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dissertation entitled

ROLE OF HEPATOCYTE GROWTH FACTOR AND ITS RECEPTOR MET IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

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Date Oct 9, 2001

ROLE OF HEPATOCYTE GROWTH FACTOR AND ITS RECEPTOR MET IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS

By

Hongyan Liang

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Molecular Biology

2001

ABSTRACT

ROLE OF HEPATOCYTE GROWTH FACTOR AND ITS RECEPTOR MET IN THE MALIGNANT TRANSFORMATION OF HUMAN FIBROBLASTS

By

Hongyan Liang

It is now generally recognized that cancer is the result of a multistep process, which involves the activation of oncogenes and the inactivation of tumor suppressor genes. In cancer cells, activation of oncogenes occurs mainly by mutations or over-expression. Conventionally, only mutated genes have been considered as candidate cancer-related genes. Recent advances in various techniques have identified many more genes that are altered in expression in cancer cells than are mutated. Non-mutated genes with stably altered expression patterns may be a key component of the cancer puzzle and may ultimately be traced back to aberrations in upstream genes, such as those encoding transcription factors.

The goal of my research has been to investigate the role of hepatocyte growth factor gene (*hgf*) and its receptor, the *met* gene, in the malignant transformation of human fibroblasts. I utilized human fibroblastic cells transformed to malignancy in culture in the Carcinogenesis Laboratory as well as human fibrosarcoma cell lines derived from patients' tumors. My first observation was that the MET protein was expressed at three to nine fold higher levels in ten out of 11 human fibrosarcoma cell lines tested, compared to several foreskin-derived normal human fibroblast cell lines. To determine whether these higher levels of expression play a causal role in the malignant transformation of human fibroblasts, I chose two human fibrosarcoma cell lines and down-regulated their expression of MET and/or HGF using chimeric transgenes consisting of U1 small nuclear RNA and hammerhead ribozymes targeting *met* and/or *hgf*. When injected into athymic mice, cell strains with reduced MET and/or HGF

expression exhibited reduced frequency and increased latency in tumor formation and growth. These results directly demonstrate a strong dependence on endogenous high levels of MET and/or HGF expression for both tumor formation and growth of human fibrosarcomas.

I also examined the mechanism(s) responsible for MET over-expression in human fibrosarcoma cell lines. Gene amplification was not found to be important for such over-expression. Instead, a majority of the fibrosarcoma cell lines with high levels of MET showed coordinately high levels of transcription factor Sp1. Deletion analysis and site-directed mutagenesis of the *met* promoter revealed that the tandem Sp1 sites in the proximal promoter region are important for the transcription of the *met* gene. Two human fibrosarcoma cell lines with high levels of MET and Sp1 exhibited much higher *met* promoter-luciferase activity than did two normal human fibroblast cell lines with low levels of MET and Sp1. Furthermore, transfection of Sp1 cDNA into a normal human fibroblast cell line resulted in a dose-dependent increase in the *met* promoter activity, whereas transfection of a human fibrobsarcoma cell line with an Sp1 decoy to interfere with and inhibit Sp1 binding to DNA led to a dramatic reduction in MET expression. These data demonstrate that transcriptional up-regulation by Sp1 is a major mechanism for MET over-expression in human fibrosarcomas. The results suggest that when highly expressed, Sp1 functions as an oncoprotein.

DEDICATION

To my mother, Xiangying Liao

for her endless love and faith in me

To my husband, Kai Li

who is the sunshine of my life

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my major professor, Dr. J. Justin McCormick, for his patience, encouragement and guidance throughout the course of my Ph.D. study. In addition, I want to express my sincerest gratitude to Dr. Veronica M. Maher for her insights, advice, and encouragement. Furthermore, I want to give special thanks to the other members of my graduate guidance committee, Drs. William R. Henry, David DeWitt and Peter Gulick, for their invaluable time and intellectual input during the preparation of this thesis. I am grateful to Dr. Katheryn Meek for her help as well.

Also I greatly appreciate the generous assistance and friendship from the past and present members of the Carcinogenesis Laboratory: Dr. Scott Boley, Dr. Wendy Davis, Dr. Susanna Kleff, Dr. W. Glenn McGregor, Dr. Sandra O'Reilly, Dr. Joseph Przybyszewski, Dr. Hong Zhang, Daniel Appledorn, Clarissa Dallas, Philip Kilanowski-Doroh, April T. Hoffman, Evan Kaplan, Suzanne K. Kohler, Zhenjun Lou, Terry McManus, Kristin McNally, Yun Wang, Lijuan Wang, and Jie Zhang. Especially, I want to sincerely thank Michele A. Battle, Jackie Dao, ZiQiang Li and Xi-De Wang for their friendship, support and company throughout my graduate study.

The love and support from my parents, my sister and brother-in-law, and my brother and sister-in-law are sincerely appreciated. Finally, I would like to thank my husband, Kai Li, for his great love and support. Without it, my adventure into science would not be so exciting and rewarding.

