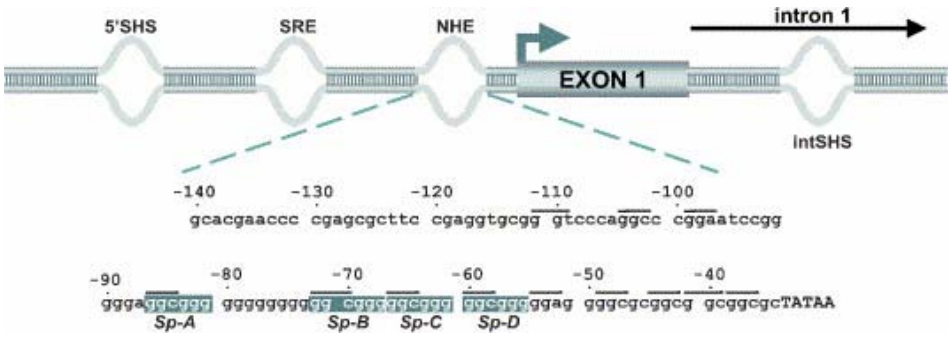
healing. PDGF expression is usually under tight repressive control but is induced by various forms of stress, such as tissue injury, inflammation, and acute exposure to mitogens (including PDGF itself). Inappropriate PDGF expression is linked to a variety of fibroproliferative disorders, with unregulated PDGF signaling representing an oncogenic stimulus in many cancers (Schilling *et al*., 1998; Shamah *et al*., 1993).

PDGF-A expression is controlled to a significant degree via regulation of its transcription rate (reviewed in Kaetzel, 2003). The PDGF-A promoter is highly GC-rich and its activity is regulated via the binding of such transcriptional regulators as the Sp/Kruppel-like factors (KLFs), early growth response gene (egr-1), Wilms tumor protein 1 (WT1), GC factor 2, and nuclear factor I-X (NFI-X) to their respective cognate response elements. The PDGF-A promoter also assumes unusual single-stranded conformations, especially in a region which has been termed the nuclease-hypersensitive element (NHE; Ma *et al*., 2002) by virtue of its hypersensitivity to single-strand-specific endonucleases (Wang *et al*., 1992). Nuclease-hypersensitive elements have also been identified in other regions of the PDGF-A gene, including two structurally similar silencer elements located upstream of the promoter (Liu *et al*., 1996) and within the first intron (Wang *et al*., 1994); these have been designated the 5′-S1-hypersensitive (5′SHS) and intSHS sequences, respectively (Figure 4). DNA binding proteins with specificity for single-stranded motifs in these promoter and silencer elements have yet to be identified.

Our previous data indicated that H1 can bind to DNA, recognizing both the 5'SHS silencer and the NHE of the *c-myc* promoter, with a high affinity for single stranded forms of elements. Thus, binding of H1 and H2 to DNA elements that mediate either enhancement or repression appears to indicate that their function is

related to the specific DNA sequence recognized. Both H1 and H2 have been shown to bind members of the ROR/RZR family of nuclear orphan receptors, suggesting a role in facilitating the docking of other transcription factors. A prominent example of other transcription factor is the PUR family (α and β) of proteins that bind single-stranded G-rich motifs. Purα was recognized as a transcription activator for several promoters, including human neurotropic JC virus (Chen *et al*., 1995), myelin basic protein, FE65, and neuronal acetylcholine receptor genes (reviewed in Gallia *et al.*, 2000). Purα can function either as a repressor or activator depending on promoter and cellular context, and has also been shown to interact with RNA (Gallia *et al.*, 2000). We proposed a model in which 5'SHS silencer activity and the promoter is mediated by the binding of single-strand specific proteins (both G-rich and C-rich strand binding proteins), which may serve to stabilize a DNA conformation required for repressive interactions of the silencer with other factors that interact with the 5'SHS silencer and the promoter of the A-chain gene.

Figure 4. Prominent nuclease-hypersensitive elements in the PDGF-A promoter and proximal flanking regions. At the top of the figure, the four nuclease-hypersensitive elements portrayed as unpaired loops are: the *5′SHS* silencer (−1438 to −1388), a serum response element (*SRE*; −477 to −468), the nuclease-hypersensitive promoter element (*NHE*; −100 to −40), and the intron SHS silencer (*intSHS*; +1605 to +1630). Expanded below the promoter region is the nucleotide sequence of the NHE, within which the boundaries of 12 consensus Purα binding sites (GGN, where N is not a G) are identified with black lines above the sequence. The four consensus Sp1 binding sites (Sp-A through Sp-D) are identified with black boxes. The TATA box is shown in bold upper case letters.



2.2. Materials and methods

2.2.1. Cell culture and transient transfection assays

Plasmids pAC261 and pACF11 have been described (Kaetzel *et al*., 1994). Transient transfections in HepG2 cells were conducted with 5×10^5 per 60 mm dish using Lipofectamine (Invitrogen). Transfection efficiency was normalized by cotransfection with RSV β-gal (1 μg), a plasmid expressing β-galactosidase (β-gal). Cells were harvested 48 h after transfection, with determination of chloramphenicol acetyltransferase (CAT) and β-gal activities conducted as described (Kaetzel *et al*., 1994). Mouse embryo fibroblast cell lines (MEFs) were derived from wild-type mice (Purα +/+) and a line harboring targeted ablation of both (Purα −/−) Purα gene copies (Khalili *et al*., 2003). Cells were transfected with pAC261-luciferase and the *Renilla* luciferase plasmid, phRL-SV40, using the liposomal reagent, Tfx-10 (Promega). After 48 h, activities of firefly and *Renilla* luciferase were assayed using the dual luciferase system as described by the manufacturer (Promega). Transfections were conducted in duplicate with three or more replicate experiments.

2.2.2. Electrophoretic mobility shift assay (EMSA)

Expression of GST-Purα in *Escherichia coli* and its purification have been described previously (Gallia *et al*., 1998). Binding reactions for EMSAs were conducted in a 20 μl volume at room temperature for 30 min. Reactions contained 20,000 cpm (20–40 fmol) of oligodeoxyribonucleotides, 100 ng of purified GST-Purα, and 1 μg of poly(dI–dC) as nonspecific competitor. Protein–DNA complexes and radiolabeled probes were resolved by electrophoresis through 6% polyacrylamide

gels in 0.5X TBE buffer (45 mM Tris borate, 1 mM EDTA, pH 8.3), with radiolabeled bands visualized by phosphorimaging (Molecular Dynamics). Oligodeoxyribonucleotide sequences employed were: 5′SHS, 5′-CTA GAG ACG TGG GGA GGG GGC CTG CAG GTG TGT-3′; PDGF-A NHE, 5′-CTA GAG GGG GCG GGG GCG GGG GCG GGG GAG GGG-3′; non-specific competitor R1, 5′-GTA CGT ACG TAC GTA CGT ACG TAC GTA CGT ACG T-3′.

2.2.3. Ethylation interference footprinting

^{32}P-endlabeled NHE (G-rich sense strand, −104 to +6) was modified with *N*-ethyl-*N*-nitrosourea as described previously (Maul et al., 1998). Binding reactions were scaled up from EMSA conditions by approximately 10-fold (200 ng of GST-Purα, 10 μg of poly (dI–dC), 0.4 pmol of probe). Protein-bound and free DNA were separated by nondenaturing electrophoresis, followed by autoradiography and electroelution of bands. Piperidine cleavage products were dried under speed vacuum and subjected to three cycles of resuspension in H_2O and drying. DNA fragments were suspended in formamide loading buffer, resolved by electrophoresis through 8% polyacrylamide sequencing gels, and visualized by phosphorimaging.

2.2.4. RNA isolation and real time PCR

Purα +/+ and Purα −/− MEFs were incubated in the presence or absence of 10% fetal bovine serum (FBS) for the times indicated in the text. One μg of total RNA was reverse transcribed using a SuperScript First strand synthesis kit for RT-PCR

(Invitrogen). DNA products from reverse transcription reactions were diluted with H_2O for measurement of PDGF-A (1:10) or 18S (1:1000) mRNAs by real-time PCR. A relative standard curve was made by diluting the cDNA product from a single sample from 1 pg to 100 ng. Analysis was conducted with the ABI Prism Sequence Detection System 7700 analyzer, with relative initial RNA concentrations determined from C_t values based on the standard curve.

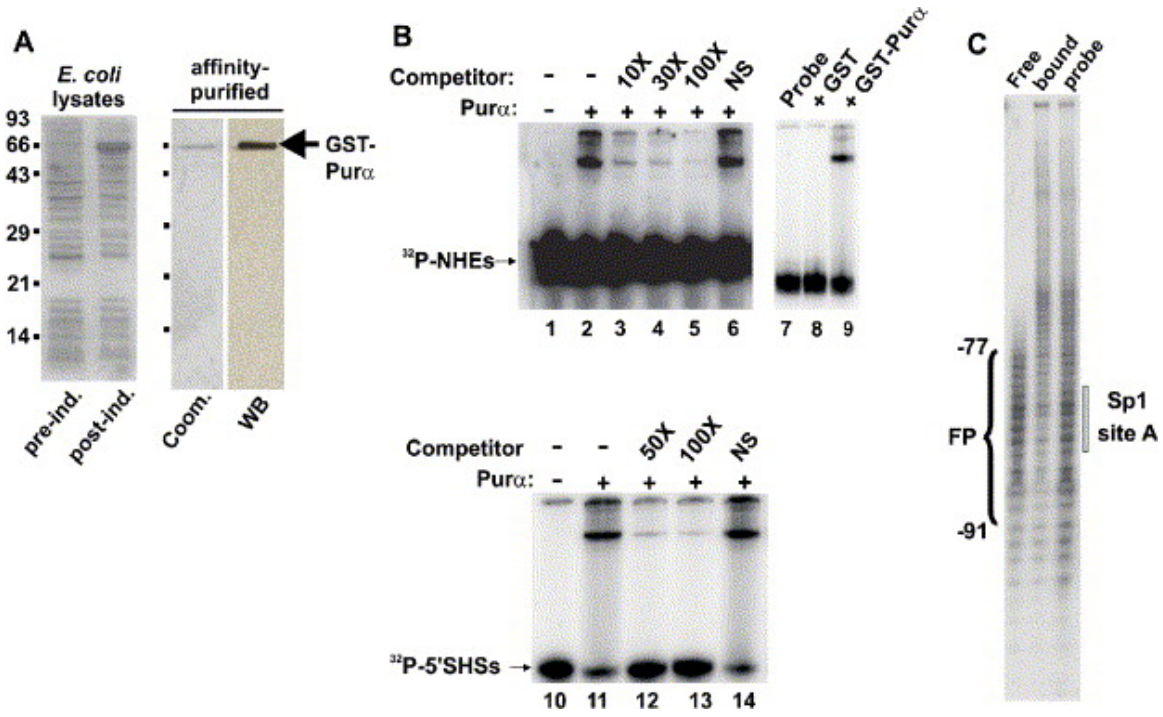
2.3. Results

2.3.1. Purα binds to G-rich strands of the 5′SHS silencer and NHE of the PDGF-A gene

The human PDGF-A gene contains at least four paranemic elements that are highly GC-rich and contain multiple GGN repeat sequences (Figure 4), consensus binding sites for Purα (Gallia *et al*., 2000). To assess binding of Purα to G-rich strands of the 5′SHS silencer (Liu *et al*., 1996) and nuclease-hypersensitive promoter element NHE (Ma *et al*., 2004), EMSAs were conducted with GST-tagged human Purα (GST-Purα). IPTG treatment resulted in strong induction of a 66 kDa band corresponding to GST-Purα (Figure 5A), which was purified using GSH-Sepharose chromatography and its identity confirmed by immunoblot analysis with anti-GST antibody (Figure 5B). Incubation of GST-Purα (100 ng) with the single-stranded, G-rich strand of the PDGF-A NHE sequence resulted in the formation of a complex (Figure 5B, lane 2) not obtained with GST polypeptide alone (Figure 5B, compare lanes 7 and 8). Unlabeled NHE efficiently displaced labeled NHE from the

complexes (IC_{50}<1 nM; Fig. 5B, lanes 3–5), indicating high affinity binding of GST-Purα to the NHE sequence. Displacement of radiolabeled 5′SHS with unlabeled homologous competitor was also efficient (lanes 11–13). Specificity of binding between GST-Purα and the NHE and 5′SHS motifs was indicated by the lack of displacement with an irrelevant DNA sequence (lanes 6 and 14).

Figure 5. Purα binds to G-rich strands of the 5′ SHS silencer and NHE with high affinity and nucleotide sequence-selectivity. (A) Expression and purification of GST-Purα. SDS-PAGE analysis of protein lysates from *E. coli* expressing recombinant GST-Purα (left), obtained before (pre-ind.) and after (post-ind.) IPTG induction. At right is the analysis of GST-Purα protein obtained by GSH-sepharose affinity purification. Purified GST-Purα was subjected to SDS-PAGE and either stained with Coomassie blue (1 μg GST-Purα, *Coom*.) or analyzed by Western blot (100 ng GST-Purα, *WB*) with anti-GST antibody. (B) EMSA of GST-Purα binding with the NHE promoter (top) and 5′SHS silencer (bottom) elements. GST-Purα (100 ng) was incubated with a ^{32}P-endlabeled oligodeoxyribonucleotide corresponding to the G-rich sense strand of the NHE element (^{32}P-*NHEs*), either in the absence (lane 2) or presence of 50- (lane 3) or 100-fold (lane 4) molar excess of unlabeled homologous competitor DNA. An irrelevant 33 nt oligodeoxyribonucleotide (*NS*, lane 5) was employed as a negative control. The same experimental paradigm was employed in the analysis of GST-Purα binding to the G-rich strand of the 5′SHS (5′*SHSs*; lanes 6–10). (C) Ethylation interference footprinting (EIF) analysis of binding between GST-Purα and the NHE. Ethylated, ^{32}P-endlabeled NHEs probe was incubated with affinity purified GST-Purα and subjected to nondenaturing electrophoresis to separate bound and free DNA. Shown are products obtained following piperidine cleavage at guanine residues of free and bound probes, and ethylated probe alone. At left are the footprint dimensions (*FP*) obtained, with the location of Sp1 site A identified at right.


A
E. coli lysates
affinity-purified
93
66
43
29
21
14
GST-Purα
pre-ind.
post-ind.
Coom.
WB
B
Competitor: - - 10X 30X 100X NS
Purα: - + + + + +
Probe
+ GST
+ GST-Purα
32P-NHEs
1 2 3 4 5 6 7 8 9
Competitor - - 50X 100X NS
Purα: - + + + +
32P-5'SHSs
10 11 12 13 14
C
Free probe
bound probe
-77
FP
-91
Sp1 site A

2.3.2. Localization of Purα binding to the PDGF-A NHE sequence by ethylation interference footprinting analysis

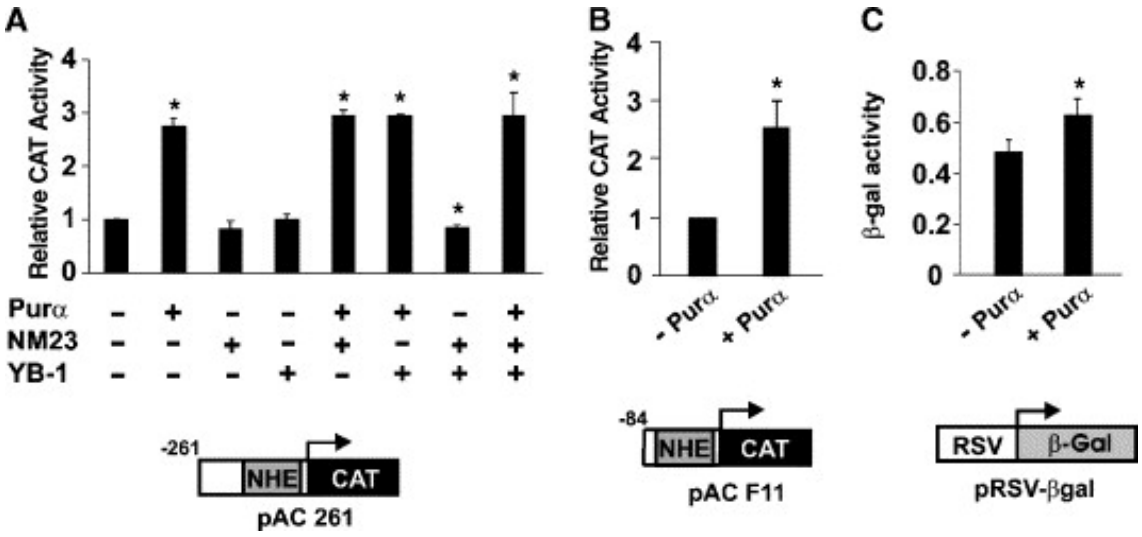
To localize nucleotide residues essential for binding of Purα to the NHE sequence, ethylation interference footprinting (EIF) was conducted. EIF permits single nucleotide resolution mapping of protein contacts with DNA, primarily at guanine residues (Büning *et al*., 1995 and Maul *et al*., 1998). A single-stranded DNA corresponding to nucleotides −104 to −6 was radiolabeled, ethylated, and incubated with purified GST-Purα. Interference with Purα-NHE complex formation was evident when residues located between −91 and −77 were ethylated, seen as a decrease in intensity of bands in the bound relative to the free lane (Figure 5C). The footprinted region contains a multitude of the GGN motifs characteristic of Purα binding sites (see Figure 4). Footprinting was most pronounced at residues −89 through −82, coinciding with two GGN repeats (GGA GGC) and one of the four consensus Sp1 sites (Sp-A) of the NHE.

2.3.3. Forced expression of Purα upregulates transcriptional activity of the NHE

Having shown Purα binds *in vitro* with high affinity and DNA sequence-selectivity to the G-rich strands of the NHE and 5′SHS elements of the PDGF-A gene, we sought to determine whether the protein could regulate the respective transcriptional activities associated with these elements. To this end, the effects of forced Purα expression on reporter constructs containing these elements were assessed by transient transfection analysis in HepG2 (human hepatoma) cells. HepG2 cells were employed as they exhibit a significant PDGF-A promoter activity

upon which either enhancement or repression could be observed (Maul *et al*., 1998). Purα expression resulted in a nearly 3-fold induction in transcription mediated by a −261 to +8 fragment of the PDGF-A promoter region, which contains the NHE (Figure 6A). A similar effect (2.5-fold) was also seen with a shorter PDGF-A promoter fragment (−84 to +8; Figure 6B) containing only the NHE and TATA motifs, localizing the region necessary for Purα induction to the NHE. Purα expression resulted in a much smaller, albeit statistically significant (1.3-fold) induction in Rous sarcoma virus promoter activity (Figure 6C), indicating the specificity of Purα-mediated induction of NHE activity. Constructs in which the 5′SHS was either relocated adjacent to the −261 promoter fragment, or studied in the context of a 1.8 kb promoter fragment, exhibited the same 2.5–3-fold enhancement to forced Purα expression (data not shown). Thus, Purα does not appear to interact functionally with the 5′SHS silencer, despite its affinity for the element *in vitro*. Induction of NHE activity by Purα dominated over the modest repressive activity of NM23-H1 (Figure 6A), an inhibitor of NHE activity in HepG2 cells (Ma *et al*., 2002). Expression of YB-1, which cooperates with Purα to activate the viral lytic control element of the human JC polyomavirus (JCV; Chen *et al*., 1995) and which binds with high affinity to the C-rich strand of the NHE in vitro (data not shown), had no effect on NHE activity alone or in combination with Purα. The ability of YB-1 to bind the NHE sequence suggests that it may be relevant in some physiological contexts, such as endothelial cells, in which the YB-1 family member DNA binding protein B (dbpB) has been shown to activate the PDGF-B promoter (Stenina *et al*., 2000).

Figure 6. Forced expression of Purα upregulates PDGF-A promoter activity. (A) HepG2 cells were transfected with the PDGF-A chain promoter-CAT reporter construct (*pAC261cat*) shown above the panel. Values (relative *CAT* activity) represent the ratio of CAT activity divided by β-galactosidase (β-gal) activity obtained by cotransfection with the control plasmid, pRSV-βgal. Cells were transfected with the various combinations of vectors driving expression of Purα, NM23-H1 and YB-1, as indicated. (B) Effects of Purα expression on activity of the −82 to +8 PDGF-A promoter subfragment. (C) Effects of Purα expression on activity of the Rous sarcoma virus enhancer-promoter. Asterisks denote means that are significantly different ($p \leq 0.05$) from that obtained with pAC261cat (panels A and B) or pRSV-βgal (panel C) alone.



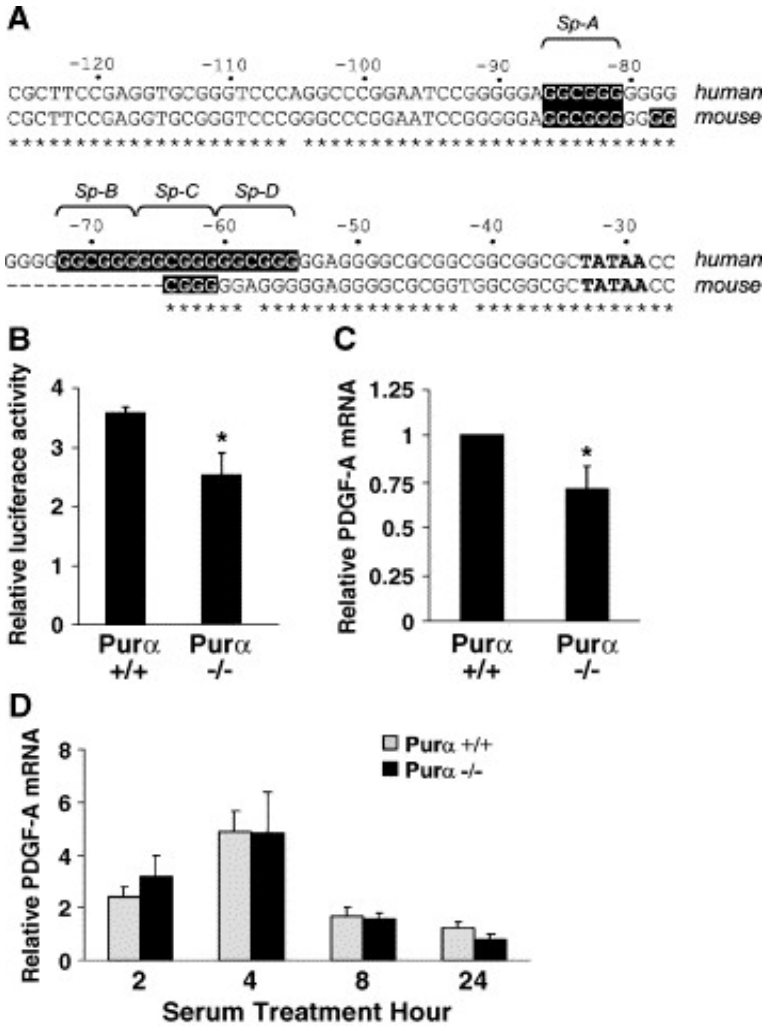
2.3.4. Purα expression is required for optimal expression of PDGF-A gene: studies in MEFs derived from Purα knockout mice

To further establish the relevance of Purα to regulation of PDGF-A gene transcription, NHE activity and PDGF-A mRNA levels were compared in embryo fibroblasts (MEFs) derived from wild-type and Purα knockout mice (Khalili *et al*., 2003). The mouse and human PDGF-A promoters are very similar in nucleotide sequence, with the mouse version also highly GC-rich and harboring at least two of the four Sp1 motifs found in the human gene (Figure 7A). Of note is the conservation of sequence in the mouse gene corresponding to −86 to −81 of the human gene, the area of strongest Purα binding identified by EIF analysis. Transcriptional activity of the −261 to +8 fragment of the human PDGF-A gene, which contains the NHE, was reduced by ~30% in MEFs derived from mice homozygous (−/−) for the Purα knockout mutation relative to the wild-type (+/+). This indicates a role for Purα in maintaining transcriptional tone of the PDGF-A promoter in fibroblasts and thus, by inference, possibly other cell types.