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LIST OF ABBREVIATIONS

ARF, alternative reading frame

APC, adenomatous polyposis coli

BPDE, benzyprene diol epoxide

BRCA, breast cancer

Cdc42, cell dependent cycle 42

CDK, cyclin-dependent kinase

CMV, cytomegalovirus

DMBA, 7,12-dimethylbenz(a)anthracene

DTT, dithiothreitol

EGFR, epithelial growth factor receptor

ERBB, erythroblastosis

ERK, extracelluar signal-regulated kinase

FAP, familial adenomatous polyposis

Gab1, Grb2-associated binder 1

GAP, GTPase activating protein

G Proteins, guanine-nucleotide binding proteins

HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

HGF, hepatocyte growth factor

HGFA, hepatocyte growth factor activator

HNPCC, hereditary nonpolyposis colorectal cancer

HNSCC, head and neck squamous cell carcinoma

HPRC, hereditary papillary renal carcinoma

hTERT, human telomerase reverse transcriptase

IL, interleukin

MAPK, mitogen-activated protein kinase

Max, Myc-associated factor X

MDM2, murine double minute clone 2

MEK, MAPK/ERK kinase

MMP, matrix metalloproteinase

MMR, mismatch repair

MT-MMP, membrane type matrix metalloproteinase

NER, nucleotide excision repair

PDGF, platelet-derived growth factor

PI3K, phosphotidylinositol 3-kinase

PKC, protein kinase C

PLC, phospholipase C

PMSF, phenylmethylsulfonyl fluoride

Rac, Ras-related C3 botulinum toxin substrate

Ral-GDS, Ras-like protein-guanine nucleotide dissociation stimulator

RB, retinoblastoma

Rho, Ras homology

RTK, receptor tyrosine kinase

SF, scatter factor

SH2, Src homology region-2

SH3, Src homology region-3

Shp, SH2 domain containing protein tyrosine phosphatase

snRNA, small nuclear RNA

SOS, son of sevenless

Sp1, specificity protein 1

STAT, signal transducers and activators of transcription

TCF/LEF, T cell factor/lymphocyte enhancer-binding factor

TF, tissue factor

TGF, transforming growth factor

TNF, tumor necrosis factor

TPA, tetra beta-phorbol 12-myristate 13-acetate

TPR, translocated promoter region

uPA, urokinase-type plasminogen activator

uPAR, urokinase-type plasminogen activator receptor

VHL, von Hippel-Lindau syndrome

VEGF, vascular endothelial growth factor

WT, Wilms tumor

XP, xeroderma pigmentosum

CHAPTER 1

LITERATURE REVIEW

I. Introduction of Molecular Mechanisms of Cancer

A. Evidence that Cancer Is a Genetic Disease

It is now generally recognized that cancer is fundamentally a genetic disease. Several lines of evidence support this theory: the detection of specific chromosome abnormalities in many types of cancer cells; the recognition of hereditary predisposition to some types of cancer; the connection between cancer susceptibility and impaired ability of cells to repair DNA damage; and the identification of alterations in cancer-related genes.

1. Chromosomal Abnormalities in Cancer Cells

Chromosome abnormalities are cytogenetically visible changes observed in almost all human cancers (1). The first chromosomal abnormality definitively associated with human cancer is the Philadelphia (Ph) chromosome, found in more than 90% of chronic myelocytic leukemia (CML) patients. The Philadelphia chromosome results from a reciprocal chromosomal translocation between chromosomes 9 and 22. Since then, more than 100 commonly occurring chromosomal translocations have been observed in leukemia, lymphoma and solid tumors. In addition to translocations, other chromosomal abnormalities found in cancer cells include chromosomal inversions, insertions, deletions, amplification and aneuploidy (2). Some of these aberrations occur only in specific cancer types. For example, deletion of 11p13 is found in Wilm's tumors and chromosome 9 monosomy is found in bladder adenocarcinoma (2, 3). Some of these aberrations are common to tumors of different types. For example, deletions of 3p13-23

region occur in small cell carcinoma, lung adenocarcinoma, renal cell carcinoma and ovarian adenocarcinoma (4).

2. Familial Cancers

Hereditary cancers represent a small fraction (1-3%) of total human cancers (5). Although rare, they have provided important insights into the origin and nature of human cancers. About 50 forms of hereditary cancers have been reported (6, 7). For example, about 40% of retinoblastomas and 20% to 40% of Wilm's tumors exhibit a dominant genetic inheritance pattern. Familial adenomatous polyposis (FAP) of the colon is transmitted as a Mendelian-dominant trait, with about 80% penetrance. Colorectal cancer will eventually develop in nearly 100% of the patients with FAP unless the colon is removed prior to cancer development (8). The Li-Fraumeni syndrome represents another hereditary neoplasia that is of an autosomal dominant trait with high penetrance (9). Members of the affected families may develop a variety of cancers at an early age, including sarcoma, breast cancer, brain cancer, and leukemia. Collectively, these hereditary diseases suggest that a number of inherited genetic aberrations predispose the patients to the onset of these cancers. The defective genes responsible for some of these diseases have been identified and cloned. For example, the *Rb* gene is linked to retinoblastomas (10, 11), the *p53* gene to the Li-Fraumeni syndrome (12) and the *APC* gene to the FAP (8). An individual with a hereditary cancer syndrome carries a germline mutation on one allele ('mutant allele') in the specific inherited cancer gene. This results in a much higher risk for cancer development in these patients, which often onset at a younger age than seen in individuals without germline mutations, and/or onset with multiple primary cancers (7).

3. Defects in DNA Repair and Predisposition to Cancer

Another convincing piece of evidence for a causal relationship between genetic mutations and human cancer comes from the fact that the incidence of cancer in the

patients with DNA repair deficiencies is greatly increased (13). Xeroderma pigmentosum (XP) is one of the most widely studied DNA repair-deficient diseases. These patients are characterized by extreme sensitivity of the skin to sunlight and usually suffer an age-specific incidence of skin cancer that is several thousand times higher than normal individuals. On sunlight-exposed area of the skin, they typically develop multiple skin tumors that lead to death from metastatic squamous or basal cell carcinoma or malignant melanoma. Genetically different forms of XP have been identified by cell fusion. Cells expressing the classical forms of XP (complementation group A through G) are deficient in nucleotide excision repair (NER) (14) and are much less efficient in removing DNA lesions induced by DNA-damaging agents, such as thymidine-thymidine (TT) cyclobutane dimers or pyrimidine (6-4) pyrimidone photoproducts caused by ultraviolet light from the sun, and therefore, are more prone to acquire genetic mutations than normal cells. In contrast, the cells belonging to the variant class of XP (XPV) are NER proficient, but have recently been found to be defective in polymerase eta, which is involved in error-free translesion synthesis past TT dimers, and therefore, are abnormally prone to acquire sunlight-induced mutations (15).

Another example is hereditary nonpolyposis colorectal cancer (HNPCC). On the basis of linkage studies, the majority of HNPCC loci were assigned to 2p22-21 and 3p21-23. Recently, several human homologues of the bacteria mismatch repair (MMR) genes *MutS* and *MutL* have been identified and matched to HNPCC locus, including h*MSH2* and h*MSH6* (*MutS* homologue, both mapped to 2p22-21) (16, 17), h*MLH1*, h*PMS1* and h*PMS2* (*MutL* homologues, all mapped to 3p21-23) (18-20). Defective MMR pathway in HNPCC patients leads to mutations in the poly (A) tracts and poly (CA) repeats, termed 'microsatellite instability' (21-23).

B. Types of Cancer-related Genes

The scientific inquiry into the molecular genetics of cancer has made remarkable advances over the past two decades. The identified cancer-related genes can be divided into two major groups: oncogenes and tumor suppressor genes (24, 25). Oncogenes are activated versions of cellular proto-oncogenes. They are dominant-acting, in that their effects can be observed when they are introduced into non-malignant cells. In cancer cells, 'activation' occurs by mutation, up-regulation, inappropriate expression, amplification, etc. (26). Tumor suppressor genes are normal cellular genes, which contribute to carcinogenesis through their loss of the ability to make functional protein by deletion, mutation, or silencing of the gene. Their behavior is recessive since both alleles must lose the ability to make functional protein (27).

1. Oncogenes

To date, more than 100 oncogenes have been identified in animal systems, but only a small subset have been found consistently as mutated genes in human cancer. Those involved in multiple tumor types include the genes that encode the growth factor receptor c-ErbB2, the membrane associated G protein Ras, the nuclear transcription factor c-Myc and the inhibitor of apoptosis Bcl-1 (28). Two examples of proto-oncogenes, *ras* and c-*myc*, are reviewed here in detail.

1.1. *ras* as a Prototype of Oncogenes

Ras is one of the most widely studied and most important oncogenes, which has been implicated in up to 30% of all human cancers (29). The human H-*ras* oncogene is homologous to the transforming sequence of the Harvey rat sarcoma virus. It is the first oncogene identified by transfecting DNA of human T24 bladder carcinoma cells into NIH3T3 mouse fibroblasts (30). H-Ras belongs to a family of small molecular-mass GTP-binding proteins (G proteins) that share the ability to bind guanine nucleotides and have intrinsic GTPase activity. The family members include H-Ras, K-Ras, N-Ras, R-

Ras and TC21, which have all been reported as potential targets for mutational activation in human cancers (31-36).

The Ras protein can be found in two states (GDP or GTP bound) and functions as a molecular switch. In cells at G_0 state, it binds to GDP since the protein has a higher affinity for GDP. Upon stimulation of appropriate signals such as growth factors, specific signal transduction leads to the release of GDP and the binding of GTP to the Ras protein (37-39). For example, the binding of epithelial growth factor (EGF) or platelet-derived growth factor (PDGF) to its corresponding receptor on the extracellular part triggers the autophosphorylation of certain tyrosine residues on the intracellluar part of the receptor. These tyrosine residues then serve as binding sites for adaptor proteins containing SH2 domains, including Grb2/Sos complex. The resulted recruitment of the guanine nucleotide exchange factor Sos to the plasma membrane leads to the release of GDP and the binding of GTP to Ras. The activated Ras protein in turn leads to the activation of the Raf1/MEK/ERK kinase cascade. The substrates of ERK include various molecules associated with cell growth and differentiation, including transcription factors (e.g., fos), cell cycle regulators (e.g. p21 and cyclin D1), and protein synthesis regulators (e.g., $p90^{rsk}$) (40, 41).

Mutations in the *ras* gene are the most frequently detected alterations of oncogenes in both animal tumor model systems and in human cancers (29). The oncogenic forms of the *ras* gene differ from their normal counter parts by having a single mutation in codon 12, 13, 59 or 61, which reduces their intrinsic GTPase activity and their ability to interact with GTPase activating proteins (GAPs) (42, 43). Thus, the oncogenic forms of Ras are persistently switched-on, and this can lead to uncontrolled cell growth through the activation of Raf1/MEK/ERK signaling pathways. The critical involvement of the Raf1/MEK/ERK cascade in mediating Ras transformation is supported by the observations that kinase-deficient mutants of Raf-1, MEKs, and ERKs

have been shown to block Ras-mediated transformation (44, 45) whereas dominant active mutants of Raf-1 and MEKs can cause tumorigenic transformation of rodent fibroblasts (46, 47).

However, recent studies indicate that Ras-induced transformation may be far more complicated. Besides controlling cell growth and differentiation, the Raf1/MEK/ERK pathway also induces the transcription of proteases involved in cell motility and invasion, such as uPA (48), and the transcription of proteins that induce angiogenesis, such as VEGF (49). In addition, the effectors of Ras have also been expanded from Raf1 to at least two more types of effectors: PI-3K and Ral-GDS (50, 51). The activation of Raf1/MEK/ERK independent pathways, which involve the activation of Rac1 and Rho A, has been shown to be required for oncogenic Ras-mediated transformation (52). These data suggest that Ras-induced transformation might result from a concerted action of multiple activities, and additional studies are needed for a complete story of Ras-induced tranformation (53).

1.2. c-*myc* as a Prototype of Oncogenes

The c-*myc* proto-oncogene was first described in 1982 (54) as the cellular homologue to the transforming sequence of the avian myelocytomatosis retrovirus MC29, and was subsequently found to be activated in various animal and human tumors (55). c-*myc* belongs to a family of *myc* genes that includes B-*myc*, L-*myc*, N-*myc* and S-*myc*; however, only c-*myc*, L-*myc*, and N-*myc* have been shown to have neoplastic potential (56-58).

The c-*myc* gene encodes a nuclear transcription factor, which contains a transactivation domain at the N-terminus and a dimerization interface consisting of a helix-loop-helix and leucine zipper (HLH/LZ) domain at the C-terminus. The proper function of c-Myc requires the presence of a partner protein: Max (Myc associated factor X), which contains a HLH/LZ motif but no transactivation domain (59). Myc-Max

heterodimer can bind to DNA and regulate transcription through the trans-activation domain of c-Myc, whereas Myc-Myc homodimer cannot bind to DNA. Immediately N-terminal to the dimerization domain is a domain rich in basic amino acids that directly contacts specific DNA sequences within the DNA major groove (60). Thus Myc-Max can bind to the consensus 5'-CACGTG-3' sequences (E boxes) and regulate the transcription of a wide variety of target genes, including genes involved in cell cycle regulation (e.g. cyclin A and cyclin E), apoptosis (e.g. p53 and ARF), immortalization (e.g. telomerase) and metabolism (e.g. DHFR and LDH-A) (61).