To confirm whether the effects of Purα expression on PDGF-A promoter activity are manifested in expression of the endogenous PDGF-A gene, steady-state concentrations of PDGF-A mRNA were measured in MEFs derived from wild-type and Purα −/− mice using real-time polymerase chain reaction (RT-PCR) analysis (Figure 7B). Following 24 h of serum-deprivation, −/− MEFs exhibited a 35% lower level of PDGF-A mRNA than wild-type MEFs. To examine whether Purα might participate in the well-documented induction of PDGF-A expression and proximal promoter activity in response to mitogen stimulation (reviewed in Kaetzel, 2003), mRNA concentrations were also measured following administration of fetal bovine

serum (FBS), a potent inducer of PDGF-A expression. In wild-type MEFs, PDGF-A mRNA levels were induced significantly by FBS within 2 h and peaked within 4 h, falling back to near baseline levels by 24 h. FBS induced mRNA concentrations in −/− MEFs to levels comparable to those seen in wild-type MEFs at 2, 4 and 8 h post-treatment. At 24 h, however, mRNA levels in the −/− MEFs fell to concentrations significantly below that of the corresponding treatment time in wild-type MEFs, consistent with their relative difference prior to FBS treatment (panel C). Thus, Purα is necessary for optimal PDGF-A expression under basal conditions, but is not required for optimal induction of the gene in response to exposure to FBS.

Figure 7. Purα is required for optimal NHE activity and expression of PDGF-A mRNA. (A) Nucleotide sequence alignment of the mouse and human PDGF-A promoters. The sequences were obtained from the human (gi 37538282_c9000-71530) and mouse (gi38081379_c148280-127560) genome databases; both were previously derived (Bonthron *et al*., 1988 and Rorsman and Betsholtz, 1992). *Sp1* motifs are identified by *shading* and *brackets*. (B) Transient transfection analysis of NHE activity in MEFs derived from wild-type (Purα +/+) and Purα knockout (Purα −/−) mice. Values represent relative luciferase activities obtained with pAC261luc, corrected for differences in transfection efficiency with the *Renilla* luciferase reporter construct phRL-SV40. (C) Steady-state concentrations of PDGF-A mRNA in MEFs from Purα +/+ and Purα −/− mice as determined by real-time polymerase chain reaction analysis. (D) Inactivation of the Purα gene does not influence induction of the murine PDGF-A gene by fetal bovine serum. Values in panels C and D are expressed relative to those obtained in untreated Purα +/+ MEFs (*fold-change*). Bars represent means±S.E.M. (n=5), with asterisks denoting significant differences (p<0.05).


A
Sp-A
-120
-110
-100
-90
-80
CGCTTCCGAGGTGCGGGTCCCAGGCCCGGAATCCGGGGGAGGCGGGGGGGG human
CGCTTCCGAGGTGCGGGTCCCGGGCCCGGAATCCGGGGGAGGCGGCGGGCC mouse
Sp-B
Sp-C
Sp-D
-70
-60
-50
-40
-30
GGGGGGCGGGGGCGGGGCGGGGGAGGGGCGCGGCGGCGGCGCTATAACC human
CGGGGGAGGGGGAGGGGCGCGGTGGCGGCGCTATAACC mouse
B
Relative luciferace activity
Purα +/+
Purα -/-
C
Relative PDGF-A mRNA
Purα +/+
Purα -/-
D
Relative PDGF-A mRNA
Purα +/+
Purα -/-
Serum Treatment Hour

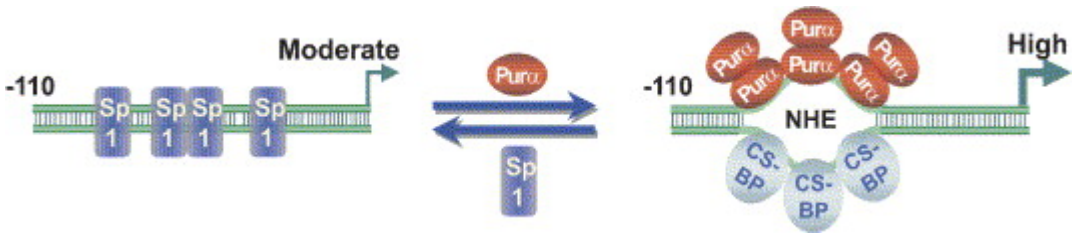
2.4. Discussion

To date, the only transcription factors shown to interact with the PDGF-A promoter, such as the positively acting Sp/Krüppel proteins and the repressive factors WT1, GCF2 and NFI-X, bind to DNA in double-stranded form (reviewed in Kaetzel, 2003). The considerable single-stranded (paranemic) character of the PDGF-A promoter, however, suggested that single-stranded DNA binding proteins might also play important roles in regulation of promoter activity. The current study has provided the novel observation that Purα, a transcription factor with an affinity for single-stranded GGN motifs, activates transcription of the PDGF-A gene via binding interactions with the G-rich strand of the promoter. The NHE region of the PDGF-A promoter contains at least 12 such GGN motifs (Figure 4), consistent with multiple potential targets for Purα interaction. A subfragment of the PDGF-A promoter (−84 to +8) containing eight of these motifs exhibits full transcriptional inducibility upon forced expression of Purα, indicating this region represents a minimal Purα response element. However, ethylation interference footprinting indicated that Purα also binds with high affinity to a region found further upstream (−91 to −77) which contains a single GGN motif, strongly suggesting these upstream sites may also be competent for Purα responsiveness. Accordingly, a comprehensive mutagenic analysis of Purα binding and transcriptional responsiveness of all 12 GGN motifs, singly and in combination, might be informative in elucidating this complex molecular interaction.

Purα mRNA is expressed in all metazoan tissues examined to date (Johnson, 2003), suggesting potential for wide-ranging roles in the regulation of PDGF-A gene transcription. The similarly ubiquitous patterns of expression for Sp1 and other Sp/Krüppel proteins suggests the PDGF-A promoter may exist in an equilibrium

between double-stranded (Sp-bound; moderate transcription) and single-stranded (Purα-bound, higher transcription) states, as shown in the model of Figure 8. This model is similar to that proposed for the myelin basic promoter (MBP) gene, where a transcriptional repressor, MyEF-2, is displaced from key response elements as Purα levels increase over the course of brain development (Muralidharan *et al*., 1997). Of additional relevance is the cell cycle-dependent pattern of Purα expression, in which nuclear levels of the protein are low in early G1 phase but increase progressively throughout S, G2, and M-phases (Itoh *et al*., 1998). This raises the possibility that Purα may play an important role in shifting the equilibrium of the PDGF-A promoter toward a single-stranded and a more transcriptionally active structure in proliferating cells, particularly at later stages of the cell cycle. In this regard, Purα is a DNA unwinding protein (Darbinian *et al*., 2001a) with the potential for both creating and stabilizing single-stranded conformations. It should also be noted that Purα may also interact with a double-stranded CCAGCA motif in the ovine placental lactogen promoter (Limesand *et al*., 2004). While such a motif is not apparent in the PDGF-A promoter, the possibility that Purα may interact with double-stranded motifs in this region cannot be formally excluded.

Figure 8. A proposed model portraying an equilibrium between the double-stranded NHE bound by Sp1 (left) and the single-stranded condition, stabilized via binding of Purα dimers and a hypothetical C-strand binding protein (*CS-BP*; right). The Sp1-bound, double-stranded state is shown to impart moderate transcriptional tone while the Purα-bound, single-stranded condition provides high transcriptional activity. The CS-BP is presented in light of a similar model described for the MCAT enhancer of the vascular smooth muscle α-actin (SMA) gene (Kelm *et al*., 1997).



Purα is a transcription-enhancing factor in the context of the PDGF-A promoter, consistent with its interactions with regulatory elements in promoters of such genes as myelin basic protein, neuron-specific FE65, and neuronal nicotinic acetylcholine receptor (nAchR). In the current study, Purα failed to augment the silencer activity of the 5′SHS element, in contrast with its high affinity for the G-rich strand of the 5′SHS sequence *in vitro*. This observation also contrasts with the silencer promoting activity of Purα in its interactions with the muscle-specific MCAT enhancer in the vascular smooth muscle α-actin gene (Kelm *et al*., 1997). The apparent lack of functional interaction between Purα and the 5′SHS silencer could be the result of competition with other silencing factors with greater affinity and/or abundance in the HepG2 cells studies.

The use of MEFs derived from Purα knockout mice yielded the observation that Purα expression was necessary for optimal expression of PDGF-A mRNA, at least in the MEF system. Close inspection of the NHE region of the mouse PDGF-A promoter reveals the presence of at least three GGN motifs within close proximity of each other (**GGA GGC** GGG GG **GGC**, analogous to −89 to −64 of the human promoter sequence), strongly suggesting this sequence is the target of Purα action in both the mouse and human. Although the loss of transcriptional activity resulting from Purα gene inactivation was statistically significant, the effect was rather modest. This is not surprising in light of the abundance of Sp1 proteins in mammalian cells, with which Purα may be competing for access to the NHE region in wild-type MEFs. Another potential attenuating factor is the aforementioned cell cycle-dependent pattern of Purα expression. The contribution of Purα to PDGF-A gene transcription and steady-state concentrations of PDGF-A mRNA might only be exerted transiently at the late stages (late S, M) of the cell cycle.

Our current model describes a competitive scenario between Purα and Sp1 for access to the NHE. However, Purα and Sp1 have also been shown to associate physically and collaborate functionally in the activation of the MB1 regulatory element of the MBP gene in mouse brain (Tretiakova *et al*., 1999). This interaction was proposed to require the phosphorylated form of Sp1, whose levels are developmentally regulated and increase over the course of brain development. Moreover, Purα and Sp1 function cooperatively to activate transcription of the *CD11c* gene during PMA-induced monocytic differentiation in U937 cells (Shelley *et al*., 2002). Thus, the potential for functional collaboration between these transcription factors at the NHE must also be considered, although an underlying mechanism that could accommodate the presence of both factors at the same site is obscure at present. One plausible notion is that phosphorylated Sp1 provides the initial interaction with double-stranded DNA, followed by recruitment of Purα via protein–protein interactions, Purα-mediated DNA unwinding, and either displacement of Sp1 or cooperative recruitment of transcriptional coactivators by both factors. The NHE is currently being employed for testing of this complex molecular model.

The demonstration that Purα is a positive transcriptional regulator of PDGF expression, the latter a long-recognized contributor to oncogenic transformation and neovascularization, represents an important addition to the known roles of Purα in cell cycle control and oncogenesis (see review, Johnson, 2003). For example, Purα associates with a number of cell cycle control proteins, including hypophosphorylated retinoblastoma protein, cyclin A (Itoh *et al*., 1998) and CDK2 in S-phase (Barr and Johnson, 2001), cyclin B1 in M-phase (Barr and Johnson, 2001), and CDK5 (Gilden and ter Meulen, 2002). Our observed enhancement of a tumor-promoting mechanism contrasts with most reports to date, however, which suggest

Purα may serve tumor-suppressor-like functions. Purα opposes growth colony formation in K-*ras* transformed cells (Barr and Johnson, 2001) and other tumor cell types (Darbinian *et al*., 2001b). An anti-oncogenic role of Purα in human cancer per se is suggested strongly by its downregulation in CML patients at diagnosis (Bruchova *et al*., 2002), and the frequent deletion of the PURA locus in myelodysplastic syndrome, a condition that often precedes AML (Lezon-Geyda *et al*., 2001). On the other hand, Purγ was recently shown to be upregulated in all members of a tumor tissue panel, suggesting at least this isoform may have tumor-promoting functions (Liu and Johnson, 2002). Addition of PDGF-A to the list of genes upregulated by Purα suggests the role of Purα in cancer may be more complex than indicated initially. Studies to further examine the potential Purα−PDGF axis of cellular transformation appear to be warranted.

Our lab's previous data showed transient transfection analyses in HepG2 cells revealed that both NM23-H1 and -H2 repressed transcriptional activity driven by the PDGF-A basal promoter (-82 to +8). Activity of the negative regulatory region (-1853 to -883), which contains the 5'-SHS, was also inhibited modestly by NM23-H1 and NM23-H2, indicating the role for NM23 proteins in repressing transcription of a growth factor oncogene, providing a possible molecular mechanism to explain their metastasis-suppressing effects. In these current experiments, we only tested a minimum transcriptional downregulation of PDGF-A chain by NM23-H1 either binding to 5'SHS (negative regulatory element) or NHE (positive basal regulatory element). Compared with the basal transcriptional regulation, there is no significant difference between two of them. When NM23-H1 cotransfected with Pura or YB-1 (single-stranded DNA binding proteins), their transcriptional regulation function on PDGF-A chain couldn't be changed by NM23-H1 overexpression. This suggests that

transcriptional downregulation of NM23 on PDGF-A chain is very weak. Considering the strong metastasis suppression of NM23, this theory of transcriptional repression of oncogene is not ideal. So, we need a new model to explain the metastasis suppressor function of NM23.

CHAPTER THREE

DNA DAMAGING AGENTS CAUSE THE ACCUMULATION OF NM23 PROTEINS IN NUCLEAR FOCI, SUGGESTING A ROLE IN DNA REPAIR

3.1. Introduction

Our current data in this project suggests that only minimum transcriptional downregulation on PDGF-A chain promoter comes from NM23-H1. Thus, the potent metastasis suppressor activity of NM23 would appear to be mediated via some other function of the molecule. A potential candidate is the 3'-5'exonuclease of NM23-H1 discovered in our lab (Ma *et al*., 2004) and nuclease of NM23-H2 (Postel *et al*., 2000). In NM23-H2, lysine-12, a phylogenetically conserved residue, was identified as the amino acid forming the covalent complex with DNA, and critical for the cleavage of the DNA phosphodiester backbone. It is also known that lysine-12 lies in the catalytic pocket, essentially involved in the NDP kinase phosphorylation reaction. These findings suggest catalysis of DNA cleavage and phosphorylation of nucleotides by NM23/NDPK share a single active site, implying a DNA repair function (Postel *et al*., 2000).

As a multifunctional enzyme, NM23 is distributed ubiquitously, though its expression may vary in a differentiation- and tissue-specific manner. The proteins are found both in the cytoplasm and in the nucleus (Rosengard *et al*., 1989). In case of stress, such as virus infection, T lymphocyte attack, or ATP depletion, cytoplasmic NM23s translocate into the nucleus alone or in combination with other proteins. NM23-H1 is predominantly localized in the cytoplasm of Epstein-Barr virus (EBV)-negative B-lymphocytes (Subramanian *et al*., 2001). However, in cells that are transfected with EBNA-3C, the viral oncoprotein of EBV, NM23-H1 signals were pronounced in the

nucleus and colocalized with EBNA-3C. In the nucleus, EBNA-3C reverses the NM23-H1 mediated suppression of tumor cell migration. These observations indicate that NM23 could sense the viral infection and translocate into the nucleus and lose its function of suppression of tumor cell migration.

Recently, NM23-H1 was found as one component of an ER-associated complex containing PP32 and the granzyme A substrates SET, HMG-2, and Ape1 (Fan *et al*., 2003). NM23 could sense the cellular changes in tumor cells and translocate into nucleus, nicking DNA and inducing caspase-independent apoptosis. When K562 cells are loading with perforin and granzyme, which are cytolytic proteins released from cytotoxic T lymphocytes (CTL) and NK cells, NM23-H1 is released from cytoplasm. Then released NM23-H1 translocates to the nucleus, and induces DNA single-stranded nicks. All these results suggest that NM23 may also function as a stress or energy sensor, sensing any cytoplasmic changes caused by virus infection, ATP depletion, or DNA damage. Translocated NM23s will either repair damaged DNA, or induce apoptosis if the DNA damage is impossible to be repaired.

To obtain direct evidence regarding the translocation of NM23 proteins into nucleus on DNA damage, two tumor cell lines, HepG2 and HeLa, were used and treated with the DNA damaging agents, cisplatin and etoposide. Western blot and immunofluorescence were employed to measure the localization and compartmentation of NM23. Also, co-localization of NM23 with Ape1 was investigated after cells were treated with DNA damaging agents.

3.2. Materials and methods

3.2.1. Cell culture and preparation of nuclear extract

HepG2, HeLa cells were grown at 37ºC under 5% CO_2 in DMEM medium supplemented with 5% fetal bovine serum (FBS) and 2 mM glutamine. Cells grown to 80% confluence were used for fractionation. Each cell line was treated with cisplatin (15 μg/ml) and etoposide (15 μg/ml) for 2 h, 4 h, and 24 h. DMSO treatment was used as control. After cells were treated for different time period, the growth medium was aspirated; the plate was rinsed 2 times with 5 ml ice-cold PBS and cells removed with a teflon-coated scraper (Costar). Cell nuclear extracts were prepared as described previously (Pines *et al*., 2005). Briefly, 10^7 cells were washed once with phosphate-buffered saline (PBS) and resuspended in 100 μl of hypotonic lysis buffer A [10 mM HEPES, 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9]. After 10 min, cells were homogenized by 10 strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation at 500 g at 4ºC for 5 min in a microcentrifuge. The supernatant was considered as the cytoplasmic fraction. Nuclei were then washed three times with the same volumes of buffer A in order to minimize cytoplasmic contamination. Nuclear proteins were extracted with 100 μl of buffer B (10 mM HEPES, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 0.5 mM PMSF, pH 7.9). After incubating for 30 min at 4ºC, samples were centrifuged at 12,000 g for 20 min at 4ºC. Nuclear extracts were then analysed for protein concentration by Bradford assay (Bio-rad) and stored at -80ºC in aliquots.

3.2.2. Western blot

Protein (40–50 mg/lane) was mixed with loading buffer and separated by 15% SDS–PAGE. Protein was transferred to membrane by semidry transfer apparutus.

Following fixation of proteins, the membrane was wetted in methanol, rinsed in distilled water, and blocked in blocking buffer containing 5% dry nonfat milk for overnight, NM23s was detected using a polyclonal rabbit anti-NM23 antibody (Santa Cruz) at a final dilution of 1:100 (final concentration 2 μg/ml) for 1 h. Membranes were then incubated with buffer containing 1:10,000 diluted goat anti-rabbit secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. Bound secondary antibodies were detected using chemiluminescence for 5 min.

3.2.3. Immunofluorescence staining

HeLa Cells ($1x10^4$) were grown on 8-chamber, polystyrene vessel tissue culture glass slide (BD) and treated with etoposide and cisplantin for different time period. Cells were washed three times with PBS and fixed with 4% formaldehyde for 10 min. Then cells were permeabilized with 0.3% Triton X-100 for 10 min and then preincubated with 10% goat serum for 2 h. Cells were incubated with an anti-NM23 (Santa Cruz) polyclonal antibody overnight. Primary antibody labeling was visualized by incubation with anti-rabbit IgG-FITC (Santa Cruz, 1:100) for 60 min. 4',6'-diamidino-2-phenylindole (DAPI) (Sigma) was used for nuclear conunterstaining. Prepared slides were then analyzed by fluorescent microscopy. For co-localization, anti-Ape1 antibody was used as another primary antibody, and goat-anti-mouse-Texas Red was used as secondary antibody.

3.3. Results

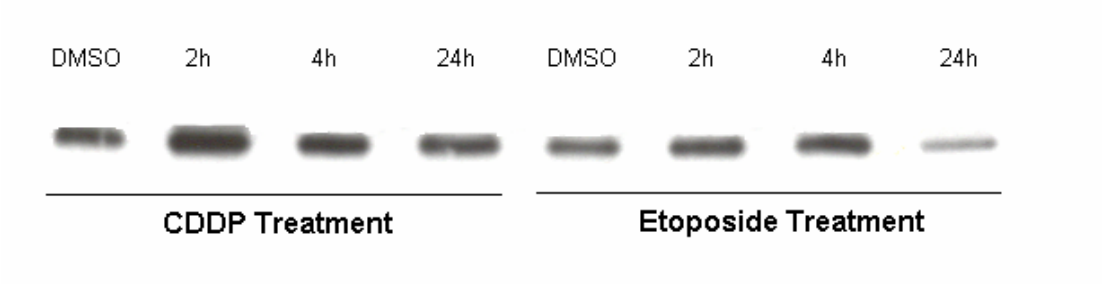
3.3.1. NM23 translocates into the nucleus following treatment with DNA damaging

agents

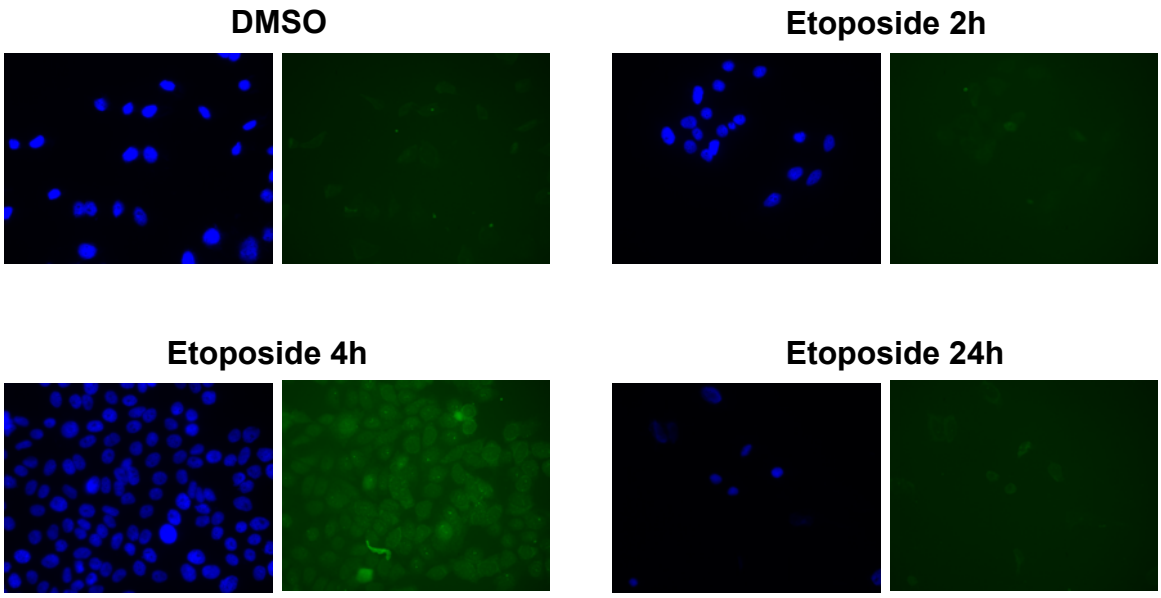
To examine whether NM23 has a role in the cellular response to DNA damage, we probed its intracellular localization by western blot and indirect immunofluorescence. After HeLa cells were exposured to cisplatin and etoposide for different time course, nuclear extract was obtained and western blot analysis was performed. Both anti-cancer reagents induced nuclear translocation of NM23 compared with untreated cells (DMSO control). It seems that cisplatin induces nuclear tanslocation of NM23 faster (peak time is at 2 hour of treatment) than etoposide (peak time is at 4 hour of treatment) (Figure 9A). To verify the results from western blot of nuclear extract, indirect immunofluorescence and confocal analysis were performed on both cell lines treated by etoposide. Immunofluorescence microscopy (Figure 9B) and confocal microscopy (Figure 9C) results are all consistent with western blot with peak time of nuclear NM23 increasing at 4 h of treatment. Obviously, increase of nuclear NM23 is after its increase in the cytoplasm, suggesting that both neosynthesis and nuclear translocation of NM23 take place at the time of DNA damage.

Figure 9. Cisplatin- and etoposide-induced expression of NM23 and translocation from the cytoplasm to the nucleus. (A) Cells were cultured in DMEM and treated with cisplatin and etoposide (15 μg/ml) for the indicated times. Western blot analysis, performed as described in materials and methods, of NM23 expression levels of nuclear extract is indicated. DMSO, solvent for cisplantin and etoposide, was used as control treatment. (B) Hela cells were grown on 8-well chamber slides and treated with etoposide (15 μg/ml) for the indicated times. Cells were then fixed, permeabilized, and stained for NM23 (green). Nuclei were conterstained by DAPI (blue). Immunofluorescence microscopy was used for visualization. (C) HepG2 cells were grown on 8-well chamber slides and treated with etoposide (15 μg/ml) for the indicated times. Cells were then fixed, permeabilized, and stained for NM23. Confocal microscopy was used for image analysis.