In human cancers, c-*myc* is activated through several mechanisms. c-*myc* is over-expressed and/or amplified in a wide variety of human tumors including breast, colon, cervical and small cell lung carcinomas, osterosarcomas and glioblastomas (56, 62). c-*myc* has also been reported to be activated through translocations that juxtapose the c-*myc* proto-oncogene at chromosome 8q24 to one of the three immunoglobulin genes on chromosome 2, 14, or 22 in B cell lymphomas (63) and through point mutations in the coding sequence in Burkitt's lymphomas (64). c-*myc* transgenic mice exhibit neoplastic premalignant and malignant phenotypes in skin (65) and hematopoietic lineages (66). The oncogenic transforming activity of c-*myc* is believed to result from the aberrant transcriptional regulation of its target genes, especially those involed in promoting cell proliferation and deregulating cell cycle control (55, 61).

The activation of the c-*myc* gene has also been shown to be central to signal transduction through other tumor suppressor protein or oncoproteins. For example, c-*myc* is the target of the adenomatous polyposis coli (APC) pathway, which negatively regulates β-catenin. β-Catenin is a co-activator for the transcription factor Tcf (T cell factor), which is able to activate c-*myc* expression directly. In human colorectal adenocarcinomas, the inactivation of the APC tumor suppressor protein results in the activation of β-catenin, which in turn results in the activation of c-*myc* (67). The activities

of human transforming proteins BCR-ABL (68, 69), TEL-PDGFR (70) and proto-oncogene c-*src* (71) have also been shown to depend on the c-*myc* gene.

2. Tumor Suppressor Genes

So far, about a dozen confirmed tumor suppressor genes have been characterized, including *p53*, *Rb*, *WT1*, *BRCA1*, *BRCA2*, *VHL*, and *APC* (28). Two prototypes of tumor suppressor genes (*p53* and *Rb*) are reviewed here.

2.1. *p53* as a Prototype of Tumor Suppressor Genes

The p53 protein was first identifed in 1979 as a cellular protein tightly bound to the simian virus large T antigen (SV40LT) (72-74). Subsequent work on p53 not only provided insights into the mechanism of transformation by DNA tumor viruses, but also led to the unexpected realization that p53 is a key player in practically all types of human cancers. More than 50% of human tumors contain mutations in the *p53* gene (75).

p53 is a member of a family that includes *p63* and *p73* (76, 77). The *p53* gene encodes a 393 amino acid nuclear phosphoprotein. The N-terminal region is highly charged and acts as a transcription activation domain. The C-terminal domain is responsible for its oligomerization. p53 proteins assemble through this domain to form stable tetramers. The core or central region of p53 is highly conserved and has sequence-specific DNA binding activity. More than 90% of p53 mutations reside in this region. Such mutations often result in a conformational change of the p53 protein or a defective contact of the protein with DNA (75, 78).

Normally, the amount of p53 protein in a cell at steady state is low because of its relatively short half-life (about 20 minutes). Several different types of DNA damage can activate p53 protein, including double strand breaks induced by γ-irradiation and the presence of DNA repair intermediates after ultraviolet irradiation or chemical damage to DNA. This results in a rapid increase in the level of p53 in the cells and the activation of p53 as a transcription factor (79). In addition to its response to DNA damage, p53 can

also mediate response to a more general stress, such as hypoxia, heat or starvation (80, 81).

One of the key functions of p53 is to induce G_1 arrest in response to DNA damage (82). One of the downstream genes turned on by p53 is *p21* (*WAF1*, *Cip-1*) (83). The p21 protein binds to a number of cyclin and Cdk complexes, inhibiting cdk activity and blocking cell cycle progression (84). The p21 protein also binds to proliferating cell nuclear antigen (PCNA), blocking its role as a DNA polymerase processivity factor in DNA replication (85). This p53-mediated G_1 arrest is believed to give cells sufficient time to repair DNA damage before the cells enter the S phase. p53 has also been implicated in a G_2/M phase checkpoint. When mitotic spindle inhibitors, such as nocodazole, are added to the cells with wild-type p53, the cells are arrested in G_2. In the absence of wild-type p53, these cells will reinitiate DNA synthesis, increasing the ploidy of the cells (86). In addition, p53 appears to be an integral part of the process that regulates the number of centrosomes in a cell. In culture, mouse embryo fibroblasts that are *p53* -/- produce abnormal numbers of centrosomes, initiate spindles with three or four poles, and rapidly become aneuploid after a few doublings (87). This p53-mediated G_2/M check point may account for the phenotype of genomic instability that is commonly associated with a *p53* mutation.

Another important function of p53 is to trigger apoptosis under several different conditions. Normal mouse thymocytes will undergo apoptosis in response to DNA damage, whereas thymocytes from *p53* null mice do not undergo apoptosis in response to the same stimulus (88). p53 can also initiate apoptosis in response to the expression of a viral or cellular oncogene, such as adenovirus *E1A*, *E2F-1* or *myc* (89-91). In addition, p53 can induce apoptosis in response to hypoxia (81). This represents yet another way that p53 may act as a gatekeeper against the formation of cancers as blood supply becomes rate limiting when tumors reach a certain size (92).

In addition to acting as a transcription factor through its DNA binding activity, p53 can also directly bind to and act on several cellular proteins (93). For example, the p53 protein has five copies of proline-X-X-proline motif localized between residues 61-94 and this motif has been shown to bind to the SH3 domains of the c-Abl protein (94). After DNA damage, the kinase activity of nuclear c-Abl protein is increased. This results in the binding of c-Abl to p53, which enhances p53 transcriptional activity (95). Such a regulated interaction between p53 and SH3 domain-containing proteins may be one way for the p53 protein to communicate with signal transduction pathways and sense oncogene activities.

Another important protein that binds to p53 is MDM-2. This association has two consequences: first, it prevents p53 from acting as a transcriptional activator (96); second, it targets p53 to rapid degradation through the ubiquitin-proteasome pathway (97, 98). Thus, MDM-2 is a potent physiological antagonist of p53. In addition, the *mdm-2* gene itself is a transcriptional target of p53 (99-101), establishing a negative auto-regulatory feedback loop between p53 and its own inhibitor MDM-2. In addition to *p53* mutations, amplification of the *mdm-2* gene has been found in several types of tumors, including 30-40% of human sarcomas (102, 103).

In summary, p53 is a cellular gatekeeper for growth and division and an important tumor suppressor protein. In response to DNA damage or other stresses, the p53 protein initiates a protective cell cycle arrest or apoptotic cell death. A number of factors influence the decision of a cell to enter a p53-mediated cell cycle arrest or apoptotic pathway. Under conditions in which the DNA is damaged, growth factors are limiting, or an activating oncogene is forcing the cell into a replicative cycle, p53-mediated apoptosis prevails. In this way, cells with unstable genomes (due to DNA damage) or cells in an abnormal environment are eliminated in a p53-dependent

apoptotic event. This is most likely the reason why so many cancerous cells select against wild-type p53 function (104, 105).

2.2. *Rb* as a Prototype of Tumor Suppressor Genes

The retinoblastoma (*Rb*) gene was the first familial cancer gene to be identified. It was isolated by positional cloning of retinoblastoma (10). The *Rb* gene encodes a nuclear phosphoprotein that exerts its cell growth inhibitory function mainly by binding to and repressing the transcriptional activities of the E2F family of transcription factors (E2Fs) (106, 107). The central 'pocket' domain of Rb is required for binding to E2Fs, and most naturally-occuring tumor-promoting mutations reside in this region (108).

Rb exerts most of its effect in a defined window of time in the first two thirds of the G_1 phase of the cell cycle. Cells entering G1 from mitosis require exposure to serum mitogens continuously until several hours before the onset of S phase; thereafter they become relatively serum-independent. The transition from serum-dependent to serum-independent state is demarcated by a discrete point in time, termed R (restriction) point (109). Before the cells reach the R point, the Rb protein is found in a hypophosphorylated form. The hypophosphorylated Rb binds to E2Fs, repressing expression of E2Fs target genes. During the last several hours of G1, the Rb protein is pohosphorylated first by cyclin D-CDK4/6 complex and then by cyclin E-CDK2 complex (110). The successive phopshorylation of Rb by both of these complexes is needed to completely inactivate Rb (111). Phosphorylated Rb protein dissociates from E2Fs, enabling them to transactivate the genes important for S phase entry, such as c-*myc*, B-*myb*, *cdc2*, thymidine kinase and dihydrofolate reductases (112).

Mutations of the *Rb* gene are frequently observed in only a subset of human tumors, such as retinoblastomas, osteosarcomas, small cell lung carcinomas, and bladder carcinomas (108). However, the disruption of Rb function is present in most tumors. For example, many tumors constitutively overexpress cyclin D1 (113), and this

in turn activates cyclin D-dependent kinases, resulting in hyperphosphorylation of the Rb protein. In a great majority of cervical carcinomas, infections of the papilloma virus lead to the inactivation of Rb through the binding of the E7 viral protein to Rb (114). The tumor suppressor function of Rb has also been confirmed by its ability to inhibit the malignant transformation when expressed in human cancer cells that had inactivated endogenous *Rb* genes (115).

C. Carcinogenesis is a Multistep Process

1. Epidemiological Studies of Cancer

The first clue to the multistep nature of carcinogenesis comes from epidemiological studies. Cancer is predominantly a disease of the elderly, with the risk of acquiring this disease increasing with age. Earlier epidemiological studies of cancer incidence as a function of age using mathematical modeling suggest that for adult human tumors 4-6 genetic changes were required for the genesis of a tumor (116-118). Recently, Renan (119) used the same method but a better-defined data set to address the question of the number of the mutational changes required for 28 different human malignancies. By plotting the log of the age-specific mortality rate against the log of the age in years of the person affected, and determining the best-fit linear regression coefficients for each tumor type, he concluded that the common adult human cancers of the lip, stomach, liver, pancreas, kidney, skin and bladder required 7-8 mutational changes, and tumors with very late onset, such as prostate cancer, required 12 changes.

2. Experimental Animal Studies: Mouse Skin Carcinogenesis

A classical model of multistep carcinogenesis is the pathogenesis of mouse skin cancer (120-123). Sequential applications of chemical agents to mouse skin can induce tumors, and tumor development can be divided into three stages: initiation, promotion and progression. Typically, tumor initiation is brought about by the single application of

a mutagen, such as 7,12-dimethylbenz(a)anthracene (DMBA); promotion is carried out by repeated applications of tumor promoters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), or by a natural promoting stimulus such as wounding. Papillomas began to appear at 12-20 weeks after the promotion began. By about one year, 40-60% of the animals had some papillomas that became squamous cell carcinomas. If a promoting agent was given alone or before the initiating agent, usually no malignant tumors occurred.

The initiation process is carcinogen-induced and produces a subtle change in keratinocyte phenotype. The biochemical basis for the initiated phenotype has been identified as mutations in the H-*ras* gene and alterations in protein kinase C. H-*ras* mutations are usually heterozygous in papillomas and can be detected in initiated skin prior to the emergence of tumors (124). Furthermore, the initiating agent used determines the existence, nature and site of H-*ras* mutations (125, 126). Differential modifications of isoforms of PKC, particularly activation of PKCα and inhibition of PKCδ, produce kerotinocytes with enhanced proliferative capacity and reduced sensitivity to signals for terminal differentiation (127).

Repeated applications of tumor promoters to the initiated epidermis cause the selective clonal outgrowth of initiated cells to produce multiple benign tumors (papillomas), each representing an expanded clone of initiated cells (128, 129). The mechanism of exogenous promotion is likely to be epigenetic in most cases since 1) most promoting agents are non-mutagens, and 2) a single genetic change in normal keratinocytes, such as *ras* mutation, is sufficient to produce a papilloma phenotype (130).