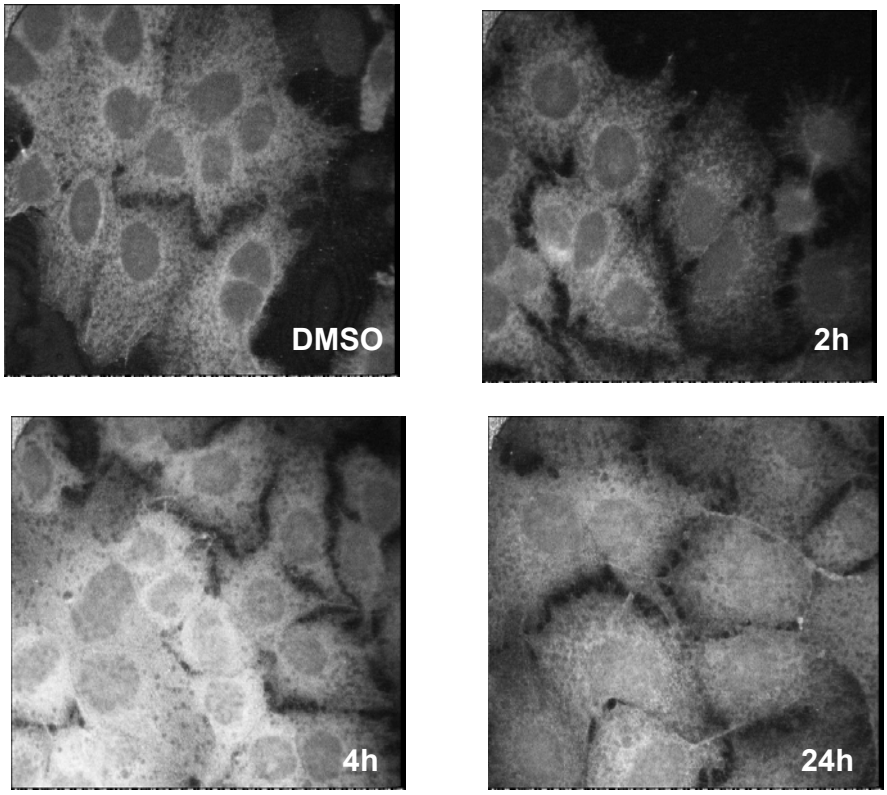
A.



B.



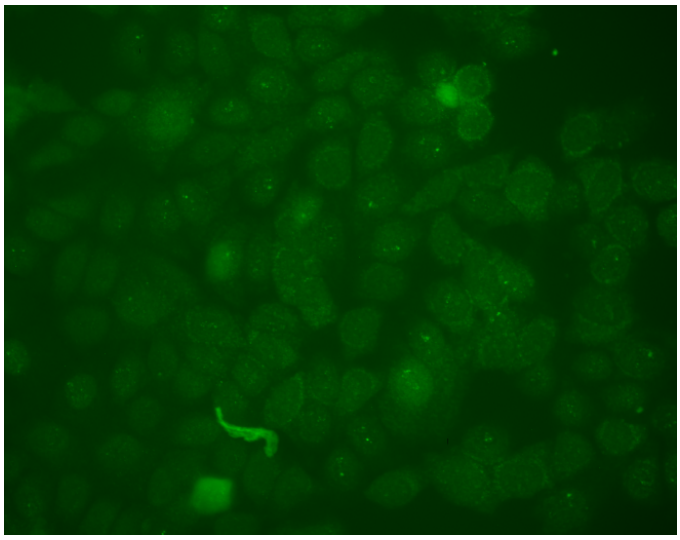
C.

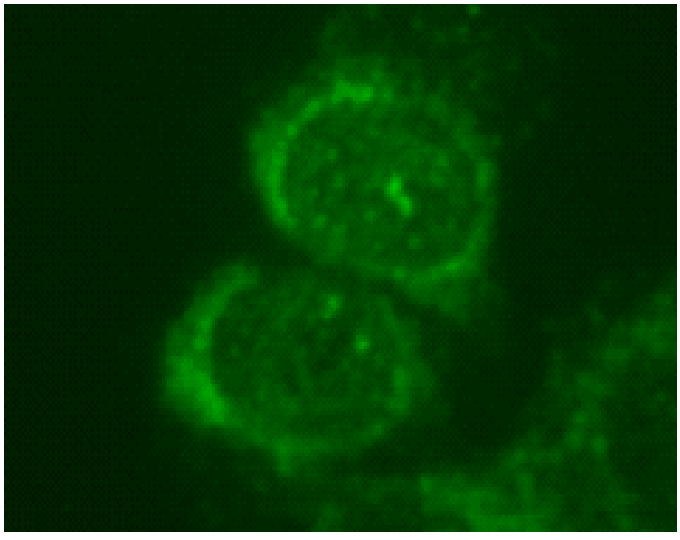


3.3.2. DNA damaging agents induces the concentration of NM23 within nuclear foci

Inhibition of the topoisomerase II function can result in DNA double-strand breaks (DSBs) and, thus, lead to chromosomal translocations. DSBs must be recognized and be able to promote the recruitment of some DNA repair proteins to the damaged sites in order to initiate repair (Kantidze *et al*., 2006). To clarify the question we have checked whether the exposure of cells to etoposide triggers the formation of NM23 nuclear foci, which so far have been taken as an indicator for the presence of DNA repair (Raderschall *et al*., 1999 and Robinson *et al*., 2005). In untreated HeLa cells, NM23 showed diffuse staining of the cytoplasm and nuclei (Figure 9C, DMSO treatment). After exposure to DNA damage reagents etoposide for 4h and 24 h, NM23s relocalized to distinct nuclear foci (Figure 10). We propose that these foci represent sites of processing of DNA double-strand breaks (DSBs).

Figure 10. Etoposide induces nuclear foci formation of NM23. HeLa cells were grown on 8-well chamber slides and treated with etoposide (15 μg/ml). Cells were fixed, permeabilized, and stained with anti-NM23 polycolonal antibody (Sigma, 1:100 dilution) and FITC conjugated secondary antibody. Immunofluorescence microscopy was used for visualization.

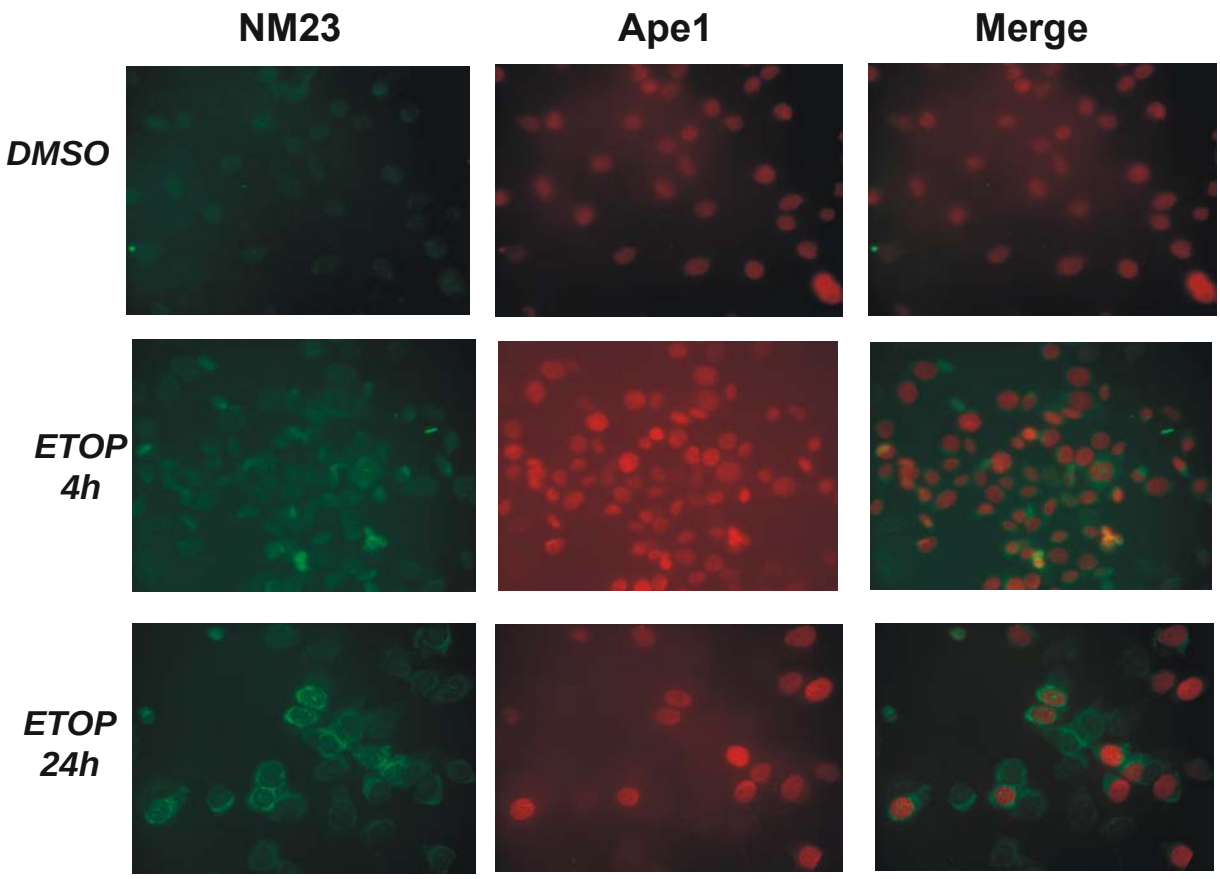




3.3.3. NM23 does not co-localize with Ape1 in response to DNA damage

To further investigate the molecular relationship between NM23 and Ape1 after DNA damage, we examined whether NM23s co-localized with Ape1, which has been reported to co-translocate into nucleus with NM23s in response to Granzyme A loading or CTL attack (Fan *et al*., 2003). Ape1 is Apurinic apyrimidinic endonuclease redox effector factor-1 (APE1/Ref-1) involved both in the base excision repair (BER) of DNA lesions and in the eukaryotic transcriptional regulation (Pines *et al*., 2005). After cells were treated with etoposide for 4 h and 24 h, cells were stained with anti-NM23 (green) and Ape1/Ref-1 (red). Both protein expressions were induced by etoposide treatment, and nuclear foci were formed at 4 h and 24 h treatment. In contrast to previous report that both proteins are in the cytoplasm, most Ape1 was found in the nucleus even before the etoposide treatment. Four hour treatment induced more Ape1 expression in the nucleus and translocation of NM23 only. But NM23 foci do not co-localize with Ape1 foci. After 24 h of treatment, even though NM23 expression are still high in the nucleus and foci are very significant, Ape1 staining and foci are lost in some cells which still contain NM23 foci (Figure 11). All these data suggest that in response to different DNA damage, NM23 and Ape1 might react differently. They may localize to their own binding sites to initiate DNA repair and function for different time periods.

Figure 11. Etoposide induces NM23 and Ape1 foci that do not co-localize. HeLa cells treated with etoposide for indicated time. After fixation, cells were stained and examined by immunofluorescence microscopy. NM23 was visualized with FITC-conjugated antibody (green) and Ape1 was visualized using a Texas red (TR)-conjugated secondary antibody (red). No colocalization (yellow) was seen when images of NM23 (green) and Ape1 (red) were merged. FITC is shown at left, TR staining red in the middle, and the merged image at right.


NM23
Ape1
Merge
DMSO
ETOP 4h
ETOP 24h

3.4. Discussion

Etoposide and cisplatin are both clinically widely used anti-cancer drugs. Cisplatin is used in the treatment of several types of cancer and is particularly effective against testicular tumors. Cisplatin introduces cytotoxic DNA damage predominantly in the form of intrastrand crosslinks between adjacent purines (Yamada *et al*., 1997). Etoposide specifically inhibits topoisomerase II (Topo II). It acts after cleavage of DNA by Topo II, leaving a lesion in which the enzyme is covalently linked to 5'-ends whereas 3' termini are free. These lesions likely develop into double-strand breaks and single-strand DNA gaps. As a consequence, etoposide triggers checkpoint activation (Rossi *et al*., 2006). Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosome segregation are completed with high fidelity. In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair (reviewed in Elledge, 1996; reviewed in Weinert, 1998).

One hallmark of the DNA damage response is the aggregation of multiprotein complexes into foci or repair centers. A large amount of evidence showed that many DNA repair proteins, such as P53 (Al Rashid *et al*., 2005), PCNA (Essers *et al*., 2005), Rad 51 (Tarsounas *et al*., 2003), MRN complex (Mre11, Rad50 and NBS1) (Stracker *et al*., 2002), accumulate at the DNA damage site to form nuclear foci. NM23s have been reported to be distributed broadly in the cell in a differentiation and tissue specific manner. The proteins are found in the cytoplasm, in mitochondria, and in the nucleus. Our western blot and immunoflurescence results suggested that NM23s mostly exist in the cytoplasm. If NM23s play an important role in DNA repair, they should go into nucleus and accumulate at the DNA damage site, either alone or with other DNA

repair proteins. In the current study, as shown in western blot, NM23 expression is induced dramatically in two tumor cell line (HepG2, HeLa) in response to etoposide and cisplatin treatment. Cisplatin elicits peak translocation within 2 h of treatment, while etoposide induces nuclear translocation peak at 4 h of treatment, suggesting different repair responses induced by two different chemical reagents. But both treatments cause NM23 expression returning to near baseline levels by 24 h. Immunofluorescence studies in Hela cells confirm the immunoblot results and demonstrate increased expression and nuclear translocation of NM23 during periods of genomic stress. Furthermore, distinct nuclear foci were demonstrated in the cells following exposure to DNA damaging agents. Taken together, these results strongly suggest the DNA repair function of NM23s in the DNA damage response.

More studies are actively underway to investigate the potential role of NM23 in DNA repair and physical interactions between H1 and other repair proteins. More DNA damaging treatments are employed in our lab to explore the repair mechanisms of NM23. In fact, we have observed that UV irradiation appears to induce NM23-H1 expression and localization in nuclear foci to an even greater extent than etoposide and cispatin. Moreover, NM23-H1 containing foci appear to colocalize with Rad1, a component of the 9-1-1 complex (Rad9, Rad1, and Hus1) implicated DNA damage induced by UV and other agents.

NM23 and Ape1 have been described as two components of the SET complex in the cytoplasm (Fan *et al*., 2003). Other reports have described that Ape1 is found in the cytoplasm at a significant level when determined by immunocytochemistry with anti-Ape1 antibodies (Ramana *et al*., 1998 and Kakolyris *et al*., 1999). Our results are consistent with the earlier report (Takao *et al*., 1998), in that Ape1 molecules were exclusively present in the nuclei. Four hour of etoposide treatment induces

significantly increased expression of Ape1 in the nucleus, and those foci formed after etoposide treatment seem to be much larger than NM23 foci in the same cells. Almost no NM23 foci and Ape1 foci colocalize. At the time point of 24 h treatment, some cells lost staining of Ape1, but NM23 foci are still contained in the nucleus, suggesting different functioning time in the process of DNA repair. All these data suggest that, even though the NM23, as an exonuclease, and Ape1, as an endonuclease, participate DNA repair, their interaction may be limited to responses to specific types of lesions.

The isoform of NM23s in this current study could not be determined because the antibodies used in immunoblot and immunofluorescence studies cross-react to both NM23-H1 and H2. More specific anti-NM23-H1 antibody should be used in order to determine whether the DNA repair function is more related to NM23-H1.

While DNA repair is an obvious potential function of the 3'-5' EXO activity, another outcome of cells after genomic insult, apoptosis, has to be considered. In this regard, the study about the relationship of NM23 and apoptosis has been performed to investigate complementary function of NM23 in our lab.

CHAPTER FOUR

THE NDPK AND, POSSIBLY, 3'-5'EXO ACTIVITIES OF NM23-H1 PLAY IMPORTANT ROLES IN SUPPRESSING PROGRESSION OF HUMAN MELANOMA CELLS TO A METASTATIC PHENOTYPE

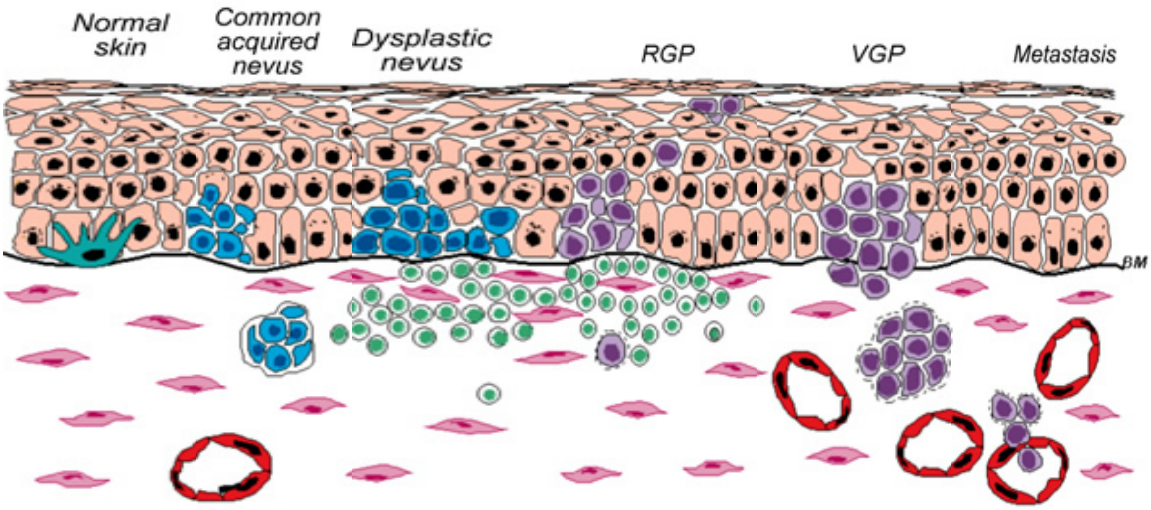
4.1. Introduction

Once the hypothesis of DNA repair function of NM23 EXO activities has been tested, we will test for the first time the extent to which exonuclease activities, and by extension, the repair activities of H1 are required for the ability to suppress tumor metastasis. In the cell culture models for studying antimetastatic activities of NM23 proteins, melanoma is a highly suitable cell to establish the study model. Because, first, the metastasis suppressor function of NM23-H1 was identified in melanoma cells (Steeg *et al*., 1988). Second, melanoma is highly metastatic compared with another two skin cancers, basal cell cancer and squamous cell cancer. Although representing only approximately 4% of skin cancers, melanoma accounts for approximately 79% of skin cancer deaths, with an annual mortality of 2.3 per 100,000 people. Third, clinical data indicates a reverse correlation between NM23 expression in primary melanomas and rate of progression to metastasis (Florenes *et al*., 1992). Last, there is the availability of a panel of melanoma cell lines, developed and characterized in the laboratory of M.Herlyn (Wistar Inst), that represent different stages of progression (Herlyn *et al*., 1985) and can be used for the study of NM23 in impairing progression of melanoma cells and suppression of metastasis.

Clinical and histologic studies have resulted in defining five major steps of melanoma development and progression (Figure 12). Step 1, common acquired and congenital nevi with structurally normal melanocytes; step 2, dysplastic nevi with

structural and architectural atypia; step 3, RGP (radial growth phase), nontumorigenic primary melanomas without metastatic competence, corresponding to this early stage of horizontally spreading growth in the epidermal and papillary dermal layers of skin; step 4, VGP (vertical growth phase), tumorigenic primary melanomas with competence for metastasis, representing a more progressed and invasive stage characterized by aggressive downward invasion into dermal layer and beyond; and step 5, metastatic melanoma, which were derived either from metastatic lesions or by repeated passaging of tumor explants of prematastatic lines in rodents (Herlyn *et al*., 1985). It is now appreciated that transformation of melanocytes into malignant melanoma involves the interplay between genetic factors, UV exposure, and the tumor microenvironment.

Figure 12. Process of melanoma development from normal melanocytes. Nevi are characterized by aberrant cell growth, consisting of enlarged, coalescent nests of nevocytes, which display different degrees of cytologic dysplasia. Further progression results in malignant cells, which grow only within or in close proximity to the epidermis (radial growth phase; RGP). Eventually, cells acquire the ability to invade deeply into dermis (vertical growth phase; VGP) and then into lymphatics and blood vessels, leading to systemic dissemination (metastatic melanoma). BM, basement membrane (Graphic is from http:// www.wistar.upenn.edu/herlyn/default).



Over 18 years of intensive study, multiple enzymatic activities of NM23-H1 have been uncovered. But the molecular mechanisms underlying its anti-metastatic activity remains controversial. To date, the strongest case for metastasis-suppressing activity has been made for the hisK of NM23-H1. Some evidence demonstrated that hisK null mutants of NM23-H1 (such as $P_{96}S$ and $S_{120}G$) lose some of the motility-suppressing activity of the wild-type protein when overexpressed in MDA-MB-435 breast carcinoma cells (Freije *et al*., 1997; Wagner *et al*., 1997). However, definitive validation of the role played by the hisK awaits detailed characterization of relevant downstream hisK substrates and their relationships to metastasis suppression. Other data indicated that there is no correlation between NDPK activity and suppression of tumor metastatic potential among control and NM23-H1 transfected murine melanoma cells (MacDonald *et al*., 1993). This has yet to be demonstrated using the forced expression model used above due to lethality associated with overexpression of NDPK mutants.

3'-5' exonuclease activity is a newly identified biochemical function of NM23-H1 in our lab (Ma *et al*., 2004). This activity is very intriguing in light of the association of these enzymes with DNA repair processes, and the mutator phenotype which often arises as a consequence of their deficiencies (reviewed in Shevelev and Hübescher, 2002). To overcome numerous barriers in the whole metastasis process, tumor cells must acquire enough genetic alterations which are usually caused by accumulation of mutations. So, a primary working hypothesis of this current project is that 3'-5' EXO activity plays a major role in metastasis suppressor function of NM23-H1 molecule. Loss of NM23 could lead to accumulation of mutation and thus progression to the metastatic phenotype. This hypothesis does not exclude contributions from NDPK and hisK activities of NM23-H1 to the metastatic process. In fact, this highly selective and

low efficient process could be completed by combination of multiple functions of metastasis suppressor.

To study the relevance of EXO or possibly other enzymatic activities to metastasis suppression, two melanoma cell lines were selected because of their abnormally low amount of NM23-H1 and NM23-H2 expression. WM793, a VGP cell line, represents a premetastatic cell line, while 1205LU, a MET cell line, represents metastatic cell line. After stable transfection and fluorescence-activated cell sorting, all the transfected cell lines are expressing equal amount of wild-type or variant mutant NM23-H1, including $H_{118}F$ mutant which was previously reported lethal to cells, was successfully transfected into both 1205LU and WM793 cells. These cell lines were used to measure the metastatic characteristics both in cell culture (motility assay, invasion assay) and *in vivo*, using standard approaches of experimental and spontaneous metastasis. Relevance of an enzymatic activity to suppression of metastasis should be apparent through a significant reduction in the antimetastatic activity of its corresponding mutant. The relevance of enzymatic activities of NM23-H1 to prevention of tumor progression in premetastatic WM793 cells was also tested.

Motility assay and invasion assay are most widely used *in vitro* methods to study cell migration and the effect of various chemoattractants. These assays are largely based on the use of Boyden chambers or Transwell culture inserts in which porous membranes separate seeded cells from a chemotactic factor supplied in the medium in the lower chamber (de la Monte *et al*., 2002). Matrigel is considered as *in vitro* counterpart of basement membrane. It has been used to characterize involvement of ECM receptors and matrix degrading enzymes which play roles in tumor progression. But *in vitro* assays of invasiveness have been less than perfect. Animal models provide a more clinically accurate mechanism to study metastasis. Spontaneous

metastasis assay in nude mice takes about 4 months to complete. It recapitulates all the metastatic steps; therefore it's the best study model even though it is time consuming. Experimental metastasis takes only 1 month. But this assay skips many early steps of metastasis and starts from survival of cells in blood stream (see Figure 1).

Based on definition, metastasis suppressor genes differ from tumor suppressor genes by which transfection of metastasis suppressor gene back to cells has no effect on cell proliferation, colony formation in soft agar, and primary tumor growth. Cell proliferation rate was determined by MTS assay in the current project. It's very important to know the growth rate of all parent cells and transfected cells. Because the tested cells stayed in animal model in such a long time, similar growth rate of all the cell lines won't have any effect on metastasis assay.