Premalignant and malignant progression of a papilloma to a carcinoma is usually a spontaneous process, but can be enhanced and accelerated by additional applications of mutagens, supporting a genetic basis for progression (131, 132). Genetic studies

indicate that nonrandom, sequential chromosomal aberrations are associated with premalignant progression of mouse skin papillomas; particularly prominent are trisomies of chromosome 6 and 7 (133, 134). Changes in two cellular genes, H-*ras* and *p53*, have been closely identified with malignant progression of skin tumors. The muated H-*ras* gene, which is heterozygous in papaillomas, is frequently homozygous in caricnomas (135). Mutations in the *p53* gene are rarely found in papillomas, but are frequently detected in caicinomas, particullay those induced by benzo[a]pyrene (136).

Such mouse skin carcinogenesis model has been useful in dissecting the complex process in skin cancer pathogenesis and the three-stage definition (initiation, promotion and progression) has been adopted to analyze stages in carcinogenesis for most epithelial neoplasms of humans and experimental animals (137-139).

3. Human Cancer Studies: Colorectal Carcinogenesis

Colorectal cancer has provided another excellent model system in which to search for and study the genetic alterations involved in the development of a common human neoplasm. For colorectal cancers, there is a well-defined progression from benign to malignant tumors, and the tumors that arise are monoclonal in origin (140). In addition, colorectal cancers exist in both sporadic and hereditary forms. In patients with familial adenoma polyposis (FAP), hundreds to thousands of adenomatous polyps exist in their colons and rectums, and a small percentage of them undergo progression to invasive adenocarcinoma. The high frequency of polyps at various stage of progression allows one to study the stage-wise pattern of colorectal cancer. Using large numbers of samples from FAP patients and sporadic tumors, Vogelstein and colleagues identified the critical genetic events driving colorectal carcinogenesis (141, 142).

The locus linked to FAP has been mapped to chromosome 5q21, and the gene is termed *APC* (adenomatous polyposis coli) (143, 144). The *APC* gene is mutated in the germline of FAP patients (145), and this alteration may be responsible for the

hyperproliferative epithelium present in these patients. In tumors arising from patients without polyposis, the *APC* gene may also be lost or mutated at a relative early stage of tumorigenesis (146). Another genetic event that happens early in the colorectal tumorigenesis is the global hypomethylation of the DNA (147). The loss of DNA methylation has been shown to inhibit chromosome condensation and might lead to mitotic nondisjunction (148), resulting in the loss or gain of chromosomes.

Mutations in the K-*ras* oncogene have been identified in about 10% of small adenomas, 50% of larger adenomas and in 50% of carcinomas (141, 149, 150). Such *ras* mutations, usually in codons 12 or 13, tend to be observed in more dysplastic adenomas. *Ras* gene mutations appear to occur in one cell of a preexisting adenoma and confer on it a growth advantage. Through clonal expansion of the cells with the *ras* mutation, a small adenoma is converted into a larger and more dysplastic one.

Loss of heterozygosity of additional tumor suppressor genes appears to be critical in later stages of colorectal cancers. The chromosomes most frequently deleted include chromosome 18q and 17p. 18q is deleted in 50% of late adenoma and more than 70% of carcinomas (141). A candidate tumor suppressor gene from this region has been identified and is termed *DCC* (deletion in colorectal cancer) (151). It encodes a protein with putative cell adhesion properties whose expression is absent or reduced in colorectal carcinomas. The loss of a large portion of one copy of chromosome 17p has been observed in more than 75% of carcinomas and is rarely seen in adenomas at any stages. The common lose on chromosome 17p contains the *p53* tumor suppressor gene (152). Nucleotide sequencing of the *p53* cDNA derived from colorectal cancers has shown that in 70% to 80% of the 17p deletion cases there is a missense mutation in the remaining p53 allele in the cancer cells (152, 153). Additional chromosome losses, including 1q, 4p, 6p, 6q, 8p, 9q and 22q, have been observed in 25%-50% of colorectal cancers.

On average, mutations in at least four to five genes are required for the formation of a malignant colorectal tumor. Fewer changes suffice for benign tumorigenesis. Although the genetic alterations often occur in a preferred sequence, the total accumulations of changes, rather than their order, is responsible for determining the tumor's biological properties (142).

4. Transformation of Human Cells in Culture: Establishment of a Human Fibroblastic Lineage MSU-1

One of the advantages of using human cells in culture to study neoplastic transformation is that it provides a means to dissect the carcinogenesis process under well-defined conditions. However, normal cells in culture have never been found to undergo spontaneous malignant transformation and have proven to be extremely difficult to be malignantly transformed by any means (154, 155). Recently neoplastic transformation of human cells in culture has been achieved in a stepwise fashion: i.e., immortalization followed by malignant conversion of the immortalized cells (155, 158). The establishment of a human fibroblastic cell lineage MSU-1 in the Carcinogenesis Laboratory at Michigan State University will be highlighted in the review.

4.1. Immortalization of Human Fibroblasts in Culture

Normal cells in culture have a limited life span, beyond which the cells enter the terminal non-dividing state referred to as senescence (156). Finite life span normal human cells can only undergo two successive clonal selections before they enter crisis and senesce (154). Earlier studies trying to transform finite life span human cells in culture were not successful, suggesting that acquiring immortality is a prerequisite if a cell is to acquire sequentially all the genetic changes needed to become malignant (157, 158). Whether this is the case for cells in the human body is not known for certain. What is known is that many malignant tumor-derived cells can be grown indefinitely in culture, whereas cells from normal tissues are never able to grow indefinitely in culture.

Human cells can be immortalized by repeated treatment with chemical and physical carcinogens, and by infection or transfection with certain viral genes. However, it occurs at a very low frequency (< 1 in 30 million) (154, 157, 159, 160). Nevertheless, immortalization of human epithelial cells, such as epidermal keratinocytes, bronchial epithelial cells, mammary epithelial cells, and prostate epithelial cells, has been achieved by infection with the AD12-SV40 hybrid virus or human papilloma virus (155, 157).

Immortalization of diploid human foreskin fibroblasts has been achieved in the Carcinogenesis Laboratory at Michigan State University following transfection with the v-*myc* oncogene. Normal human fibroblasts derived from the foreskin of a neonate, designated LG1, were put into culture and transfected with v-*myc* oncogene. Clonally derived cell strains expressing the v-Myc protein were propagated, and most entered crisis and senesced. However, one clonal population escaped senescence and gave rise to an infinite life span cell strain designated MSU-1.0. A faster growing, spontaneous variant (designated MSU-1.1) overgrew the MSU-1.0 culture. The MSU-1.1 cell strain has a stable, near-diploid karyotype composed of 45 chromosomes, including two maker chromosomes. Both MSU-1.0 and MSU-1.1 cell strains are immortal but not tumorigenic (161). It is now clear that at least one more genetic/epigenetic change, in addition to unregulated v-*myc* expression, was involved in generating the MSU-1.0 cell strain and at least two more genetic changes were involved in generating the MSU-1.1 cell strain (158).

The exact genetic changes that occurred in the MSU-1.0 and MSU-1.1 cell strains have not been completely characterized. However, both cell strains are positive in telomerase activity, whereas the parental cell line, LG1, is negative in telomerase activity. In light of recent advances which identified that human telomerase reverse transcriptase (hTERT) is sufficient to immortalize human cells, including human

fibroblasts (162, 163), and that c-Myc activates h*TERT* transcription (164, 165), it is highly likely that the expression of v-Myc oncoprotein in the LG1 cells increased the chance of activating telomerase activity in the cells, which eventually resulted in the generation of the infinite life span cell strain MSU-1.0.

4.2. Neoplastic Transformation of Immortalized Human Fibroblasts

While immorlization is not sufficient for transformation, most immortalized cells have an increased sensitivity for spontaneous, carcinogen- or oncogene-induced neoplastic progression. For example, AD12-SV40 hybrid virus-immortalized human epidermal keratinocytes can be malignantly transformed by retroviral oncogenes such as H-*ras*, *fms*, *erbB* and *src*; they can also be transformed by chemical carcinogens or x-ray irradiation followed by suitable selections (155).

In the Carcinogenesis Laboratory at Michigan State University, the infinite life span cell strain MSU-1.1 has been successfully converted into malignant cells by either oncogene transfection or carcinogen treatment. Over-expression of the activated H-Ras (166) or N-Ras oncoprotein (167) can convert MSU-1.1 cells into malignant cells. Expression of the same *ras* oncogenes at the level found for the endogenous H-*ras* or N-*ras* proto-oncogenes did not cause malignant transformation. However, a subsequent transfection of v-*fes* oncogene into the MSU-1.1 cells that expressed a transfected H-*ras* or v-*sis* oncogene at low levels resulted in malignant transformation (168). These malignant oncogene-transformed cell strains maintain the same karyotype seen in the MSU-1.1 cells and also retain p53 trans-activating function. These studies suggest at least two genetic/epigenetic changes are needed for the malignant transformation of MSU-1.1 cells. They also demonstrate that these transforming oncogenes must play complementary roles.

The MSU-1.1 cells can also be transformed by a variety of carcinogens, including benzo[a]pyrene diol epoxide, *N*-methyl-*N*-nitrosourea and γ-radiation (169-171). After a

single dose of the carcinogen, distinctive focal areas of overgrowth (termed foci) were observed in the MSU-1.1 monolayer culture. Cells isolated and re-cloned from these foci grew to a higher final density than the parental cells. 4/8 focus-derived cell strains from benzo[a]pyrene treatment, 10/29 from MNU treatment and 8/13 from γ-radiation formed malignant tumors in athymic mice, and they also lacked p53 trans-activating function. However, some focus-derived cell strains lacked p53 activities as well, but did not form tumors (170, 171). It appears that the inactivation of p53 is required but not sufficient for the malignant transformation. In addition, unlike the oncogene transfectants, the cells malignantly transformed by carcinogen treatments exhibited additional chromosomal changes besides the two stable maker chromosomes seen in the MSU-1.1 cells (169). Thus besides the inactivation of p53 function, additional genetic/epigenetic changes are required for the malignant transformation of MSU-1.1 cells by carcinogens.

In summary, these studies on the MSU-1 lineage suggest that the malignant transformation of human fibroblasts requires six or more genetic changes (172). They support that carcinogenesis is a multistep process and provide insights into the molecular mechanism underlying the process.

D. Carcinogens as Mutagens

Epidemiological studies suggest that human cancer is a multi-factorial disease. Most cancers are in principle preventable and many could be avoided by a suitable choice of lifestyle (e.g. avoiding smoking, preventing obesity and choosing a suitable diet) and environment (e.g. infectious agents and carcinogens, including chemical and physical carcinogens) (173-175). Though virus infections may play an important role for a few types of cancers (e.g. papilloma virus for cervical cancer, hepatitis B virus for liver cancer, and Epstein-Barr virus for Burkitt's lymphoma and nasopharyngeal cancer)

(114), it appears that carcinogens, especially chemical carcinogens, are of major importance in the induction of human cancers (176).

The evidence that chemicals can induce cancer in human beings has been accumulated for more than two centuries (177). Most carcinogens to which human are exposed require metabolism to their active derivatives. The ultimate reactive and carcinogenic forms are strong electrophiles, which react with DNA and cause mutations (178). The mutagenicity of an agent can be assayed by various methods. For example, the Ames test (179, 180) uses several strains of the bacteria *Salmonella typhimurium*, which are histidine auxotrophs and have a poor nucleotide excision repair mechanism and an increased permeability to exogenously added chemicals. Using this system, Ames and his colleagues have estimated that about 90% of all carcinogens tested are mutagens, whereas few non-carcinogenic agents show significant mutagenicity in this test system (181). On the other hand, chemical reagents that cause genetic changes are frequently carcinogenic (182).

The mouse skin carcinogenesis model has provided elegant proof of the cause-effect relationship between carcinogen exposure and cancer development. In this model, the *ras* gene is consistently activated and *ras* mutations are already present at the early benign tumor stage (124). The strongest evidence that *ras* gene point mutations were directly induced by the initiating carcinogens came from the studies of the mutation spectra of skin tumors induced by different initiating agents. For example, mouse skin tumors initiated with 7,12-dimethylbenz(a)anthracene (DMBA) showed predominantly mutations at the middle adenosine residue of H-*ras* codon 61 (C<u>A</u>A) which resulted in the conversion to thymidine (C<u>T</u>A) and the introduction of an activating missense mutation (125, 183). Such mutation was not seen in tumors induced by *N*-methyl-*N*-nitrosourea (MNU). Instead, MNU-induced tumors had the expected G→A mutation (126).