4.2. Materials and methods

4.2.1. Cell culture, whole cell extract, and western blot

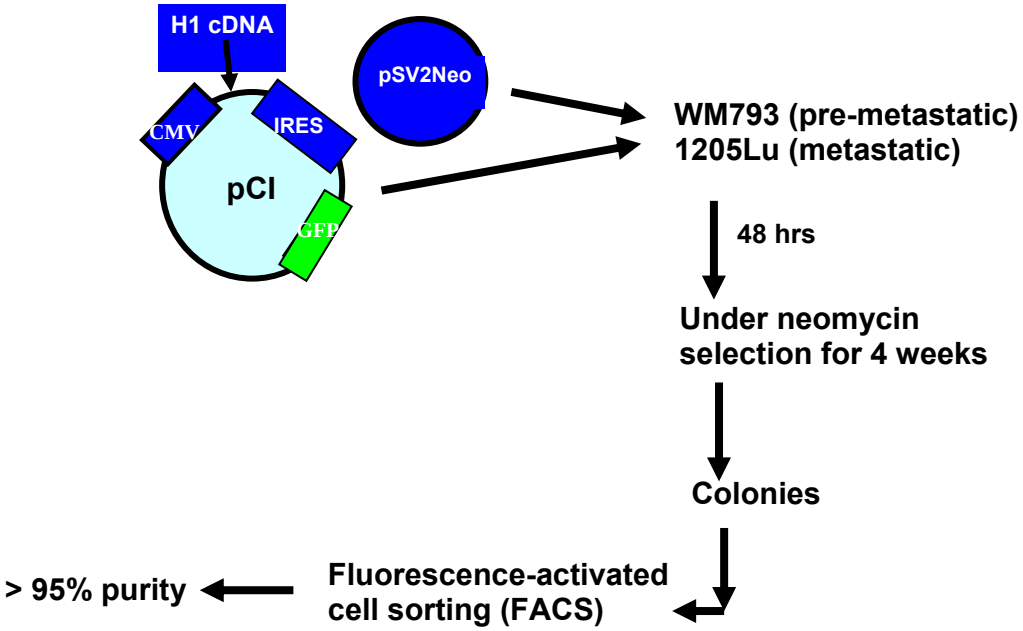
Melanoma cell lines were cultured in MCDB153/L15 (Sigma) medium (v/v: 4/1) supplemented with $CaCl_2$ (2 mM), insulin (5 mg/ml) and 2% fetal bovine serum (FBS) (Gibco). At confluence, cells were centrifuged at 500 g for 5 min at 4ºC and supernatant was discarded. The cell pellet was loosened by gently vortexing for 5 s followed by addition of equal volume of M-Per (Pierce) with vigorous shaking. After incubation in 4ºC overnight, an aliquot was examined under a microscope to ensure that cells had uniformly lysed. The supernatant was aspirated after spinning for 30 min at maximum speed at 4ºC. The protein concentration was measured by Bradford assay (BioRad). Protein (40-50 mg/lane) was mixed with loading buffer and

separated by 15% SDS-PAGE. Protein was transferred to a nitrocellulose membrane by a semidry transfer apparatus. NM23s was detected using a polycolonal anti-NDPK antibody (Lab vision) at a final dilution of 1:500 for 1 h, and goat anti-rabbit secondary antibody conjugated to HRP for 1 h at room temperature. Bound secondary antibodies were detected using chemiluminescence (Amersham) for 5 min.

4.2.2. Plasmid construction, stable transfection, and flow cytometry

Wild-type and variant mutant NM23-H1 cDNA were cloned into the pCI vector. Other features in this vector include CMV promoter, green fluorescence protein (GFP) cDNA, and an internal ribosome entry sequence (IRES) interposed between the cDNAs to permit cotranslation of NM23-H1 and GFP from a bicistronic mRNA. Melanoma cells were seeded in six-well plates with 2 x 10^5 cells/well. Cells at a confluence of 70% were incubated with DNA (wt or mutant NM23-H1 cDNA) and Fugene 6 (Roche) complex (3:1 ratio) for 48 h at 37ºC. pSV2Neo was cotransfected as a selection marker. After 48 h incubation, cells were split in a 1:4 ratio to 100 mm dishes. Twenty-four later, geneticin (G418, Life technologies; 250 µg/ml) was added for selection. After 4 weeks selection, all the cell colones were trypsinized and pooled together. Cells were sorted by fluorescence-activated cell sorting (FACS) using a FACS Calibur flow cytometer. The sorting procedure has yielded more than 95% GFP fluorescent cells (Figure 13).

Figure 13. Procedures of stable transfection of melanoma cells.



4.2.3. Two-dimentional gel electrophoresis

Materials for the first and second dimension were obtained from Bio-Rad. Approximately 50 μg of purified proteins and whole cell extracts from transfected 1205Lu cells were loaded in 350 μl of rehydration buffer containing 8 M urea, 4% CHAPS, 100mM DTT, 0.2% Bio-lytes, and 0.001% bromophenol blue. ReadyStrip IPG strips (17 cm, PH 4-7) were placed side down into the rehydration buffer in a tray channel with 3 ml of mineral oil applied on the top. Rehydration was carried out for 12 h at 50 volts. The first dimension of electrophoresis was run in a Protean IEF cell, according to the manufacturer's instructions. To solubilize focused proteins and allow SDS binding, IPG strips were equilibrated prior to second dimension electrophoresis with 375 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 2% DTT, 20% glycerol for 10 min to reduce sulfhydryl groups, followed by 375 mM Tris–HCl, pH 8.8, 6 M urea, 2% SDS, 2% iodoacetamide, 20% glycerol for an additional 10 min period to alkylate sulfhydryl groups. Second dimension electrophoresis was performed in 15% acrylamide gels. Then western blot was performed as previously described.

4.2.4. Motility assay

Transwell 24-well plates (6.5 mm diameter, 8.0 μm pore size) (Corning Inc, Life Sciences) were incubated at 37ºC overnight before $1x10^4$ melanoma cells (in 0.1 ml of medium) were plated in the upper compartment. The lower compartment was loaded with 0.6 ml of medium plus 10% FBS as chemoattractant. After 24 h of incubation at 37ºC, non-invading cells in the upper compartment were removed with a cotton swab. The microporous membrane was fixed in 70% ethanol for 1 h and cells reaching the membrane surface of the lower compartment were stained with

hematoxylin for 2 h. Stained cells were counted using an inverted light microscope. Five random high power fields (10x10) were counted to get an average number for one well. The experiments were repeated in triplicate wells for at least three times.

4.2.5. MTS assay

Cell viability was assessed with the nonradioactive cell proliferation 3-(4,5-dimethyl-thiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay using CellTiter96 AQ_{ueous} Reagent (Promega) as described by the manufacturer. Briefly, cells were cultured in 96-well plates at a concentration of 2 x 10^3/well. On day 1, day 2, day 3, day 4, and day 5, 20 µL of CellTiter96 AQ_{ueous} Reagent (MTS/PMS mixture solution) were added into each well of 96-well plate containing 100 µL of cells in culture medium. Then the plate was incubated for 1 hour at 37°C, 5% CO_2, and formazan absorbance was measured at 490 nm on a ELISA plate reader. Each measurement was done in triplicate and the mean value was calculated.

4.2.6. Soft agar assay

For anchorage-independent growth, $5x10^4$ parent and transfected cells were resuspended in 3 ml of growth medium containing 0.3% Nobel agar and plated on each well of 6-well plate containing a solidified bottom layer made of 1% Nobel agar in medium. After the 0.3% Nobel agar solidified, 2 ml of growth medium was added to the plates and replaced every 3 days. 28 days after plating, colonies were stained with crystal violet (0.005%) and imaged at 10X magnification. Each experiment was performed in triplicate and the mean value was calculated.

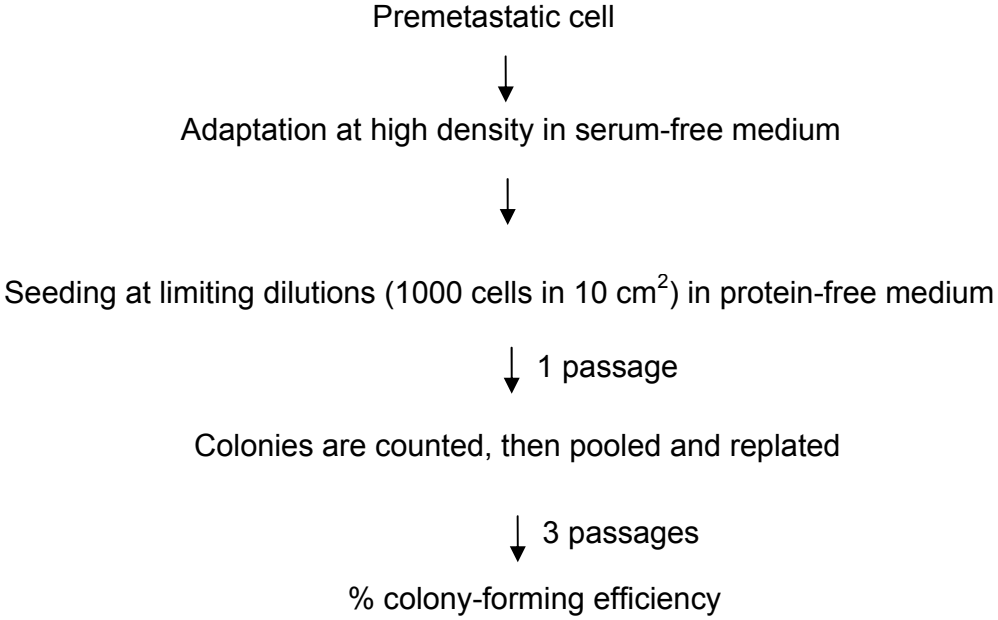
4.2.7. Invasion assay

BD BioCoat™ Matrigel™ invasion chamber (24-well format) were incubated at 37ºC overnight before $1x10^4$ melanoma cells (in 0.1 ml of medium) were plated in the upper compartment. The lower compartment was loaded with 0.6 ml of medium plus 10% FBS as chemoattractant. After 24 h of incubation at 37ºC, non-invading cells in the upper compartment were removed with a cotton swab. The microporous membrane was fixed in 70% ethanol for 1 h and cells reaching the membrane surface of the lower compartment were stained with hematoxylin for 2 h. Stained cells were counted using an inverted light microscope. Five random high power fields (10x10) were counted to get an average number for one well. The experiments were repeated in triplicate wells for at least three times. The standard error of the mean was calculated from independently performed experiments. The statistical significance of the data was analyzed using the Student *t* test.

4.2.8. Progression of WM793 in protein-free medium

This is a modified method from development of invasive and growth factor-independent cell variants from primary human melanomas (Kath *et al*., 1991). WM793 parent and transfected cells were plated at high density ($2x10^4$) in 24-well plates precoated with 0.1% gelatin in serum free medium. After 1 week adaptation, cells were trypsinized and reseeded at low density (1000 cells/well of 6-well plate). Two months later, colonies formed in each well from each cell line were counted, and then pooled and replated in the protein free medium at low density for next passage. Total 4 passages were performed. Results were expressed as the % colony formation efficiency (# of colony yield per 1000 seeded cells) at each selection cycle (Figure 14).

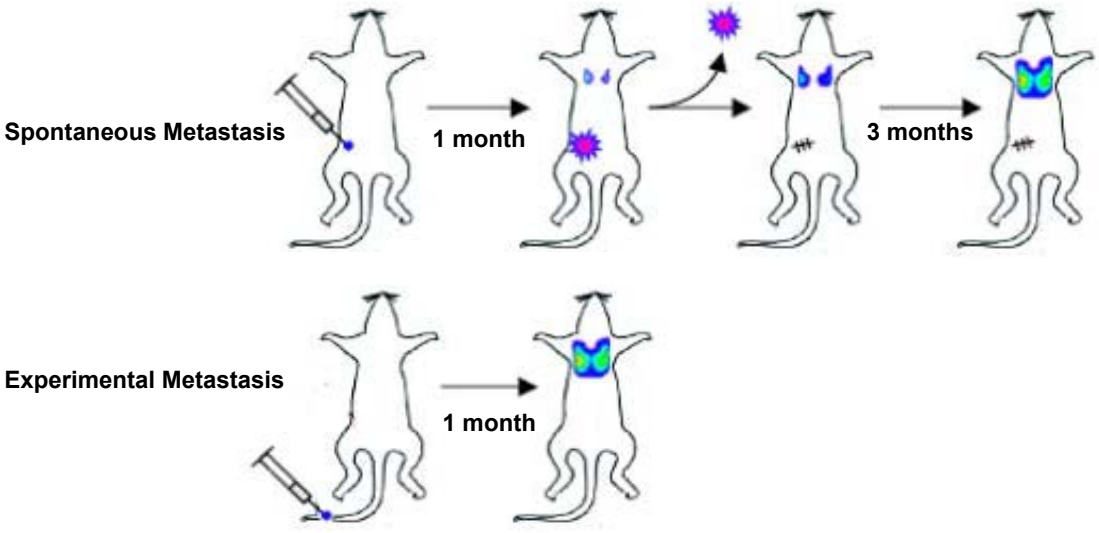
Figure 14. Progression of premetastatic melanoma cells in protein-free medium.



4.2.9. *In vivo* metastasis assays

Care of mice, surgery and injection protocols were approved by the IACUC at the University of Kentucky Medical Center (protocol # 00319M2001 and 00801M2004) and followed the National Institutes of Health guidelines. To produce spontaneous lung metastasis, a total of 2 million 1205LU parent or transfected cells in 200 μl HBSS were injected subcutaneously into the flank of athymic nude mice (Harlan, *nu/nu*) using a 1 ml tuberculin syringe. Tumor volumes were recorded every 3 days from two-dimensional measurements obtained with vernier calipers, using a standard formula (Tomayko and Reynolds, 1989). After 28 days, when tumors reach 800 mm^3 (~1 cm diameter), mice were anathesized under isofluorene and primary tumors were removed and checked for fluorescence (Kodak Image Station 4000). Nude mice were kept for another 3 months to permit metastatic growth. At the end of the experimental period, mice were euthanatized and lung metastasis was determined by counting metastatic nodules under a dissection microscope. Freshly dissected lungs were mounted in OCT (Tissue Tek) and frozen in a bath of liqud nitrogen. A total of 10 μm lung sections were obtained using a cryo-microtome (Leica 3050S) and were mounted onto glass slides (Super Frost Plus, Fisher). All the slides were stained by H.E. staining. Photographs were taken at ×10 and ×20 magnification. To produce experimental lung metastasis, $2x10^6$ cells were injected directly into the lateral tail vein as described previously (Kath *et al*., 1991). Lungs were scored for metastasis nodules and frozen for section as described above (Figure 15).

Figure 15. Illustration of procedures of *in vivo* metastasis assays. In spontaneous metastasis assay, $2x10^6$ 1205LU parent or transfected cells were injected subcutaneously. After one month, the primary tumors were removed and mice were kept alive for another three months to let lung metastases formed. At the end of the experiment, mice were sacrificed and lung nodules were counted. In experimental metastasis assay, $2x10^6$ 1205LU parent or transfected cells were injected through tail vein, and lung nodules were counted after one month (graphics from Minn *et al*., 2005).

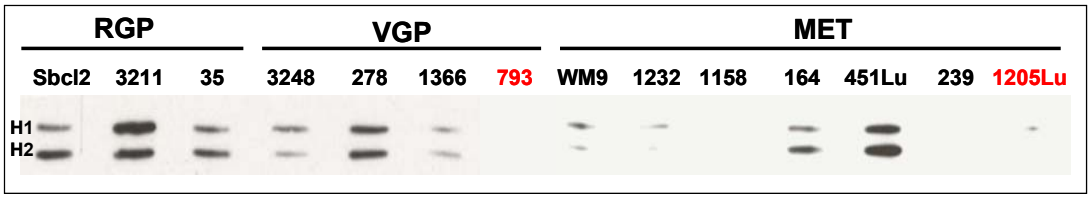


4.3. Results

4.3.1. Expression of NM23-H1 and NM23-H2 is frequently lost in many metastatic melanoma cell lines

A panel of melanoma cells, including different stages of RGP, VGP, and MET, was screened by immunoblot for NM23 expression. A significant loss of expression for both NM23-H1 and NM23-H2 has been demonstrated in five out of seven total MET cell lines (Figure 16). Another two MET cell lines, WM164 and 451LU, are demonstrated marked expression of NM23-H1 and NM23-H2, suggesting the multifunctional enzymatic activities of NM23s instead of metastasis suppression. Interestingly, WM793, a cell line from VGP group, was also found the total loss of NM23-H1 and H2 expression, suggesting the potential malignancy and invasiveness of this cell line. Two cell lines were employed for overexpressing wild-type or mutant NM23-H1 by stable transfection due to their very low expression of endogenous NM23s. 1205LU from MET represents highly metastatic melanoma cells, and WM793 from VGP represents premetastatic models of melanoma. 1205LU cells were originally derived from WM793 cells in animal models. Forced expression of wild-type and mutant NM23-H1 in these two cell lines provides useful tools to determine the extent to which each of the enzymatic activities of NM23-H1, the 3'-5' EXO in particular, suppresses metastatic progression.

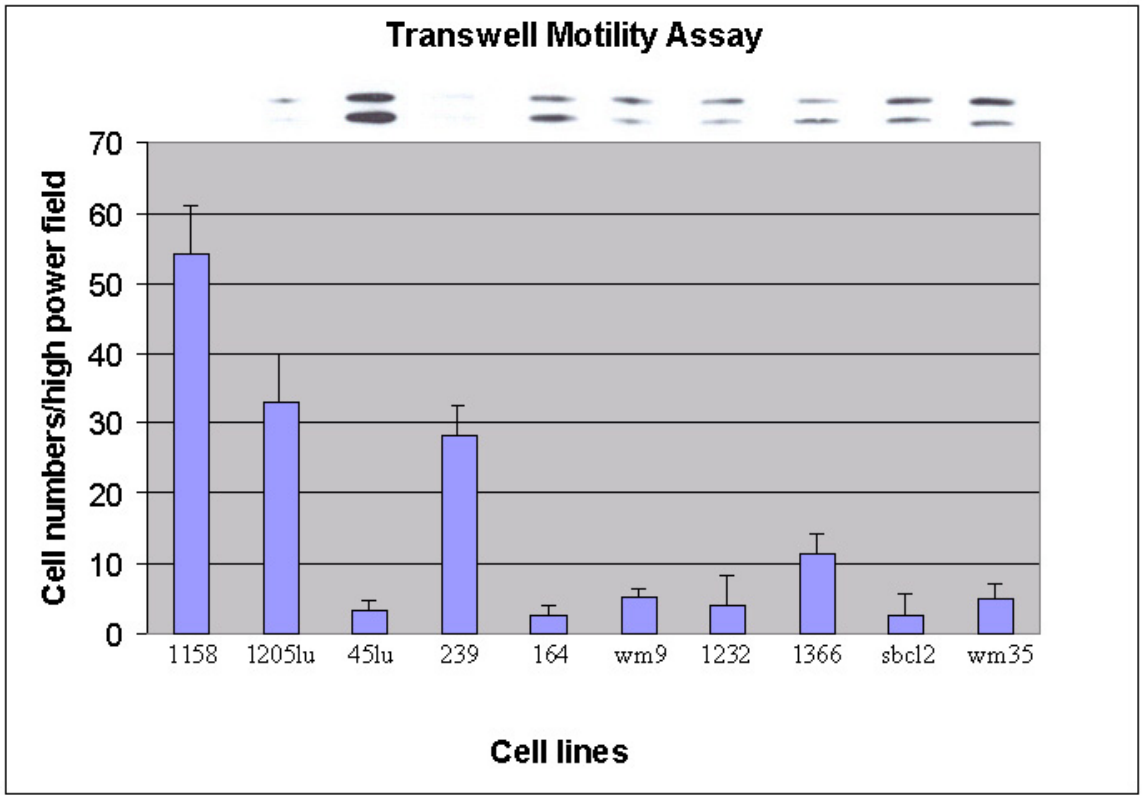
Figure 16. Expression of NM23-H1 and H2 in melanoma cell lines. Whole cell extract from each melanoma cell line was obtained and 40 μg total protein was loaded for each lane. Following SDS-PAGE gel and semidry transfer of protein, western blot was performed by using anti-NDPK antibody (Lab vision, 1:500 dilution) and goat-anti-rabbit secondary antibody (Pierce, 1:10,000 dilution). RGP, radial growth phase; VGP, vertical growth phase; MET, metastatic



4.3.2. Motility of melanoma cells is inversely correlated to the expression of NM23s

Since the expression of NM23 is related to clinical outcome of tumor patients (Ohba *et al*., 2005; Wang *et al*., 2004; Terada *et al*., 2002), it is highly likely that the expression of NM23s is also correlated with tumor cell invasiveness. To investigate the relationship between NM23s and motility of melanoma cells, transwell motility assay was carried out among different melanoma cell lines. As we expected, cell motility is inversely correlated with the expression of NM23-H1 and NM23-H2. Three MET cell line, WM1158, 1205LU, and WM239, exhibited the highest motility compared with other melanoma cell lines, while other VGP or RGP cells demonstrated minimum of motility. Interestingly, 451LU from MET with extraordinarily high expression of NM23 exhibited the lowest motility, suggesting NM23 is not exclusively correlated to metastasis suppression, but highly correlated to cell motility (Figure 17).

Figure 17. Motility of melanoma cells is inversely correlated to the expression of NM23s. Melanoma cells ($1x10^4$ cells per well) were plated into upper chamber of 24-well transwell plate. Cells were incubated at 37ºC for 24 h with 10% FBS in the medium in the lower chamber as chemoattractant. Cells were removed by cotton swab at the end of the experiment, and cells migrated through the pores of membrane were fixed and stained with hematoxylin. Stained cells were counted under an inverted light microscope. Five random high power fields (10x10) were counted to get an average number for one well. The experiments were repeated in triplicate wells for at least three times.



4.3.3. Wild-type and variant mutant NM23-H1 are stably expressed in WM793 and 1205LU cells

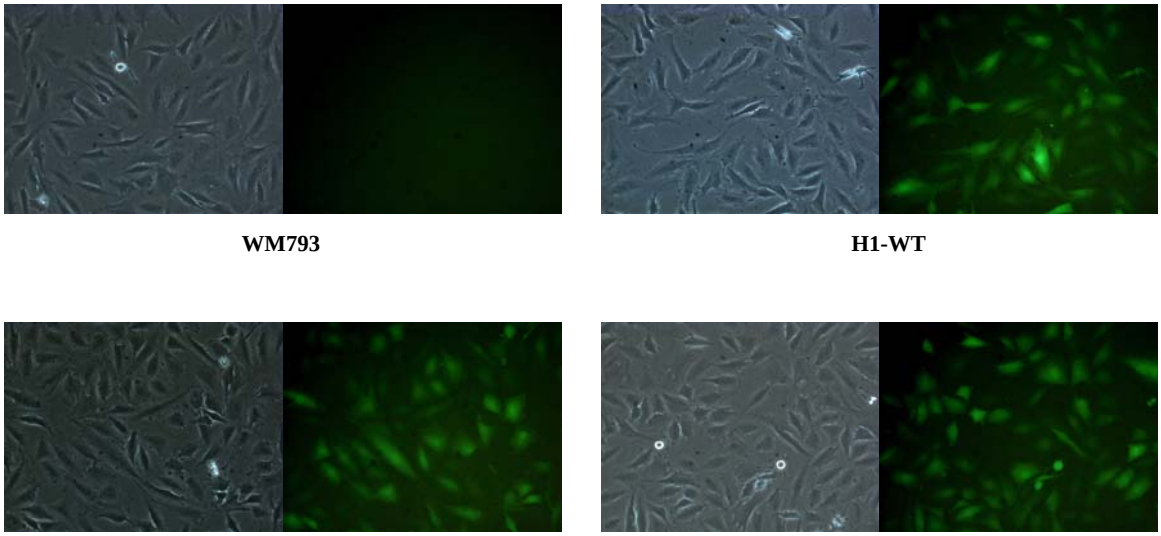
Transiently transfected cells were selected by G418 (geneticin, 250 μg/ml) for about 4 weeks. After drug selection, cell colonies are full of the whole 100 mm dishes. Not all of those colonies are GFP-positive. Those GFP-negative colonies are probably from G418 resistant cells, or more possibly, are from pSV2Neo only transfected cells. Some colonies are partially green: part of cells is green, but another part of cells in the same colony is not green, suggesting those tumor cells are heterogeneous even under the situation of cell culture. Some colonies are unanimously GFP positive. The intensity of GFP is varied in different green cells, suggesting either the difference of cDNA copy numbers in stable transfection or the difference of transcriptional or translational level. To make sure that all the forced expressions of NM23-H1 are at the same level, fluorescence associated cell sorting (FACS) was employed to obtain all GFP positive cells with similar intensity of green signal. The sorting procedures have yielded more than 95% GFP fluorescent cells (Figure 18A and B) and intensity of GFP in each transfected cell line is at the range of 10^2 to 10^4 compared with the non-transfected parent cells.

To further compare the protein expression in each transfected cell line, western blot of whole cell extract from each cell was performed immediately after cell sorting (passage 2 and passage 3). After several passages, stably transfected cells (passage 8) were measured again for protein expression. In the early passage, transfected cell obtained significant overexpression of NM23s compared with parent cells. This was more obvious in WM793 cells with almost no endogenous NM23s expressed in parent cells (Figure 18C). After several passages, 1205LU transfected cells were demonstrated fading GFP signal under the UV microscope. So, we are

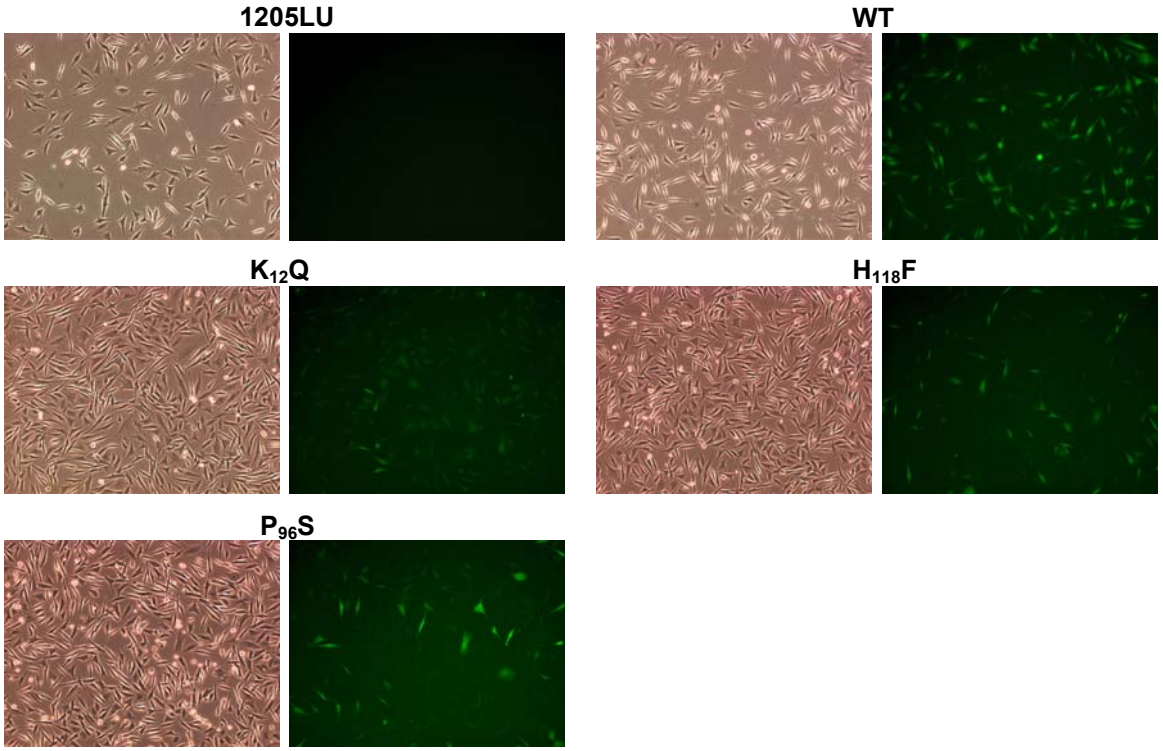
concerned about that the transfected 1205LU cells are losing NM23 expression. But transfected WM793 cells are very stable in showing the percentage and intensity of green cells. To investigate whether transfected 1205LU cells are still expressing NM23-H1 at later passage level, western blot was performed again on whole cell extract of transfected 1205LU cells at passage 8. Different from the result of passage 3, all the mutant transfected cells demonstrated the same amount of protein expression as parent cells while wild-type NM23-H1 transfected cells express minimally increased proteins (Figure 18D). There are two possibilities explaining this. It could be the transfection that is not stable enough, so cells keep losing inserted NM23 copy. Another possibility is that NM23 expression is tightly controlled in this cell line. So, if they are expressing mutant NM23, the cell will downregulate the endogenous NM23 expression. Western blot could not tell between the endogenous NM23 and mutant NM23, but 2-D gel would provide this informaton based on their changed pI value of those mutants.

Figure 18. Wild-type and variant mutant NM23-H1 are stably expressed in WM793 and 1205LU cells. (A) Stably transfected WM793 cells sorted for GFP fluorescence represent highly enriched transfectant populations. Shown is GFP fluorescence in WM 793 cells that express wild-type or mutant variants of NM23-H1. (B) Stably transfected 1205LU cells. (C) Western blot of whole cell extract of transfected cells at passage 3. P, parent cell. W, wild-type. (D) Western blot of whole cell extract of transfected 1205LU cells at passage 8.

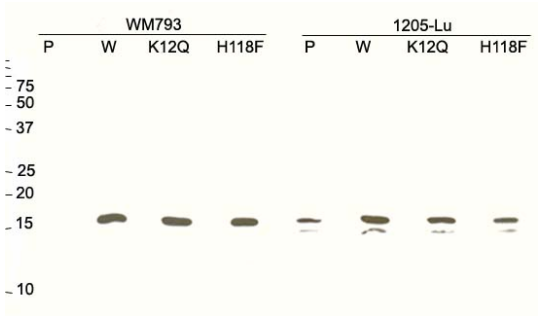
A



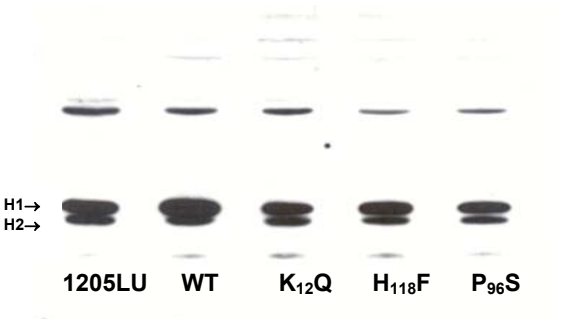
B.


1205LU
WT
K12Q
H118F
P96S

C.


WM793
1205-Lu
P
W
K12Q
H118F
P
W
K12Q
H118F
75
50
37
25
20
15
10

D.

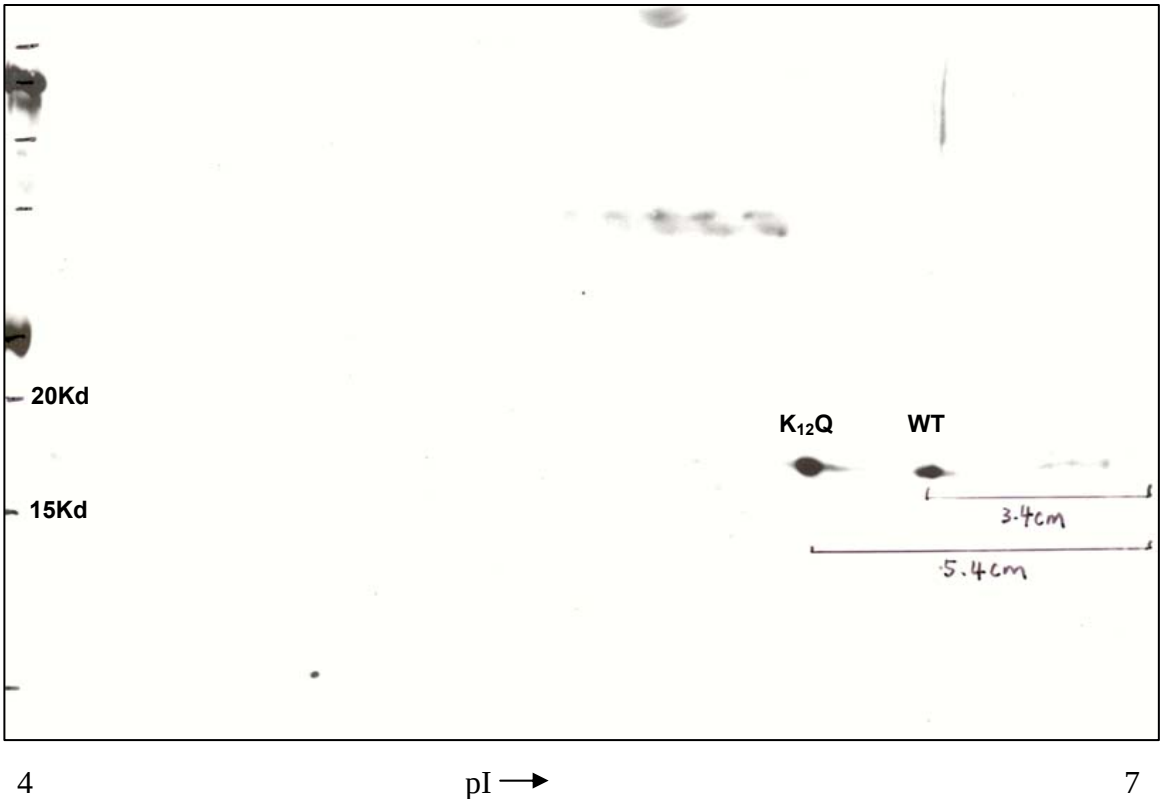

H1→
H2→
1205LU
WT
K12Q
H118F
P96S

4.3.4. Endogenous and mutant NM23s are equally expressed in transfected 1205LU cells

Wild-type or mutant NM23s were overexpressed in stably transfected 1205LU cells at early passage number (P3). After several passages, the expression of NM23 went down (Figure 18D) to the level of endogenous protein level, suggesting that NM23 expression is strictly limited to some extent. To measure the origin of the protein expressed in transfected 1205LU cells, 2-D gel was employed to separate the wild-type (endogenous) and all mutant NM23s. Single amino acid change causes significant change of pI value of each mutant. Purified wild-type NM23-H1 and $K_{12}Q$ mutant were run on 2-D gel to test their pI value. The $K_{12}Q$ mutant (theoretical pI 5.60) was separated markedly from wild-type NM23-H1 (theoretical pI 5.83) (Figure 19A). To study the persistence of forced NM23 expression in stably transfected 1205LU cells, 50 μg whole cell extract from parent cell line and three mutant transfected cell lines were loaded and run on 2-D gel followed by western blot. In parent cell, different from purified wild-type NM23-H1, endogenous NM23-H1 was demonstrated as two separate dots, suggesting the existence of phosphorylated NM23-H1. These two protein dots have been demonstrated in every mutant transfected cell line at decreased level compared with parent cells. Furthermore, all mutant NM23-H1s have been demonstrated in each cell line at the same expression level of endogenous protein, indicating mutant protein and endogenous protein are equally expressed in each mutant transfected cell lines (Figure 19 B).

Figure 19. Mutant NM23-H1 is stably expressed in transfected 1205Lu cells. (A) Purified wild-type NM23-H1 (3 μg) and $K_{12}Q$ mutant protein (6 μg) were loaded on 17 cm IPG strips. Gels were run on first dimensional and secondary dimensional gel as described in methods. Proteins were transferred to nitrocellulose membrane by semidry methods followed by detection with western blot. (B). Whole cell extract from 1205LU cells and three transfected cell line (50 μg) was tested in the same way as (A).

A.



B.

1205LU-parent

1205LU-$K_{12}Q$

1205LU-$H_{118}F$

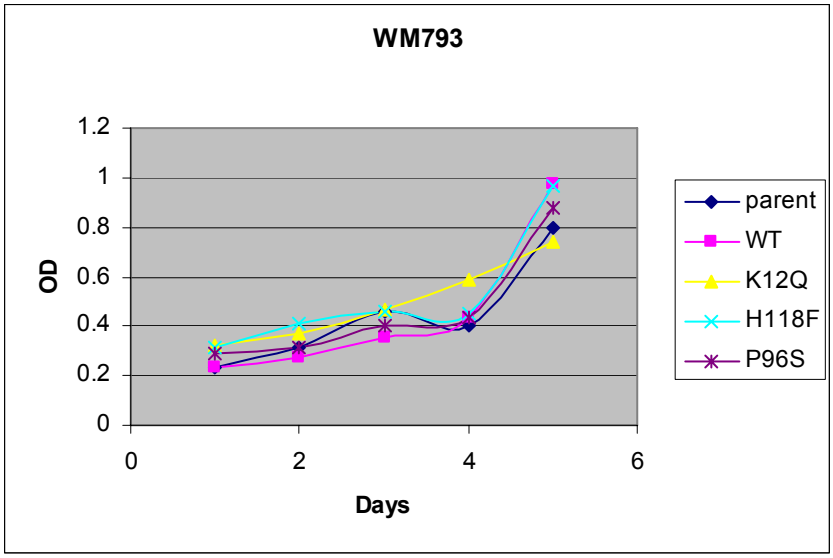
1205LU-$P_{96}S$

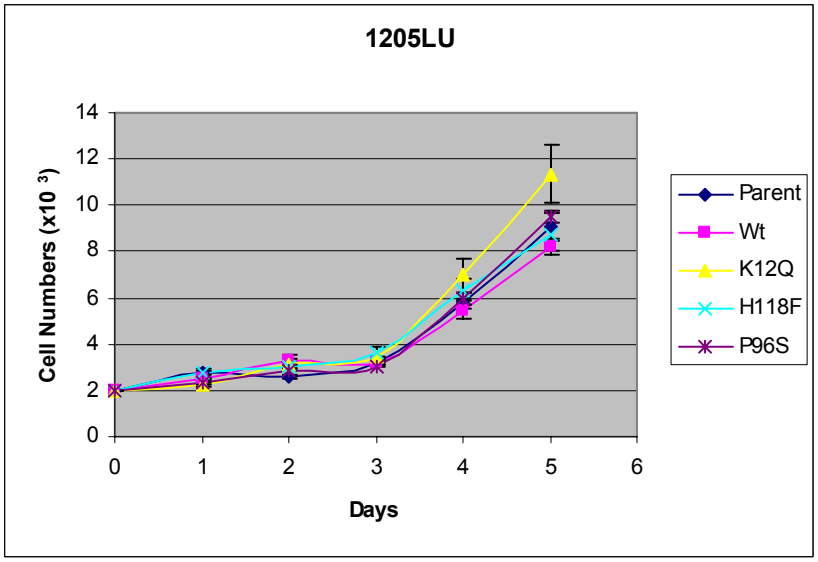
4.3.5. Overexpression of NM23-H1 in melanoma cells has no effect on transformation phenotype

NM23-H1 is a metastasis suppressor protein and should not have any effect on transformation phenotype. To test whether NM23 transfection has no effect on cell proliferation, a serial of MTS assay was performed from day 1 to day 5 on both cell lines. Either wild-type NM23-H1 or mutant NM23-H1 transfection has no any effect on proliferation of both cell line compared with parent cells, consistant with the definition of metastasis suppressor gene (Figure 20A). Anchorage independent growth assay in soft agar is considered as a stringent assay for detecting malignant transformation of cells. Colony formation in soft agar of transfected cell lines was compared and transfected WM793 cells showed similar colony formation ability as parent cells. For 1205LU cells, wild-type and $K_{12}Q$, $H_{118}F$ mutant overexpression has no effect on anchorage independent growth in soft agar compared with parent cells. But to our surprise, $P_{96}S$ mutant transfected cells had significantly decreased colony numbers compared with other 1205LU transfected cells, suggesting adominant-negative function of this mutant (Figure 20 B).

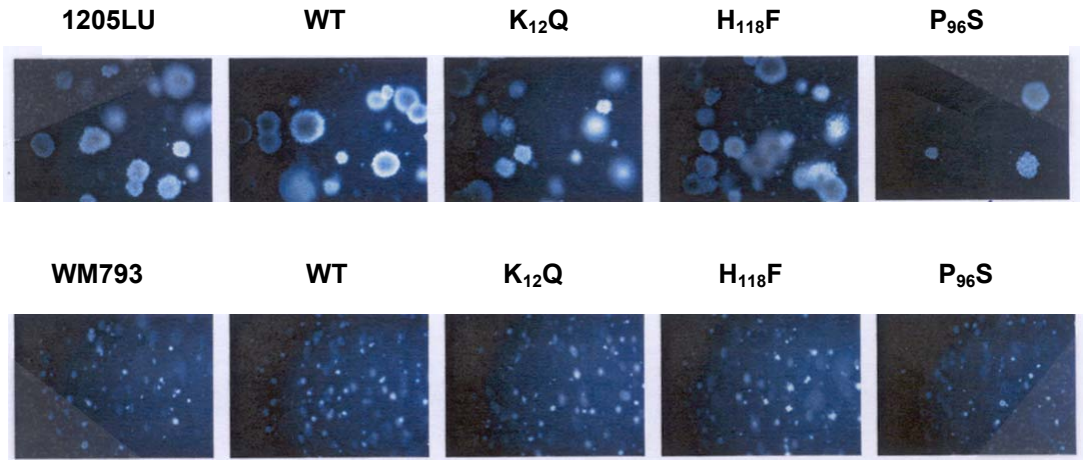
Figure 20. Overexpression of NM23-H1 in melanoma cells has no effect on the transformed phenotype. (A) Cell proliferation rate was determined by MTS assay. Cells were plated in 96-well plate from $2x10^3$ on day 0, and were measured on day 1 to day 5. (B) Cells ($5x10^4$) were seeded in the top medium and stained by 0.005% crystal violet for 1 h after 28 days. Colony numbers were counted by five random high power fields to get an average number. The experiments were repeated in triplicate wells for at least three times.

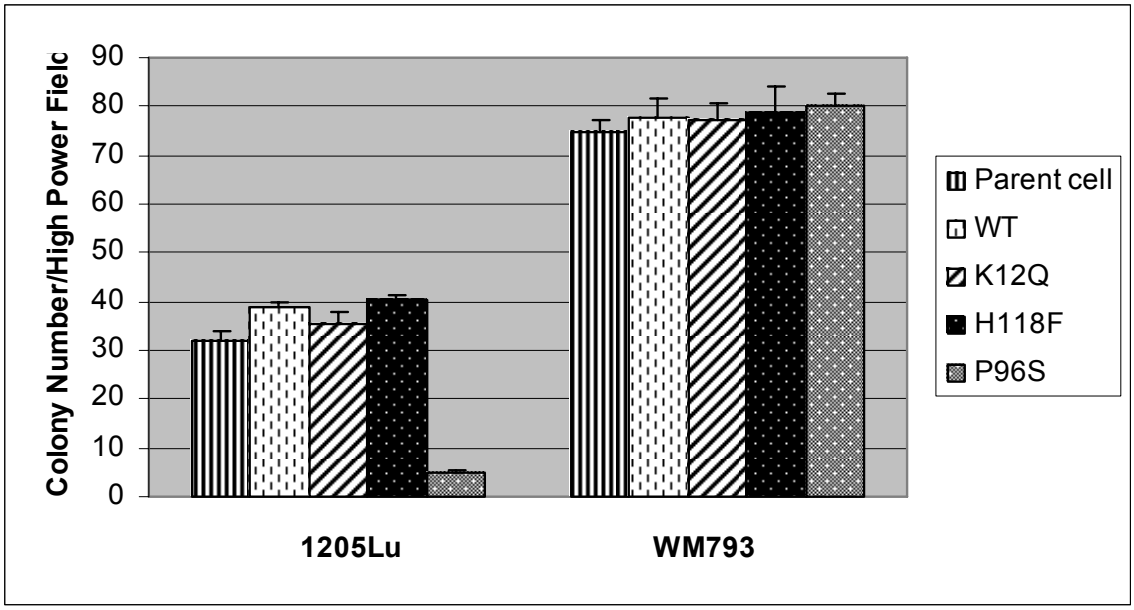
A.





B.

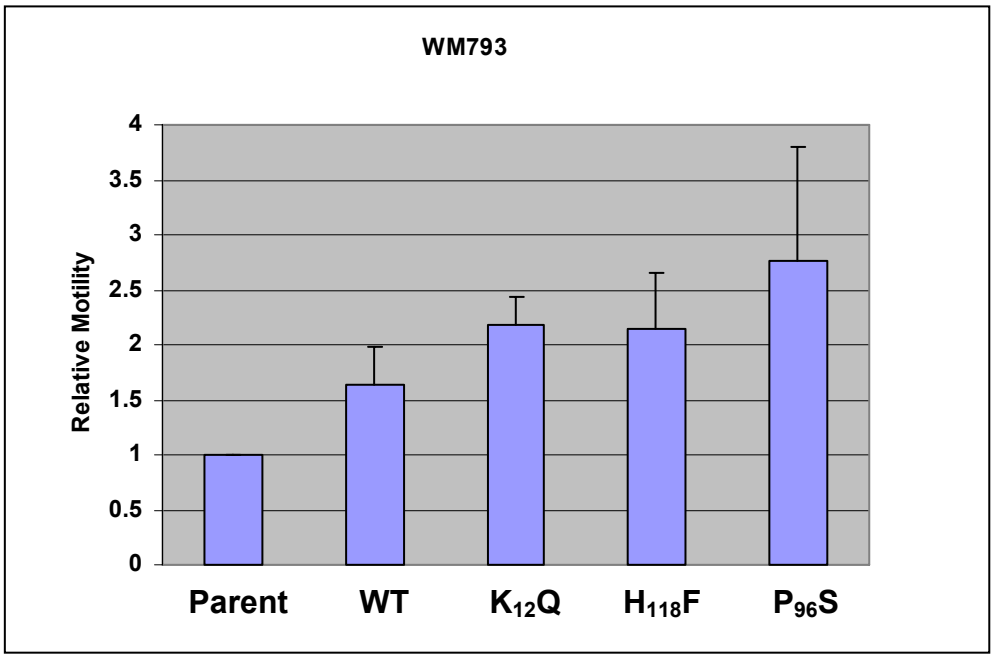

1205LU
WT
K12Q
H118F
P96S
WM793
WT
K12Q
H118F
P96S

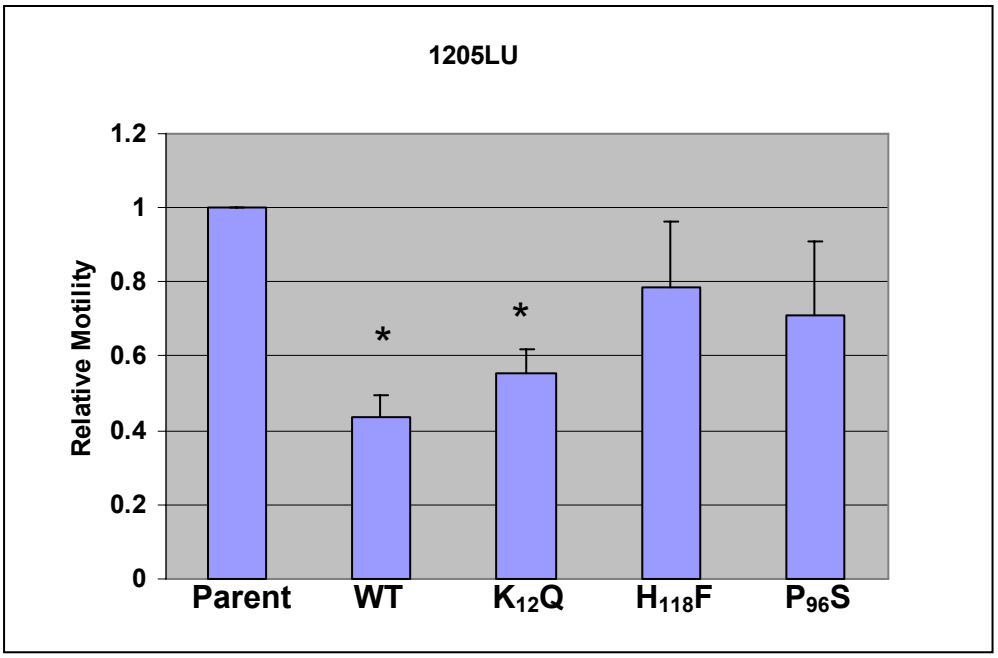

Colony Number/High Power Fielc
90
80
70
60
50
40
30
20
10
0
1205Lu
WM793
Parent cell
WT
K12Q
H118F
P96S

4.3.6. Forced expression of wild-type and $K_{12}Q$ mutant NM23-H1 decreased motility in 1205LU cells

Cell motility is a complex cellular function that is altered through changes in cell adhesion and is also reflected through cellular migration (Sattler *et al*., 2003). Enhanced motility is one of the characteristics of malignant cells. To determine that the relationship between motility and NM23 as well as the contribution of enzymatic functions of NM23-H1 to motility, transwell motility assay of parent and transfected WM793 and 1205LU cell lines was performed. Wild-type NM23-H1 transfected cells were expected to lower the motility of WM793 cells based on the metastasis suppression nature of NM23-H1. Surprisingly, wild-type NM23-H1 transfected WM793 cells as well as all the H1 mutant-transfected WM793 cells had a tendency to enhance cell motility compared with parent cells, with significance in $K_{12}Q$ and $H_{118}F$ mutant tranfected cell lines. For 1205LU cells, wild-type and $K_{12}Q$ mutant transfected cells decreased cell motility markedly, while $H_{118}F$ and $P_{96}S$ mutant transfected cells have no significant difference with parent cells (Figure 21).

Figure 21. Overexpression of NM23-H1 wild-type and $K_{12}Q$ mutant decreases motility in 1205LU cell. $1x10^4$ cells were plated in upper chamber with 0.1 ml of medium plus 2% FBS. 0.6 ml of medium plus 10% FBS was added into lower chamber as chemoattractant. Cells were incubated at 37°C for 24 h to let cell migrate. At the end of the experiment, non-migrated cells were removed by cotton tips, and migrated cells were fixed and stained with hemotaxyolin for 2 h. Cells were counted by five random high power fields to get an average number. The experiments were repeated in triplicate wells for at least three times.

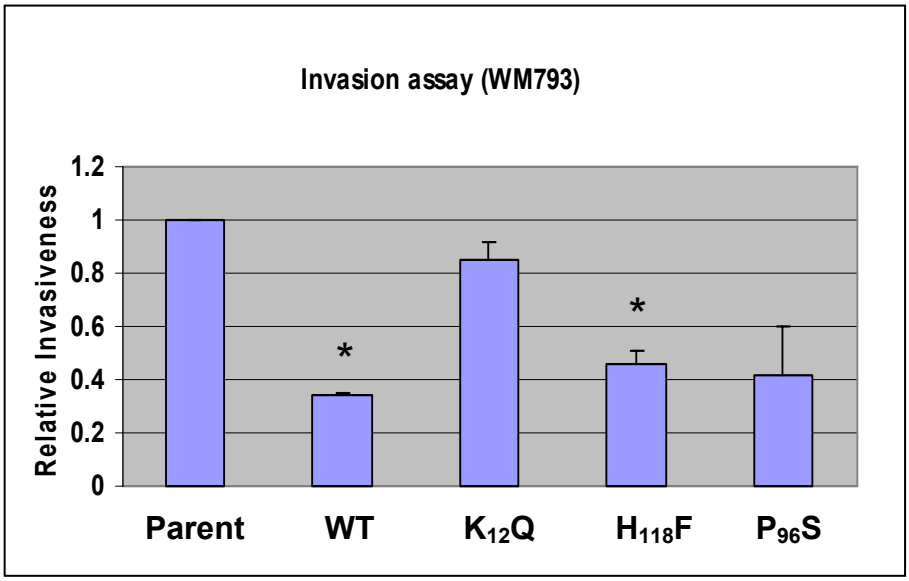

WM793
Relative Motility
4
3.5
3
2.5
2
1.5
1
0.5
0
Parent
WT
K12Q
H118F
P96S

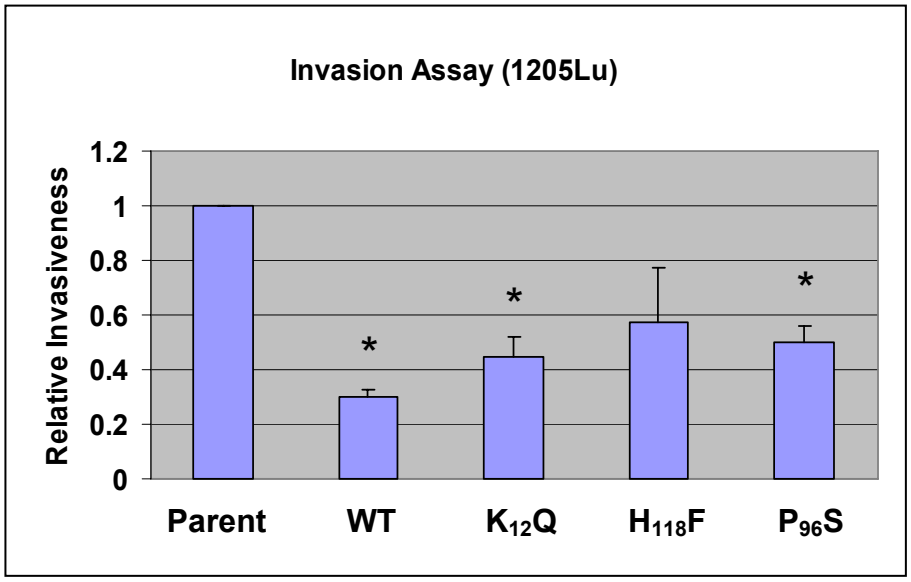

1205LU
Relative Motility
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Parent
WT
K12Q
H118F
P96S

4.3.7. NM23-H1 $K_{12}Q$ mutant lost invasion-suppressor activity in WM793 cell but not in 1205LU cell

Measurement of the tumor cell invasion through a microporous membrane coated with BD Matrigel™ Matrix in Boyden-like chambers is a widely accepted *in vitro* metastasis assay. Matrigel is a reconstituted basement membrane, which simulates the basement membrane *in vivo*. Degradation of these matrix membranes is a feature of invasive cells and has been used in an attempt to understand the process regulating critical steps of tumor cell invasion. To investigate the effect of NM23-H1 on cell invasion activity, all the transfected WM793 and 1205LU cells were measured for invasiveness compared with the parent cells. Inconsistant with motility assay, wild-type, $H_{118}F$ mutant and $P_{96}S$ transfected WM793 cells all exhibited decreased invasiveness compared with parent cells, while $K_{12}Q$ mutant NM23-H1 transfection lost the function of invasion suppression, suggesting exonuclease function of NM23-H1 may play a role in malignancy and tumor progression. For metastatic cell line 1205LU, transfection of NM23-H1 and variant mutants decrease invasiveness of metastatic melanoma cells, consistent with the results of motility assay. Loss of exonuclease function of NM23-H1 has no effect on invasion activity compared with wild-type H1 and other mutant H1 (Figure 22).

Figure 22. NM23-H1 $K_{12}Q$ mutant lost invasion-suppressor activity in WM793 cell but not in 1205LU cell. $1x10^4$ cells were plated in upper chamber with 0.1 ml of medium plus 2% FBS. 0.6 ml of medium plus 10% FBS was added into lower chamber as chemoattractant. Cells were incubated at 37°C for 24 h to let cell invade the matrix and migrate. At the end of the experiment, non-invaded cells were removed by cotton tips, and invaded cells were fixed and stained with hemotaxyolin for 2 h. Cells were counted by five random high power fields to get an average number. The experiments were repeated in triplicate wells for at least three times.


Invasion assay (WM793)
Relative Invasiveness
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Parent
WT
K12Q
H118F
P96S


Invasion Assay (1205Lu)
Relative Invasiveness
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*
Parent
WT
K12Q
H118F
P96S

4.3.8. NM23-H1 impairs the progression of premetastic melanoma cell WM793 and exonuclease and NDPK activities are all involved.

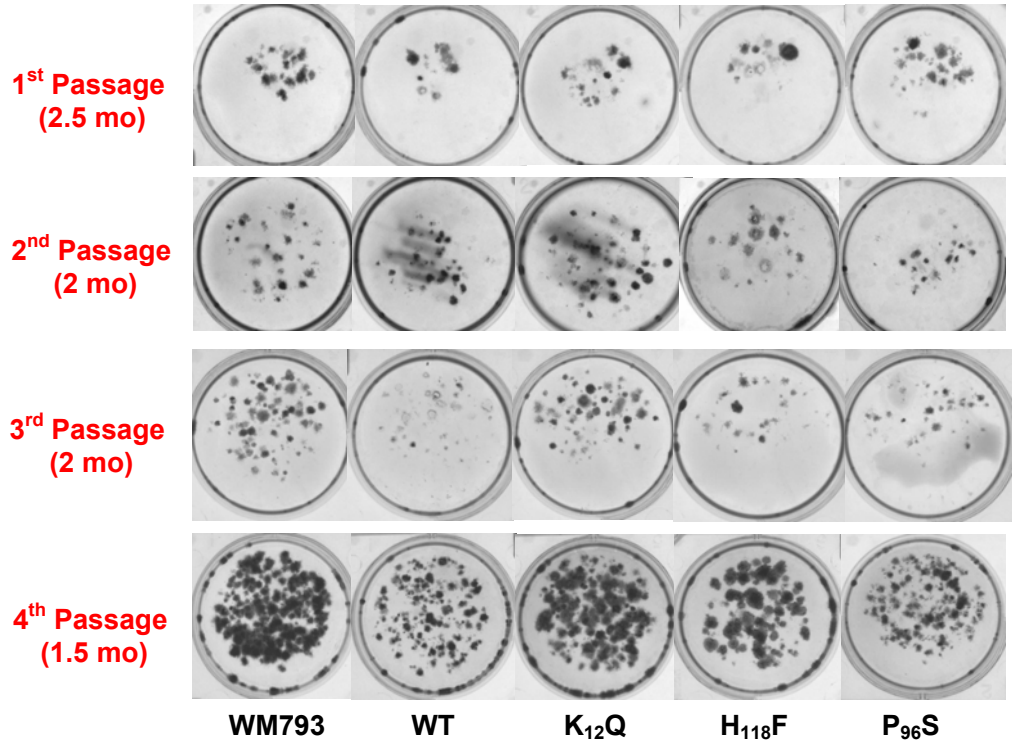
The hypothesis of the current project is that exonuclease function of NM23-H1 may play an important role in DNA repair. Loss of this function leads to accumulation of mutations and genomic instability, and thus the progression of tumor cells. WM793 is the early stage of 1205LU (see 5.1 introduction). There is a progression of WM793 cells *in vitro* and *in vivo*, to become 1205LU cells. To investigate whether replacing NM23-H1 back into WM793 cells can prevent progression of this cell line in serum-free medium, diluted WM793 cells were plated in six-well plate with protein free medium (w/o either serum or insulin). After 8-10 weeks, cell colony numbers were counted and cells were pooled and replated for next cycle (as described in methods). First, WM793 cells progressed in protein free medium. The colony formation efficiency was about 20 out of 1000 seeded cells. This efficiency increased to 200 out of 1000 cells by the end of fourth cycle, suggesting the WM793 cell line progressed and became more malignant after cultured in protein free medium. Wild-type NM23-H1 significantly suppressed this progression process, ending up with less number of colonies and smaller colony size than that of other mutant transfected cells at 3rd and 4th cycle. But $K_{12}Q$ mutant (exonuclease deficient) lost the function of suppression of cell progression. By the end of 3rd and 4th cycle, colony numbers and size were both similar to that of parent cells and significantly increased by comparing with wild-type H1 transfected cells. Interestingly, $H_{118}F$ mutant (NDPK deficient) transfection also demonstrated the similar results as $K_{12}Q$ mutant by the end of 4th cycle (similar colony size but less colony numbers) even though it has no difference in colony number and size with wild-type H1 transfected cells by the end of 3rd cycle, suggesting NDPK may also be involved in the suppression of tumor progression.

Surprisingly, $P_{96}S$ mutant H1 (hisK deficient) prevents WM793 progression as effectively as wild-type H1, with the least and smallest colonies formed by the end of 3rd and 4th cycle, suggesting that hisK is not associated with suppression of WM793 cell progression (Figure 23A). Summary of colony numbers of each cell line at each cycle are shown in Figure 23B.

Since this experiment took such a long time to complete, the stability of the overexpression level of NM23-H1 and variant mutants was concerned. To test the consistency of GFP expression in each transfected WM793 cells, 30 colonies from each transfected cell line were counted to compare the ratio of GFP-positive and GFP-negative colonies. The result showed that wild-type, $K_{12}Q$ and $P_{96}S$ H1 transfected cells kept consistent GFP expression, with only small fraction of colonies with GFP expression lost. H118F H1-transfected cells were somewhat less stable, but 50% of its colonies retained detectable GFP expression (Table 1).

Figure 23. The NDPK, and possibly, 3'-5'exonuclease activities of NM23-H1 are associated with suppression of progression of premetastatic WM793 melanoma cells to a growth factor-independent phenotype. (A) After adapted in serum-free medium at high density for 1 week, cells were plated at low density in protein free medium (serum free medium plus no insulin). Each cycle took about 8-10 weeks. Colony numbers were counted as colony formation efficiency at the end of each cycle, and cells were pooled and replated for the next cycle. Representive formed colonies from each cell line were fixed with 4% formaldehyde and stained with 0.005% crystal violet. (B) Summary of colony formation efficiency of each cell line at each cycle.

A.


1st Passage (2.5 mo)
2nd Passage (2 mo)
3rd Passage (2 mo)
4th Passage (1.5 mo)
WM793
WT
K12Q
H118F
P96S

B.

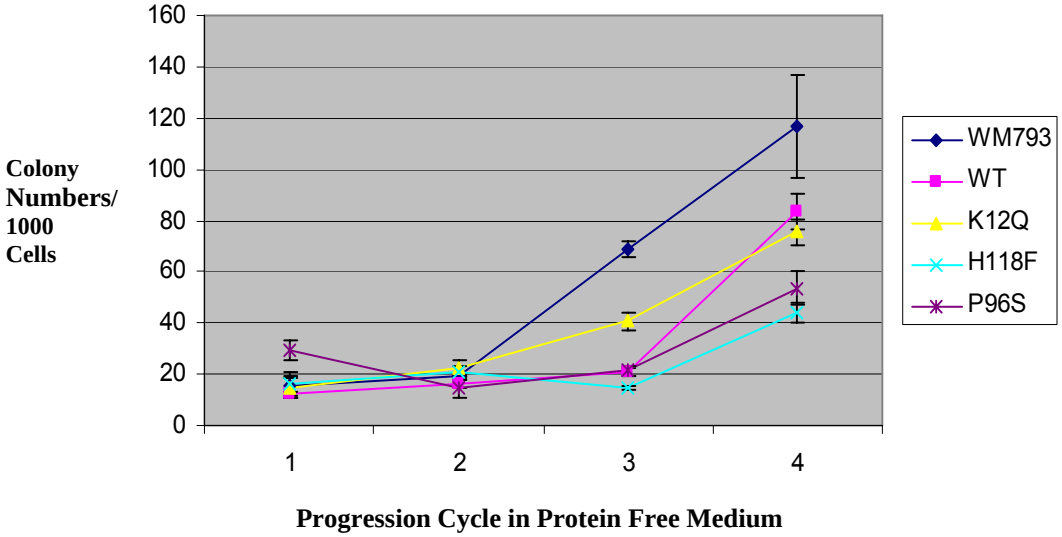

Colony Numbers/ 1000 Cells
160
140
120
100
80
60
40
20
0
1
2
3
4
WM793
WT
K12Q
H118F
P96S
Progression Cycle in Protein Free Medium

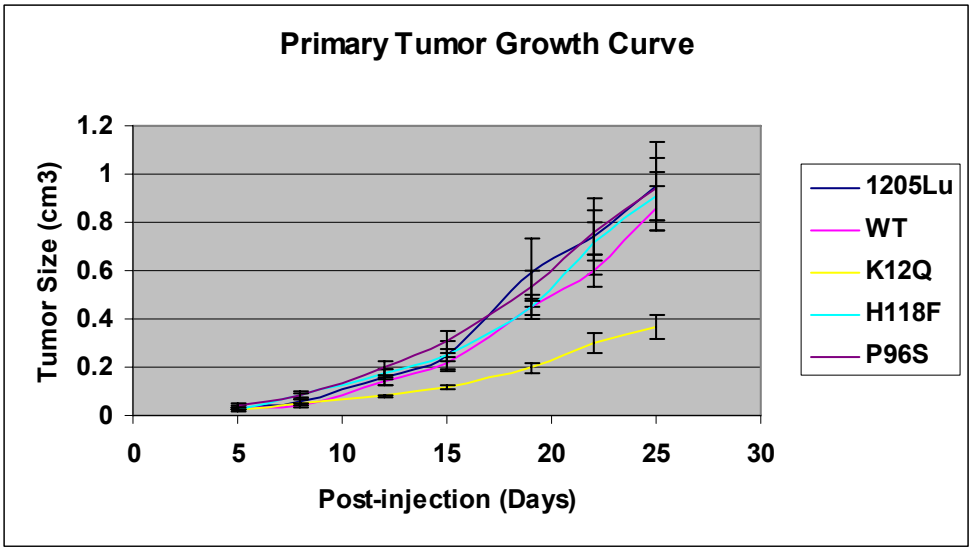
Table 1. Expression of heterologous gene products is maintained for the WM793 cell panel over the course of four cycles of selection in protein-free culture medium. A total of 30 colonies from each cell line were counted.

	GFP+	**GFP-**
WM793-WT	21	9
WM793-$K_{12}Q$	25	5
WM793-$H_{118}F$	15	15
WM793-$P_{96}S$	25	5

4.3.9. Wild-type and $P_{96}S$ NM23-H1 suppress spontaneous lung metastasis of 1205LU cells while $K_{12}Q$ and $H_{118}F$ mutant H1 lost metastasis suppressor activity

In vitro metastasis assays test only one or two characteristics of metastatic tumor cells. *In vivo* models provide a more clinically accurate mechanism for studying tumor development and progression. To determine which enzymatic function of NM23-H1 is associated with metastasis suppression, spontaneous lung metastasis of 1205LU was performed on athymic nude mice. $2x10^6$ parent and transfected 1205LU cells were injected subcutaneously on the left flank of each mouse. Four weeks later, when skin primary tumor volume reached 0.8-1.0 cm^3, tumors were removed and mice were kept for another 3 months for lung metastases formation. Removed tumors were scanned with Kodak Image Station 4000 for GFP expression. Generally, the primary tumor grew at the same rate, suggesting that transfection of wild-type H1 and mutant H1 into 1205LU has no effect on primary tumor growth, consistent with the definition of metastasis suppressor gene. $K_{12}Q$ mutant H1 transfected tumor cells grew a little slower. But the primary tumors in this group reached 0.8 cm^3 5 days later and excised at that time (Figure 24).

Figure 24. Transfection of wild-type and mutant H1 has no effect on primary tumor growth. Two million cells were injected subcutaneously on the flank of each mouse. After 4 weeks, when primary tumors reached 0.8-1.0 cm^3, tumors were excised and mice were kept for another 3 months for lung metastses formation.



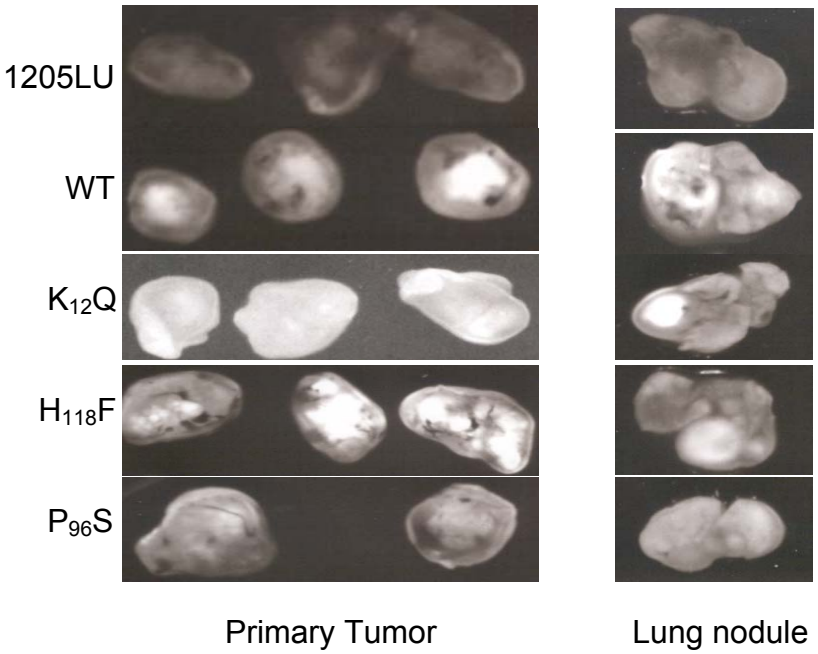
After primary tumors were removed, the mice were kept for another 3 months for lung metastasis. Three months later, all the mice were sacrificed and lungs were examined for nodule formation. Metastasis rate was calculated by number of mice with lung metastasis/total number of mice in this group. In parent cell group, 50% metastasis rate has been observed, suggesting the malignancy and invasiveness of this melanoma cell line. Wild-type H1 transfection significantly suppresses metastasis with metastasis rate down to 15%. $K_{12}Q$ and $H_{118}F$ mutant H1 lost metastasis suppression function with similar metastasis rate as parent 1205LU cells, suggesting that exonuclease and NDPK are both involved in metastasis suppression *in vivo*. Unexpectedly, $P_{96}S$ mutant, previously reported as contributor of increased cell motility, still as strongly inhibit lung metastasis as wild-type H1, suggesting this mutant may not be associated with metastasis suppression on this melanoma cell line in *in vivo* model (Table 2).

Table 2. Metastasis rate of 1205LU parent cell and transfected cell in spontaneous metastasis model. After primary tumors were excised, mice were kept for another 3 months to permit formation of lung macrometastasis. When mice were sacrificed, lungs were examined for metastasis nodules. Metastasis rate was calculated as number of mouse with lung metastasis/ total mouse number in this group.

Cell lines	# of mouse with lung metastasis	Metastasis rate
1205LU	6/12	50%
1205LU-WT	2/13	15%
1205LU-$K_{12}Q$	5/8	62.5%
1205LU-$H_{118}F$	5/10	50%
1205LU-$P_{96}S$	2/9	22%

To determine the primary tumors and metastasized nodules are consistently expressing GFP, indicating exogenous proteins expressed in these tumor cells, the excised primary tumors and lungs with macrometastasis were scanned for GFP fluorescence. Compared with 1205LU parent cell with no fluorescence, all the transfected cell-induced primary tumors and metastasized lung nodules demonstrated high intensity of fluorescence, suggesting the stability of transfected cells *in vivo*. Compared with other transfected cell-induced primary tumors and lung nodules, $P_{96}S$ mutant H1 transfected cell-caused tumors were observed with relatively low fluorescence, even though the high intensity of GFP signal of the cells in cell culture system (Figure 25).

Figure 25. GFP is consistently expressed in both primary tumors and metastasized lung nodules. Removed primary tumors and lungs with macrometastasis were scanned for fluorescence with Kodak Image Station 4000. Tumors in parent cell group which do not harbor a GFP transgene were used as negative controls.

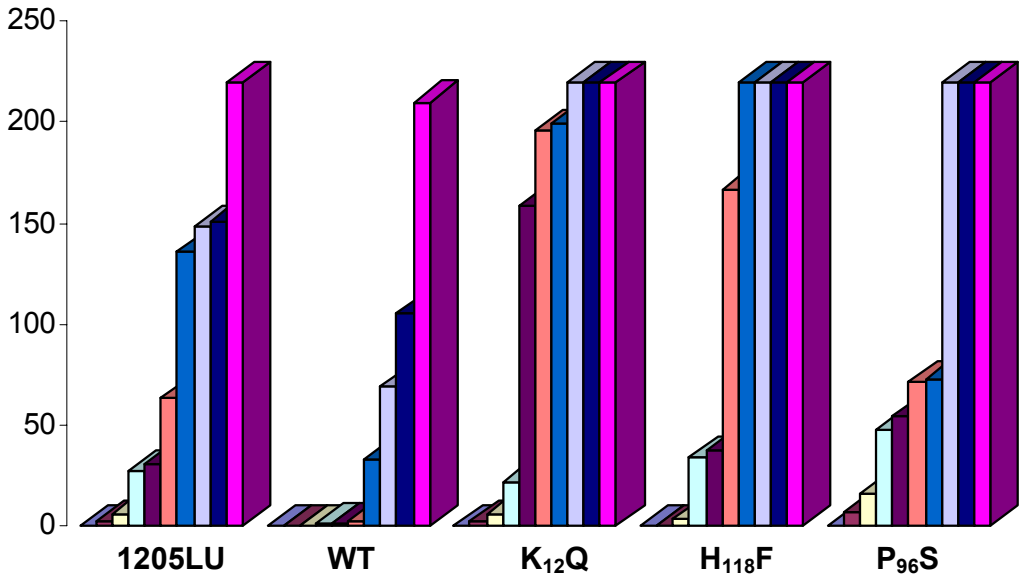


4.3.10. Experimental metastasis assay demonstrated similar results as in spontaneous metastasis assay

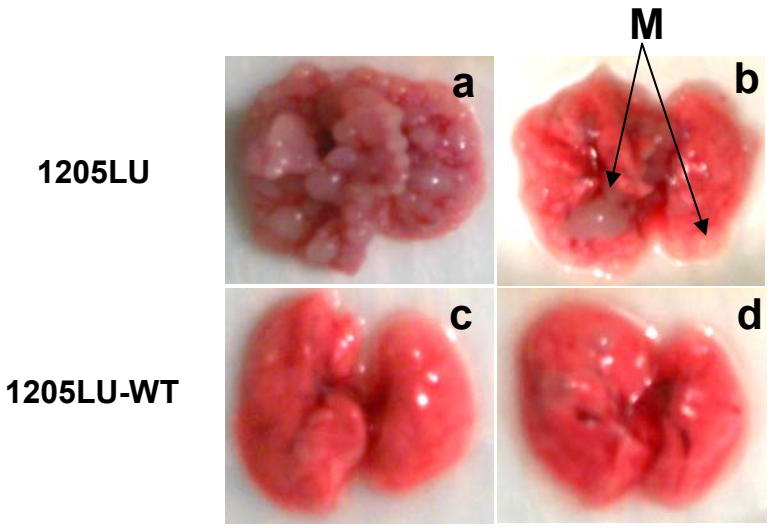
To confirm the results observed in spontaneous metastasis assay described above, experimental metastasis assay was performed on nude mice with the same 1205LU parent cells and transfected cells. $2x10^6$ cells were injected through tail vein, and one month later, mice were sacrificed and lungs were examined for macrometastasis. Similar results have been observed as the results of spontaneous metastasis. Wild-type NM23-H1 suppresses lung nodule formation while $K_{12}Q$ and $H_{118}F$ mutant H1 lost suppression function of tumor metastasis, the similar results that have been observed in spontaneous metastasis assay. Inconsistent with the results of spontaneous metastasis, $P_{96}S$ mutant H1 also lost metastasis suppression function. Unfortunately, since the standard error in each group is very high, all the results in this experiment have no statistic significance (Figure 26).

Figure 26. Experimental metastasis assay demonstrated similar results as in spontaneous metastasis assay. (A) Lung nodule numbers in each group. (B) Representative picture of metastases on the lung from parent cell group (a and b) and wild-type H1 group (c and d). (C) Histological images of normal lung and metastasized nodules stained by hematoxylin and eosin.

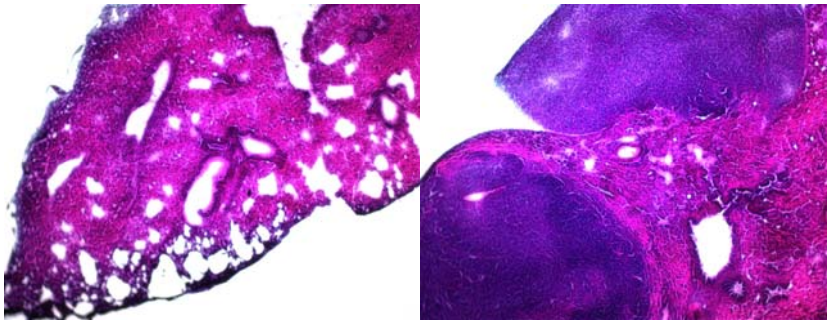
A.



B.



C.



Normal lung **Metastasized nodules**

4.4. Discussion

Our lab has obtained some data supporting the hypothesis of DNA repair role of NM23 in yeast. These observations need to be validated in cells derived from higher vertebrates, preferably in human cell lines. A panel of melanoma cells, including different stages of RGP, VGP, and MET, was screened by western blot to identify human cell lines devoid of NM23 expression in which DNA repair activity can be assessed, and complementation experiments conducted with a minimum of background NM23 expression.

A significant loss of expression for both NM23-H1 and NM23-H2 has been demonstrated in five out of seven total MET cell lines (Figure 16), suggesting that the expression of NM23 is highly related to metastasis. This relationship was further testified by motility assay of melanoma cell lines (Figure 17). Interestingly, WM793 cell from VGP group which has been testified non-metastatic in experimental rodent models has also been observed to be devoid of detectable expression of either H1 or H2. WM793 was established from the vertical growth phase of a primary lesion of patient 793. Cell line 1205LU, the metastatic variant of WM793, was established after repeated passages of WM793 cells both *in vitro* and *in vivo* in nude mice (Samasundaram *et al*., 2005). Because of their very low expression of NM23s, and also the fact that they stands for premetastatic and metastatic cell lines, respectively, these two cell lines were chosen as our study model for stable transfection.

The major goal of the project is to elucidate the molecular mechanisms underlying metastasis suppressor activity of NM23 proteins. To determine which enzymatic function is relevant to metastasis suppression, a panel of NM23-H1 has been developed in our laboratory in which the primary biochemical activities of the molecule (EXO, NDPK, and hisK) have been individually inactivated. Lysine-12 has

been reported as the most critical amino acid of NM23-H2 that cleaves the phosphodiester bond by transiently forming a covalent bond with DNA (Postel *et al*., 2000). In NM23-H1, $K_{12}Q$ mutant is also demonstrated deficient of exonuclease function. So far $K_{12}Q$ mutant is the only mutant we have which is exonuclease-deficient. So the transfected cells should lose metastasis suppression if exonuclease function and DNA repair activity are involved in metastasis suppression. Unfortunately, lysine-12 is equally critical for the NDP kinase reaction, suggesting a connection between the two seemingly disparate enzymatic activities, NDP kinase and nuclease. NM23 seems to use a single active site both for the cleavage of the DNA phosphodiester backbone and for the phosphorylation of nucleotides. So, $K_{12}Q$ mutant is also NDPK- and hisK-deficient. This would make the interpretation very difficult and complicated. To address this issue, another two mutants, $H_{118}F$ (NDPK- and hisK-deficient) and $P_{96}S$ (hisK-deficient) have been used for stable transfection and later *in vitro* and *in vivo* experiments. Comparison of the results derived from the three mutants will provide indirect evidence for studying the mechanisms of NM23 in metastasis suppression. E_5A is a promising mutant because it was reported exonuclease-deficient only with intact NDPK (Yoon *et al*., 2005). Results from this mutant would give us direct evidence to support the relationship between exonuclease and metastasis suppression. Now E_5A mutant has been constructed, and all three enzymatic functions have been measured. EXO is deficient in this mutant, while NDPK and hisK are intact. Table 3 is the summary of biochemical activities of wild-type and mutant NM23s.

Table 3. Enzymatic function of wild-type and mutant NM23-H1s.

	EXO	NDPK	hisK
WT	+++	+++	+++
$K_{12}Q$	+	+	-
$H_{118}F$	+++	-	-
$P_{96}S$	+++	+++	-
E_5A	+	+++	+++

Transfected cells were selected under G418 for about 4 weeks to get rid of non-stably transfected cells. Acquired colonies contain cells with GFP expression differently: 1) all the cells in one colony are GFP positive, suggesting that all cells are stably transfected; 2) cells in one colony are partially green, suggesting that some cells are devoid of plasmid DNA insert after multiple cell divisions; 3) all the cells in one colony are GFP negative, suggesting that those cells are either antibiotic resistant or pSV2Neo-transfected only. Existence of high percentage of GFP negative cells requires further selection. To obtain cells consistently expressing wild-type or mutant NM23, fluorescence-activated cell sorting (FACS) was performed on all the transfected cell lines. The sorting procedure has yielded more than 95% GFP positive cells in each transfected cell lines. Furthermore, the sorting procedure has yielded GFP positive cells with similar intensity of fluorescence, indicating that equal amount of NM23-H1 is expressed across cell lines (Figure 18A and B). Immunoblot analysis further validated the forced expression of wild-type and mutant NM23-H1, which was achieved at much higher levels than the endogenous NM23-H1 expression in the parent 1205LU and WM793 cells (Figure 18C).

After G418 selection and cell sorting, the stably transfected cell lines were ready for *in vitro* and *in vivo* study. But after several passages, 1205LU mutant transfected cell lines have exhibited decreased expression of GFP, suggesting decreased NM23-H1 mutant as well. This was confirmed by immunoblot result. Western blot of WCE from 1205LU and transfected cells at passage 8 demonstrated decreased expression of NM23 mutants as low as endogenous NM23 in parent cells while wild-type transfected cells are expressing 30-50% more NM23 proteins than parent cells (Figure 18D). Compared with the immunoblot result from passage 3, this result suggested that NM23 expressions are decreased in all the transfected cell lines after a while. This

could be caused by cell line which tightly limits NM23 expression in the cell. NM23 expression must be precisely controlled in 1205LU cells. In the early passage, forced expression broke the balance of this control system. So cells overexpress wild-type or mutant NM23s. After a serial passage, cells have gradually gained back the balance again and express much less amount of NM23s. This has been demonstrated by 2-D gel which allows the separation of wild-type NM23-H1 and variant mutant NM23-H1 because of their different pI. Compared with parent 1205LU cells expressing a certain amount of endogenous NM23-H1, all mutant transfected cell lines express equal amount of proteins, including 50% of endogenous NM23-H1, and 50% of mutant NM23-H1(Figure 19B). All these data suggested that 1205LU cells downregulated endogenous NM23 expression when overexpressing mutant NM23s, without change the total amount of NM23 expression in the cell.

After the mutant NM23 proteins were confirmed to be persistently expressed in the transfected cells, these cells were used to perform *in vivo* and *in vitro* studies as designed. Even though there is strong evidence to support the role of hisK function in anti-metastatic activity, other enzymatic functions of NM23-H1 should also be considered. It is reasonable that multiple functions of this protein are involved in the metastasis suppression which could be a multi-step process. Our hypothesis proposed that 3'-5' exonuclease function of NM23-H1 play a major role in DNA repair and maintenance of genomic stability and loss of this protein could lead to accumulation of mutations, and thus tumor progression. Since NDPK function of NM23-H1 is also important in keeping NTP pool in balance while imbalanced NTP pool is the reason to induce mutations, loss of NDPK may be involved in tumor progression. So, all these three primary enzymatic activities of NM23-H1 were

determined systematically the relevance to antimetastatic action through both *in vitro* and *in vivo* studies.

To study the relevance of an enzymatic function to metastasis suppression, two human melanoma cell lines were chosen for proposed studies. 1205LU and WM793, represent highly metastatic and premetastatic cell line, respectively. Wild-type and variant mutant forms of NM23-H1 were stably transfected into two cell lines. Then, parent and transfected cells were used to measure the metastatic characteristics both in cell culture (such as motility, invasion) and *in vivo*, using standard approaches of experimental and spontaneous metastasis.

Before the test of metastatic characteristic of two cell lines, transformed growth in cell culture was determined. *In vitro* cell transformation is associated with certain phenotypic changes such as loss of contact inhibition and anchorage independence, allowing cells to form colonies in soft agar. The definition of metastasis suppressor gene describes the way how those genes work. Re-expression of those genes back into tumor cells should have no effect on primary tumor size, but should reduce metastasis formation significantly. A constitutive murine *nm23-1* expression construct was transfected into highly metastatic K-1735 TK murine melanoma cells, and these *nm23-1*-transfected TK clones exhibited no significant differences in intrinsic tumor cell growth, i.e., primary tumor size *in vivo*, anchorage-dependent growth rate *in vitro*, and anchorage-independent colony formation in soft agar *in vitro* while they exhibited significant reductions in tumor metastatic potential independent of tumor cell growth (Leone *et al*., 1991).

Consistent with the data previously described, transfection of wild-type and mutant NM23-H1 has no impact on proliferation rate of two human melanoma cell

lines. This is very important to know because different cell proliferation rates would confound the result of metastasis assay, such as migration assay, invasion assay, in particular, spontaneous metastasis assay because of the long time period of this study. For colony formation in soft agar, all transfected WM793 cell lines formed colonies as many as parent cells. But for 1205LU cell line, wild-type, $K_{12}Q$, and $H_{118}F$ H1 transfected cells exhibited similar colony formation ability as parent cells; only $P_{96}S$ H1 transfection demonstrated dominant-negative function and significantly reduced colony formation. The reason is unclear because $P_{96}S$ mutant is hisK deficient, while $K_{12}Q$ and $H_{118}F$ mutant are both hisK deficient. It is also unclear whether the deficiency of colony formation ability in soft agar is the reason to cause less lung metastases formed in nude mice in spontaneous metastasis assay compared with another two mutants (Table 2). Another result we found is the colony size in soft agar of two melanoma cell lines. 1205LU cell lines formed much bigger colonies which are consisting of 100-200 cells in each single colony, suggesting the high malignancy of this tumor cell line, while WM793 cell lines formed much smaller colonies which are consisting of 20-50 cells in each single colony, suggesting the much lower malignancy of this cell line (Figure 20). Because of this low tumorigenicity of WM793 cells, we didn't get primary tumors or lung nodules formed in all the animal experiments, including both spontaneous metastasis assay and experimental metastasis assay by injecting WM793 parent and transfected cells into both nude mice and SCID mice.

Tumor cells must be able to detach from the primary tumor and invade the surrounding tissue at the beginning of metastasis. To accomplish these steps, they utilize a serial of proteinase, such as matrix metalloproteinases (MMPs), serine proteinases, and cathepsins, to degrade and remold the surrounding stroma or ECM.

In this project, both motility assay and invasion assay were employed to measure whether transfection of variant H1 forms will have any effect on these two characteristics of tumor cells. At room temperature, BD Matrigel™ Matrix polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane. Cells behave as they do *in vivo* when they are cultured on BD Matrigel™ Matrix. It provides a physiologically relevant environment for studies of migration and invasion. For metastatic 1205LU cells, transfection of wild-type NM23-H1 inhibits both motility and invasion significantly, consistent with its classical metastasis suppressor actions. All the enzymatic deficient mutant H1s inhibit motility and invasion ability of 1205LU cells as much as wild-type H1, even though $P_{96}S$ mutation (hisK- deficient) was previously described to be able to disrupt NM23-H1 mediated inhibition of motility and invasion (Freije *et al*., 1997). These results suggested that any of these enzymatic functions may not have direct effect on cell motility and invasion activities. Surprisingly, for premeatstatic WM793 cells, transfection of wild-type or mutant H1 did not suppress cell motility. This is a result inconsistent with metastasis suppressor activity of H1 and the reason is unknown. Different from motility assay, transfection of wild-type H1, $H_{118}F$ and $P_{96}S$ mutant H1 markedly inhibit invasion activity of WM793 cells, but $K_{12}Q$ mutant H1 disrupts this inhibition function by wild-type and other mutant H1. $K_{12}Q$ is the only mutant we have that lost exonuclease function, suggesting this enzymatic function may play some roles in cell invasion activity directly or indirectly.

NM23 proteins may resist genetic and epigenetic changes by regulating chromatin structure to preserve genomic integrity. We propose that 3'-5' exonuclease may mediate the transactions with genome to oppose progression of premetastatic cells to metastatic cells. This progression-inhibiting activity of NM23-

H1 could be the primary role in metastasis suppression activity. WM793 cell, a VGP cell line, makes an appropriate model system for our proposed studies because it's highly invasive but non-metastatic as well as the NM23s level in this cell line is under detection. Replacing back wild-type and enzymatically-defective forms of NM23-H1 into the cell made quite stable cell lines and helped us to find out the contributions of enzymatic functions of NM23-H1 to the process of progression. WM793 cells reproducibly give rise to metastatic forms when placed under selective pressure, such as cultured in protein-free (growth factor-free) medium, or repeated passaging *in vivo* (Rodeck *et al*., 1987: Herlyn *et al*., 1990). Complementation of wild-type NM23-H1 significantly blocks the progression process of WM793 cells which did progress markedly after 4 cycle's passage in protein-free medium. Constructs of the 3'-5' EXO-deficient mutant, $K_{12}Q$ lost the function of progression blockage exhibited by wild-type H1, possibly by disrupting DNA repair or chromatin remodeling activities of small endogenous NM23 pools via a dominant-negative mechanism. To our surprise, $H_{118}F$ mutant H1 also opposes progression of WM793 to the more malignant phenotype after multiple cycles of selection. $H_{118}F$ mutant is both NDPK- and hisK-deficient. If histidine kinase is not correlated to anti-progression action, then NDPK must be involved in progression-inhibiting action. It is reasonable considering the importance of maintenance of balanced NTP pool by NDPK to preserve genomic integrity.

In vitro assay can only determine one or two features of cells associated with metastasis. *In vivo* metastasis assay is more important because it mimics the whole process of cancer metastasis, which is more clinically associated. In particular, spontaneous lung metastasis recapitulates most of the steps of cancer metastasis. So, this assay would provide more accurate and more clinically related information

over the course of the proposed experiments. Transfection of wild-type or mutant NM23-H1 has no effect on primary tumor growth even though $K_{12}Q$ mutant H1 transfected 1205LU cells reached target tumor size 5 days later. Despite the high invasiveness and highly metastatic features of 1205LU cells, it took another 3 months for the formation of lung metastases, implying the low efficiency of metastasis process. Interestingly, the *in vivo* metastasis results of 1205LU cell lines are in very similar pattern as *in vitro* progression assay of WM793 cells. As shown in table 3, wild-type H1 and P96S mutant H1 inhibit lung metastasis, while $K_{12}Q$ and $H_{118}F$ failed to oppose metastasis of 1205LU cells, suggesting exonuclease and NDPK, instead of hisK are the primary enzymatic functions contributing to metastasis suppressor activity. In this animal model, all cell lines must survive and grow in a comparatively growth factor-deprived environment, so the selective pressure placed on these cells would cause the similar effects as in progression assay of WM793 cells. Injection of 2 millions of parent or transfected 1205LU cells through tail vein set up a high barrier to overcome by treatments. In this experiment, even wild-type H1 transfected cells formed Lung nodules in 4 out of 10 mice, with whole lung metastases formed in one mouse. This “overdose” injection makes less difference between different treatments. Fewer amounts of tumor cells should be injected when this experiment is repeated.

CHAPTER FIVE

GENERAL DISCUSSION

5.1. Summary of findings

Both tumorigenesis and metastasis are induced by genetic instability which is a result of multiple mutational “hits”, including misregulation of gene transcription as well as the loss of DNA repair enzymes (reviewed in Hartsough and Steeg, 1998). Some of the well-studied oncogenes, such as *ras* and *myc*, induce both cell transformation and metastatic competence (reviewed in Bernards and Weinberg, 2002). When tumor cells obtain further genetic alterations, some cells acquire sufficient capacity to dissociate from the primary tumor, survive in the bloodstream, spread through the circulation, and proliferate at the secondary site. Eventually these tumor cells progress from benign tumor to malignant tumor with accumulating mutations.

The hypothesis that loss of gene functions could also induce tumor progression prompted the idea of metastasis suppressor gene. As the first metastasis suppressor, NM23 was identified in 1988 (Steeg *et al*., 1988). Since then more than fifteen suppressor genes have been identified by using many techniques, such as differential display, microarray, microcell-mediated chromosome transfer, subtractive hybridization, etc (reviewed in Shevde and Welch, 2003). These genes affect many aspects of signal transduction, including pathways that are involved in invasion (TIMPs), growth factor receptor signaling (KAI-1), the RAS-MAPK pathway (NM23) (reviewed in Steeg, 2003), some metastasis suppressors affects cell-cell adhesion (Cadherins) (reviewed in Jiang, 1996; Berx *et al*., 1996), and transcription (Brms1)

(Meehan *et al*., 2004: Cicek *et al*., 2005), or metastatic colonization (MKK4) (Hickson *et al*., 2006).

To identify differentially expressed genes that might be essential for metastasis, murine melanoma cells were used to perform differential colony hybridization. NM23-H1 was discovered on the basis of its much higher level in cells with low metastatic activity than in their highly metastatic counterparts (Steeg *et al*., 1988). Low NM23-H1 protein and mRNA expression correlated with a variety of tumor types, including breast, melanoma, and gastric, ovarian carcinomas (reviewed in De la Rosa *et al*., 1995). But in general, cancers containing overexpressed, mutated NM23-H1 protein are rare events (Leone *et al*., 1993; Chang *et al*., 1994), whereas loss of expression of NM23-H1 is a common feature of aggressive, poorly differentiated tumors (reviewed in Hartsough and Steeg, 1998).

Over 18 years of intensive study, multiple and diverse enzymatic activities (e.g. NDPK, hisK, and 3'-5'EXO) and regulatory activities have been ascribed to NM23s, but the molecular mechanism underlying the metastasis suppressor activity of NM23-H1 remains unclear. HisK is the only enzymatic activity that correlates tightly with the motility-suppression effect of NM23-H1 even though the downstream substrates have not been identified yet. NDPK appears to be not related to`metastasis suppression. Our lab has demonstrated that NM23 proteins can modestly repress gene transcription, possibly in the manner of a classical transcription factor (Ma *et al*., 2002). Later, we also demonstrated NM23-H1 possesses 3'-5' exonuclease function (Ma *et al*., 2004). This function is appealing as the metastasis suppressor activity because it might have the effect on multiple aspects of the metastatic phenotype. The most direct function would be an antimutator activity through which genetic alterations are opposed, and thus,

malignant progression is blocked. From our previous studies, some important questions arise: Does NM23-H1 downregulate oncogene, such as PDGF, directly or assist other negatively regulatory proteins to downregulate PDGF? Is the 3'-5' EXO function of NM23-H1 involved in DNA repair and, if so, what kind of DNA repair mechanism is this protein involved? Is NM23-H1 associated with suppression of tumor progression, and which enzymatic function is involved? Is the 3'-5' EXO a key mediator of the metastasis suppressor activity of NM23-H1? Are NDPK and hisK functions also related to this activity?

To address all these questions systematically, first, transcriptional regulation of NM23-H1 and another single-DNA-stranded binding protein Purα on PDGF-A chain was determined to study the direct and indirect regulations of NM23 on oncogene PDGF. Then, two tumor cell lines (HepG2 and HeLa) were employed to examine the nuclear translocation and nuclear foci formation of NM23s on DNA damage. Finally, two melanoma cell lines (prematastatic WM793 and metastatic 1205LU) were constructed to study the impact of wild-type and enzymatically-defective forms of NM23-H1 on metastatic phenotype and the process of metastatic progression. Resolving these questions would add greater knowledge on relationship of cancer metastasis and NM23, and aid in the development of mechanism-based therapeutic strategies for cancer metastasis treatment and prevention.

5.1.1. NM23-H1 minimally downregulates PDGF-A chain promoter and has no effect on transcriptional regulation of Purα on PDGF-A chain promoter

NM23-H2 activates transcription of *c-MYC* promoter by binding to its nuclease-hypersensitive element (NHE) (Postel *et al*., 1993; Berberich and Postel, 1995; Lee *et al*., 1997). NM23-H1 was first identified as a DNA-binding protein with

transcriptional repressor activity on the PDGF-A chain promoter in our lab (Ma *et al*., 2002). Basal transcription of the PDGF-A chain gene is mediated by the interplay of positive and negative regulatory elements in the promoter region, which are GC-rich and possess non-B DNA structure. Screening of a HeLa cDNA expression library with the C-rich strand of a PDGF-A silencer sequence (5'-S1 nuclease-hypersensitive site (SHS)) yielded three cDNA clones encoding NM23-H1, the known protein implicated as a suppressor of metastasis in melanoma and breast carcinoma. Transient transfection analyses in HepG2 cells revealed that NM23-H1 modestly repressed transcriptional activity driven by the PDGF-A basal promoter (-82 to +8) as well as the negative regulatory region (-1853 to -883), indicating a potential role for NM23 proteins in repressing transcription of a growth factor oncogene, and providing a possible molecular mechanism to explain their metastasis-suppressing effects.

Recently, cDNA microarrays were used to investigate the downstream genes involved in NM23-mediated suppression of metastasis (Zhao *et al*., 2004). Microarray analyses revealed significant as well as consistent alterations in the expression (up- and downregulation) of more than 2000 genes which are involved in different cellular functions: invasion and metastasis, apoptosis and senescence, signal transduction molecules and transcription factors, cell cycle and repair, adhesion, and angiogenesis. The results suggest the role of NM23 may play in transcriptional regulation of a large amount of downstream genes. Furthermore, analyses by chromatin immunoprecipitation (ChIP) in viable M14 cells (human melanoma cell) showed DNA sequences bound to NM23 are correlated to oncosuppressor gene *p53*, *WT1, ING1*, and *NM23-H1* (Cervoni *et al*., 2006), suggesting NM23 binding is involved in the transcription regulation of these genes.

To further investigate whether NM23 facilitates other transcription factors to bind to silencer region of PDGF-A chain promoter, and form functional silencer complex, Purα was purified and examined for its binding and functional regulation of PDGF-A chain promoter alone and with NM23-H1. Purα is a single-stranded DNA binding protein and can function either as a repressor or activator depending on promoter and cellular context. The original model proposed in our lab hypothesized that 5'SHS silencer activity is mediated by the binding of NM23-H1, and other single-strand specific protein, such as Purα, may serve to stabilize a DNA conformation required for repressive interactions of the silencer with transcriptional of A-chain gene.

Despite the sequence-specific binding of Purα with NHE fragment as well as 5'SHS silencer region *in vitro* (Figure 5B), transient transfection analyses demonstrated that Purα does not appear to interact functionally with the 5'-SHS silencer (Data not shown). Induction of NHE activity by Purα dominated over the modest repressive activity of NM23-H1 (Figure 6A). GST-pull down assay indicated no physical interaction between NM23-H1 and Purα (data not shown), which is not supportive for our original hypothesis of functional silencer complex. All these results, taken together, suggested that NM23-H1 has very weak transcriptional regulation on growth factor oncogene PDGF, and has no effect on another positive transcription factor Purα of PDGF-A chain promoter. Considering the broad transcriptional regulation fuction of NM23-H1, may be its transcriptional regulation on other genes, such as some oncogenes, or tumor/metastasis suppressor genes, should be investigated.

Because the effect of transcriptional regulation of NM23-H1 is very subtle, it seems not to be an ideal model to address the profound metastasis suppressor activity. A new hypothesis should be proposed to explain its anti-metastasis function.

5.1.2. NM23s may play a role in DNA repair

Some hereditary cancer syndromes are associated with DNA repair deficiencies and increased chromosomal fragility (reviewed in Fearon, 1997). Xeroderma pigmentosum (van steeg *et al*., 2000), Fanconi's anemia (reviewed in Strathdee and Buchwald, 1992), and ataxia telangiectasia (Swift *et al*., 1991) are inherited human disease associated with a predisposition to cancer, chromosomal instability, and DNA repair defects. Specifically, DNA repair gene inactivation seems lead to a "mutator phenotype", with a resultant increased rate of mutations in other cellular genes, so initiation and progression of a cancerous cell may be greatly accelerated by the inactivation of DNA repair genes (reviewed in Fearon, 1999).

During tumor progression, cancer cells must exhibit a mutator phenotype. Everyday, approximately 10^5 lesions are introduced in genomic DNA in one single cell (reviewed in Ames *et al*., 1995). Most of these lesions are recognized and corrected by cellular repair mechanism. Only a very small fraction of lesions escape and result in mutations at the time of DNA replication. These early-formed mutations could be random and may be involved in genes maintaining genetic stability, such as DNA repair enzymes. If so, a cascade of mutations would be ensued, and then multiple mutations throughout the genome will be accumulated. When tumor cells accumulate enough mutations which allow them to invade surrounding tissues, evade from immune recognition and destruction, and to proliferate at the secondary site, they become metastatic (reviewed in Bielas and Loeb, 2005).

3’-5’ exonuclease activity of NM23-H1 was identified by our lab (Ma *et al*., 2004). This enzymatic activity of other known 3’-5’ exonucleases can proofread for DNA polymerase and excise the incorrectly incorporated nucleotides during DNA replication. So, enzymes that contain 3’-5’ exonuclease activities are involved directly in maintaining genome stability (reviewed in Shevelev and Hübscher, 2002). Loss of this function could lead to strong mutator phenotype. In *E.coli*, when *ndk*, the gene for NDP kinase, was disrupted, no effects on cell growth or morphology were found surprisingly. However, a mutator phenotype was found in *ndk*-disruption strains with significantly increased frequencies of spontaneous mutations to rifampicin and nalidixic acid resistance (Lu *et al*., 1995). In Drosophila, mutation in *abnormal wing discs (awd)* gene, 78% identical to the *nm23* gene of mammals, causes morphologically abnormal wing discs and poorly differentiated leg and eye-antenna discs, as well as defects in brain cells and ovary cells (Dearolf *et al*., 1988). In mice, *nm23-M1* gene knockout is not teratogenic and the pups can grow to adult age without apparent health problems. However, they undergo a growth retardation and knocked out females cannot feed their pups (Arnaud-Dabernat *et al*., 2003). Human breast and ovarian carcinoma cells transfected with *nm23-H1* exhibit increased sensitivity to cisplatin, which is typically a phenotype of DNA repair defects (Ferguson *et al*., 1996; Aebi *et al*., 1996). Taken together, all these phenotypic properties listed above and the nature of NM23-H1 as a 3’-5’ exonuclease indicate that NM23 is an obvious DNA repair protein candidate, even though the involvement of NM23 in DNA repair has not been shown directly.

Upon damage of DNA in eukaryotic cells, several repair and checkpoint proteins undergo a dramatic intranuclear relocalization, translocating to nuclear foci thought to represent sites of DNA damage and repair (Barr *et al*., 2003). To further

investigate the involvement of NM23 in DNA repair, nuclear translocation and nuclear foci formation of NM23 on DNA damage were examined by using two tumor cell lines, HepG2 and HeLa cells. Etoposide and cisplatin were used as DNA-damaging agents to treat cells for different time courses. Western blot, immunofluorescence microscopy, and confocal microscopy results unanimously demonstrated that nuclear relocalization of NM23 in two cell lines in response to DNA damage. Nuclear foci bodies were also obvious in Hela cells after 4 h and 24 h etoposide treatment even though no colocalization of NM23 and Ape1, an endonuclease described previously interacting with NM23 in a complex (Fan *et al*., 2003), was seen. All these evidence strongly suggests that NM23s play a very important role in DNA repair.

After the role of NM23 in DNA repair was approved, a new question comes out: what DNA repair pathway is NM23 participated in? First, base excision repair (BER) is a possible pathway based on the nuclease feature of NM23. Site-directed mutagenesis identified that Lys12 is the critical amino acid responsible for the cleavage activity by NM23-H2. Substitution of lysine with glutamine completely abrogates DNA cleavage activity (Postel *et al*., 2000). Similar result was found in NM23-H1 in our lab. Enzymes that use a lysine-amine to cleave DNA belong to a subtype of BER enzymes known as bifunctional glycosylase/lyase (reviewed in Postel, 2003; Nash *et al*., 1997). It is highly likely that NM23-H1 and –H2 cleave DNA via the glycosylase/AP lyase like mechanism. Secondly, mismatch repair (MMR) should also be considered. MMR proteins are known to mediate the cellular response to cisplatin damage (reviewed in Postel, 2003). Cells deficient in DNA MMR show moderate cisplatin resistance (Fink *et al*., 1996; Drummond *et al*., 1996). NM23 was observed to increase and translocate into nucleus in response to cisplatin

treatment in tumor cell lines in our study, suggesting MMR is a candidate repair mechanism for NM23; however, our previous data demonstrated that purified recombinant NM23-H1 only digests overhanging mismatched 3' termini from double-stranded DNA templates, and does not cleave internal mismatches (Ma *et al*., 2004), suggesting the limitations of the role of NM23-H1 in MMR. Finally, since NM23 was shown to respond to etoposide damage and etoposide is a topoisomerase II usually inducing double-strand breaks (DSB), DSB repair (DSBR) could also be one repair pathway NM23 using as a cellular defending mechanism for DNA damage. In mammalian cells, DSBR is mediated either by the error-free homologous recombination (HR) or the more predominant error-prone nonhomologous end joining (NHEJ) pathways (reviewed in Jackson, 2002). We are currently investigating which DNA repair mechanism is NM23 involved in repairing DNA damage. It's possible that three pathways could be induced individually or all together depending on different insulting. More DNA damage methods, such as ultraviolet light, ionizing radiation, hydroxyurea, and MMS, etc, should be used to trigger cell DNA damage to study the accurate repair pathways.

5.1.3. NM23-H1 opposes the progression of premetastatic melanoma cells, and NDPK and/or 3'-5' exonuclease could be the major enzyme associated with this activity

Tumors have long been known to become more aggressive in clinical behavior and more 'malignant' in their characteristics over time. This has been termed 'tumor progression' and includes, among other properties invasion and metastasis, as well as more efficient escape from apoptosis and host immune surveillance (reviewed in Nowell, 2002). Tumor progression is driven by enhanced mutagenesis and clonal

evolution, in which single cells expand by stepwise selections to populate a tumor (reviewed in Bielas and Loeb, 2005). Clonal evolution of more and more aggressive subpopulations involves multiple sequential genetic changes in a variety of genes. Perhaps the best documented example of clonal evolution in a human malignancy is colon cancer, in which Vogelstein and coworkers (Kinzler and Vogelstein, 1996), as well as other laboratories, have shown a series of genetic changes associated with the clinical progression from benign colonic polyps to invasive and metastatic adenocarcinoma.

The clinical and histologic progression observed in the growth phases of melanoma is hypothesized to correspond to the accumulation of genetic mutations critical for cell proliferation, differentiation, and cell death (Clark *et al*., 1984). Primary melanoma cells in vertical growth phase (VGP) are phenotypically and genetically different from metastatic melanoma cells (MET). VGP cells must go through progression to develop MET cells. To examine whether NM23-H1 opposes the progression of VGP cells *in vitro* and whether 3'-5' exonuclease of NM23-H1 is the major mediator of this activity, premetastatic melanoma cell WM793 was chosen due to the loss of expression of NM23-H1 and H2 in this cell line and stably transfected with wild-type and variant mutant NM23-H1. Then all the transfected WM793 cell lines alone with parent cells were exposed to protein- free medium (FBS- and insulin-free) for selection of growth factor independence. The whole experiment took 4 rounds of selections to be finished (Figure 23). The first two selections didn't make significant difference between parent cell and all the transfected cell lines. The marked difference in colony formation numbers among cell lines was obvious after the third round of selection, and the difference in colony size was obvious after the fourth round of selection. Significant progression of WM793 cells cultured in protein

free medium has been demonstrated by about 10 folds increase in colony numbers (20 colonies out of 1000 cells after first round of selection vs 200 colonies out of 1000 cells after fourth round of selection). Wild-type NM23-H1 significantly opposes this progression process as well as $P_{96}S$ mutant (HisK-deficient), suggesting NM23-H1 may block the accumulation of mutations as a DNA repair protein, and histidine kinase is not involved in DNA repair function of H1 and thus prevention of tumor progression.

As expected, $K_{12}Q$ mutant (EXO-deficient and NDPK-deficient) almost totally lost the activity to oppose tumor progression. The colony formation efficiency and colony size of $K_{12}Q$ mutant transfected cells were very close to that of parent cells. These results suggested that both NDPK and/or exonuclease may be involved in decreasing mutation rate and opposing tumor progression. Exonuclease activity of H1 decreases mutation rate through DNA repair mechanism while NDPK activity of H1 may lower mutation rate by keeping balanced dNTP pool to prevent misincorporation of nucleotide during DNA replication. The involvement of NDPK in opposing tumor progression was confirmed by the partial blockade of progression of $H_{118}F$ transfected WM793 cells. Histidine kinase activity of H1 may have effects on tumor cell motility or invasion ability, it seems to have nothing to do with decreasing mutation rate and progression suppression.

After 4 rounds of selection in protein free medium, all the parent WM793 and transfected WM793 cells progressed to be more aggressive and more growth factor-independent, which is one of the phenotype metastatic melanoma cells possess. But it’s not for sure whether these cell lines have acquired enough abilities to metastasize as 1205LU cells. It is estimated that between six and ten clonal successions may be required to generate highly malignant human cancer cells

(reviewed in Bernards and Weinberg, 2002). It would be interesting if those selected cell lines could be used for *in vitro* and *in vivo* metastasis assay compared with metastatic 1205LU cells, and also used for microarray to find out the genetic alterations compared with the counterparts before selections.

5.1.4. NDPK and/or 3'-5' exonuclease activities could be the mediators of metastasis suppression

The major goal of this project is to identify the underlying molecular nature of metastasis suppression of NM23-H1. Two melanoma cell lines were chosen for our study model because of their very low amount of expression of NM23-H1 and H2. After stably transfected with wild-type and variant mutant H1 and verified the protein expression by western blot and 2D-gel, two cell lines were performed both transformation phenotype assays and metastasis phenotype assays. Due to the very low tumorigenicity and metastasis ability of WM793 and transfected WM793 cells (about 5% tumor formation rate and 5% metastasis rate), we failed all the *in vivo* animal experiments (including spontaneous metastasis assay and experimental metastasis assay) with these cells by using either athymic nude mice and more immunocompromised SCID mice.

As a metastasis suppressor gene, nm23-H1 transfection has no effect on cell proliferation rate (Leone *et al*., 1991; Russell *et al*., 1998). However, some data provide evidence that the *nm23* genes are involved in cell proliferation (Cipollini *et al*., 1997; Caligo *et al*., 1997), and they are preferentially expressed in the S-phase of the cell cycle (Sorscher *et al.,* 1993; Caligo *et al.,* 1995). To find out whether transfection of wild-type and mutant nm23-H1 has any effect on WM793 and 1205LU cells, MTT assay was performed and both cell lines exhibit similar proliferation rate

for transfected cells compared with parent cells. This is very important to know because some of our experiments, such as selection for growth factor independence and spontaneous metastasis assay, are very time-consuming. They took 8 months and 4 months, respectively, to be finished. So, if the proliferation rate is different among cell lines, then it may change or complicate the interpretation of metastasis results.

The dislodgement of tumor cells from the primary tumor, and followed by subsequent invasion into neighboring tissue is the first step in the formation of tumor metastasis. Tumor cell invasion requires specific enzymes (proteases, collagenases) to break up the barrier and to migrate through the basal lamina. In *in vitro* Boyden chamber assay, only cells with ability to secret proteolysis enzymes and ability to migrate can reach the lower chamber. As we expected, transfection of wild-type NM23-H1 inhibited cell invasion in 1205LU and WM793 cells, consistent with the definition of metastasis suppressor and previous data (Cantor *et al*., 1993). Interestingly, in 1205LU cells, $K_{12}Q$ mutant H1 didn't lose the inhibition of invasion as we expected, but the inhibition of invasion was lost in WM793 cells, suggesting the critical role of exonuclease activity of H1 in preventing progression of premetastatic melanoma cells. For 1205LU cells which are metastatic cells, since the genetic changes have occurred before H1 was overexpressed, transfection of exonuclease deficient H1 did not cause loss of inhibition function. While in the premetastic WM793 cells, genetic changes responsible for metastasis phenotype haven't taken place, so overexpression of mutant H1 had different effect on invasion inhibition. Furthermore, $P_{96}S$ mutant H1 didn't lose the invasion inhibition in both cell lines, inconsistent with previous data from Steeg group (MacDonald *et al*., 1996; Freije *et al*., 1997). This inconsistency could be caused by different cell types, or caused by

having no effect of H1 transfection on proteolytic enzyme releasing in those cells even though the motility ability may have changed.

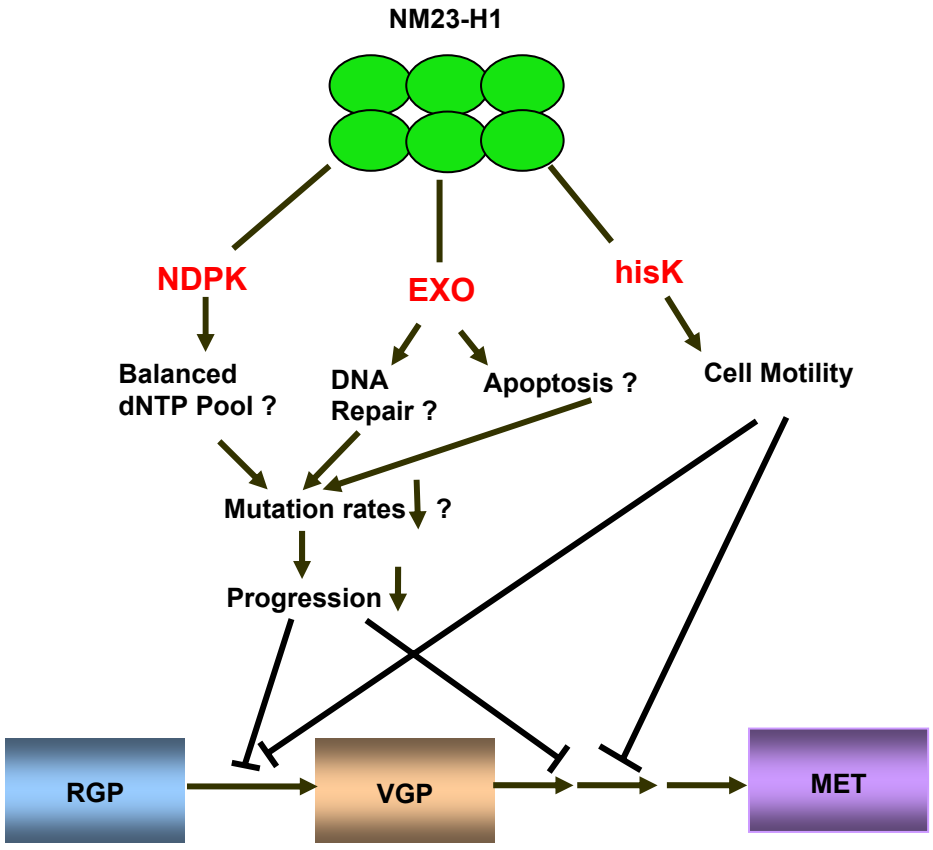
Metastasis is a complex physiologic process and investigation of the process has proven a challenging task. *In vitro* metastasis assays can only capture one or two aspects of metastatic tumor cells instead of whole complex physiologic process. So they are by no means comprehensive and accurate. *In vivo* models provide the most physiologically relevant models for studying metastasis, especially, the spontaneous lung metastasis, which plausibly mimics the clinical situation of metastasis both in pathophysiology and location. Experimental metastasis (through tail vein injection) does not recapitulate all the necessary steps that a cell needs to perform in order to metastasize from an ectopic site. It tests the ability of the cells to survive in the blood stream, to colonize, and to grow in the lung. In our spontaneous lung metastasis assays, two millions of 1205LU parent cells and all the transfected cells were injected under the skin. Five to seven days later, primary tumors formed in every group. The growth rate of primary tumors in each group is quite similar except $K_{12}Q$ mutant H1 transfected cells. Tumors in this group grew slower than other groups after 10 day postinjection, but still reached 800 mm^3 (the size ready to be removed) on day 30 postinjection. The reason to cause this slightly slow growth is not clear. After all the tumors reached 800 mm^3, they were removed and all the mice were kept for another 3 months to let tumor cells to metastasize to the lung. At the end of this experiment, all the mice were sacrificed and lung metastasized nodules were counted. As shown in table 2, wild-type H1 inhibited lung metastasis as well as $P_{96}S$ mutant H1, while $K_{12}Q$ and $H_{118}F$ mutant H1 lost metastasis suppression activity, suggesting the involvement of exonuclease and NDPK in the metastasis suppression.

The lung metastasis assay has the similar result as that of selection of growth factor independence assay even though two different cell lines were used in these two experiments respectively. There is one common point for these two experiments: long period. Selection of growth factor independence assay took 8 months, and lung metastasis assay took 4 months. This long period of the whole experiment means all the tumor cells were selected under the influence of NM23-H1 or individually enzymatic deficient mutant H1. So cells progressed with genetic changes if their mutation rate is increased (imbalanced dNTP pool) or loss of function of DNA repair (loss of exonuclease).

NDPK activity has long been thought as a function not relevant to metastasis suppression. This is the first time that we provide evidence to show the relevance of this enzymatic function to metastasis suppressor activity. The evidence includes the involvement of $H_{118}F$ mutant and $K_{12}Q$ mutant in anti-progression of premetastatic WM793 to metastatic phenotype in cell culture, as well as anti-metastasis in animal model. Both of the two mutants lack NDPK activity, suggesting this enzymatic function is essential in maintaining metastasis suppressor activity and anti-progression activity. Even though hisK is also deficient in $H_{118}F$ and $K_{12}Q$ mutants, $P_{96}S$ mutant H1 (hisK-deficient only) still keeps anti-progression activity in cell culture and anti-metastasis activity in spontaneous metastasis assay, strongly suggesting hisK is not associated with these two activities. EXO is deficient in $K_{12}Q$ mutant only and NDPK is also deficient in this mutant, so the involvement of EXO in anti-progression and anti-metastasis activities could be alone or in concert with NDPK. The direct evidence will be provided when E_5A mutant H1 is tested for anti-progression and anti-metastasis activities.

Taken all the results together, a working model is proposed to address the molecular mechanisms that mediate the metastasis suppressor activity of NM23-H1 (Figure 27).

Figure 27. Molecular mechanisms that mediate metastasis suppressor activity of NM23-H1 in melanoma cells.



5.2. Future studies

This project is mainly focusing on the study of underlying mechanisms of metastasis suppression of NM23-H1. Even though some questions have been answered by our preliminary data, intensive investigations should be done to understand the functions of NM23 in metastasis as well as the development of novel therapeutic strategies to combat progression to the metastatic phenotype.

5.2.1. To further study exonuclease function of NM23-H1 in blocking tumor progression and metastasis suppression

$K_{12}Q$ mutant is the only exonuclease-deficient mutant used in the current project. But this mutant is also NDPK and hisK deficient. Since NDPK may be associated with metastasis suppression and progression inhibition, it's necessary to use exonuclease deficient only mutant to confirm our current results. We have a candidate mutant E_5A. The mutant is previously described as exonuclease deficient but NDPK intact. We already constructed this mutant and the biochemical functions are under active test. Once the biochemical activities are tested and the E_5A mutant genes are stably transfected into cell lines, all the *in vitro* and *in vivo* metastasis assays as well as progression assay will be performed to examine the role of exonuclease in NM23-dependent metastasis suppression.

5.2.2. To compare the gene profile and protein expression profile in NM23-H1 deficient and proficient cell lines and to functionally validate some of these NM23-dependent genes

Previously, WM1158, another nm23-deficient metastatic melanoma cell line, and WRO82 cell line were infected with the Ad5-H1 adenoviral expression vector. Analysis of mRNA expression profiles revealed that 13 out of total 46 downregulated genes were associated with transcription, DNA repair, and replication. Microarray analysis will be performed for more cell lines, such as WM793, 1205LU, in pair wise, to compare the gene profile, as well as parent cells compared with transfected cells. Furthermore, WM793 parent and transfected cell line could be compared with the counterpart cell lines after 4 cycles of selection. Since cell progression was seen obviously after 4 cycles of selection, genomic alterations should be found by comparing cells before and after selection. Besides genomic technologies, proteomics may also be applied to identify molecular factors involved in nm23-associated metastasis suppression. After the nm23-dependent genes or proteins are found, functional validation will be performed by transfecting back those genes (downregulated genes in metastatic cells) or knocking out those genes (upregulated genes in metastatic cells) to see whether metastasis function will be suppressed in the cells.

5.2.3. To investigate whether introduction of NM23-H1 facilitates apoptosis following genotoxic stress

Recently, NM23-H1 was proposed as an endpoint effector of apoptosis (Fan *et al*., 2003). When cytotoxic T cell or natural killer cell attacks target cell by releasing granzyme A, granzyme A cleaves NM23-H1 inhibitor, SET, and unleashes NM23-H1 (activated DNase) to induce caspase-independent apoptosis. This finding suggests the possibility that immune surveillance may be compromised as a consequence of NM23-deficiency. To investigate whether introduction of NM23-H1 induces apoptosis under genotoxic stress, activation of caspase-8 will be measured across the 1205LU cell panel as an early indicator of apoptotic activity. Apoptosis will be measured under basal conditions and in response to genotoxic insult, such as etoposide, cisplatin and UV light. These assays should determine the extent to which 1205LU cells might be resistant to programmed cell death in the absence of NM23-H1 and its attendant 3'-5' EXO activity. In *in vivo* assay, examination of melanoma cell apoptosis at early times after lung tissue seeding of melanoma cells provides insight into tumor outgrowth at later stages. 1205LU cell panel will be injected into nude mice through tail vein. One week later, lung tissues will be excised and the levels of apoptotic cells will be examined by Dead-End TUNEL Assay in frozen tissue sections (Ervin and COX, 2005).

Appendix

20 July 2006 Our ref: CT/SS/Jul 06/J009

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Dear Dr Zhang

GENE, Vol 348, 2005, pp 25-32, Zhang et al, "Pura activities…"

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