Other examples of carcinogen-specific mutations came from the mutation spectra studies of the *p53* gene, which has been frequently studied because it has a large number (nearly 300) of mutation sites available for analysis. Data from such studies provide strong evidence of a direct link between carcinogen exposures and the following types of human cancers. 1) UV exposure and skin cancers. Cyclobutane dimers and pyrimidine (6-4) pyrimidone photoproducts are the two major adducts produced by UV exposure and induce C→T or CC→TT mutations. Such UV signature mutations are observed in the *p53* gene in human nonmelanoma skin cancers (184) and in mouse skin tumors induced by UV exposure (185). 2) Grilled food and colon cancers. The heterocyclic amine 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) contained in grilled meat or fish can induce deletion of a G residue in the sequence 5'-GGGA-3' in the *lacI* gene in transgenic mice. The same mutation is found in the *p53* gene of rat colon cancers induced by this agent and also in some human colon cancers (186). 3) Smoking and lung cancers. Benzo[a]pyrene (B[a]P) in tobacco smoke is metabolically activated into reactive form, B[a]P diol epoxide (BPDE). BPDE then forms DNA bulky adducts that lead to G→T transversions. Such mutations at codon 157, 248 and 273 in human lung cancer are the *p53* mutational hotspots, which are uncommon in other types of cancers, including lung cancer of individuals who never smoked (187).

Collectively, these studies demonstrate that specific carcinogens or carcinogen mixtures (like tobacco smoke) may leave a fingerprint, or a signature mutation spectrum, on relevant oncogenes or tumor suppressor genes (188, 189). Such actions are consistent with the finding of multiple genetic changes in cancer-related genes in human cancer cells. In addition, though most carcinogens are genotoxic, some carcinogens do not exhibit direct interaction with DNA and are non-genotoxic. Evidence exists that these carcinogens alter gene expression and stimulate cell proliferation by epigenetic

mechanisms (190-192). These studies are also consistent with the current understanding of molecular basis of multistep carcinogenesis, which involves both genetic and epigenetic changes.

E. Summary

In summary, the current view of carcinogenesis is that:

1) Carcinogenesis is a multistep process, which involves the activation of oncogenes and inactivation of tumor suppressor genes. The accumulation of 4-12 genetic changes in a single tumorigenic cell is obtained through sequential clonal expansion. It is believed that a mutational event in a cancer-related gene in a cell will give the cell a selective growth advantage, resulting in subsequent clonal expansion. This expansion increases the chance of a second mutational event occuring in the cells that already have the first mutation. Additional mutations are presumed to occur in a similar manner (24, 142).

2) Most cancers exhibit genetic instability. In a small subset of human cancers, the instability is observed at the nucleotide level and results in base substitutions, deletions or insertions of a few nucleotides (including microsatellite instability (MIN) and nucleotide excision repair (NER) -associated instability (NIN)). In most other cancers, the instability is observed at the chromosomal level and results in losses and gains of whole chromosomes or large portions thereof (193). It is likely that genetic instability is required at an early stage of carcinogenesis to give the cell a higher mutation rate and allow the generation of multiple mutations in a relatively short time (194, 195). But there is also evidence that normal rates of mutation formation, coupled with waves of clonal expansion, are sufficient for the carcinogenesis process to occur in humans (196).

3) The carcinogenesis process involves genetic alterations with changes in DNA sequences and/or epigenetic alterations without changes in DNA sequences. Genetic alterations include mutations, deletions/insertions, rearrangements and amplifications of

segments of genetic materials. Several definitions exist for epigenetic alterations. A strict definition defines it as a modification of DNA that is inheritable (e.g. alteration of DNA methylation pattern) and rules out simple changes in expression levels of various genes. Epigenetic mechanisms of gene inactivation may provide additional pathways to inactivate tumor suppressor genes (197-199).

4) Cancer is a multi-factorial disease. Chemical carcinogens are of major importance in the induction of human cancers. Most carcinogens are mutagens, and many can leave a fingerprint or a signature mutation spectrum on relevant oncogenes or tumor suppressor genes, while some carcinogens are non-genotoxic and may alter gene expression and stimulate cell proliferation by epigenetic mechanisms. The actions of carcinogens are consistent with the multistep model of carcinogenesis (176, 188, 189).

The goal of the research in the Carcinogenesis Laboratory at Michigan State University is to identify the molecular mechanisms underlying the multistep carcinogenesis process, using human fibroblastic cell lineage MSU-1 as a major tool. Although human fibroblastic tumors are relatively rare, the wealth of information available on the transformation of animal fibroblasts in culture and the fact that human fibroblasts grow well in culture and clone with high efficiency support the choice of this cell type for our studies. The successful transformation of human fibroblasts in culture and the establishment of MSU-1 lineage in the laboratory provide a well-defined system to study the carcinogenesis process. The cell strains/lines in the system are isogenic, which allows direct comparison of the levels of expression between malignant cell lines and their non-tumorigenic precursors. In contrast, tumor samples/cell lines derived from patients usually do not have their well-matched normal counterparts. Previous study in the Carcinogenesis Laboratory by Qing et al. (200, 201) using differential mRNA display between MSU-1.1 cells and 6A/SB1, a malignant cell line derived from a fibrosarcoma

formed in athymic mice by BPDE-transformed MSU-1.1 cells, identified *fibulin-1D* and a novel gene, *ST7*, as tumor-suppressor genes for the malignant transformation of human fibroblasts. In my study, MET, the receptor of hepatocyte growth factor/scatter factor (HGF/SF), was found to be over-expressed in six out of six malignant cell lines derived from fibrosarcomas formed in athymic mice by various carcinogen-transformed MSU-1.1 cells, suggesting HGF-MET pathway might be important for the malignant transformation of human fibroblasts. Therefore, literature on HGF/SF and its receptor MET in carcinogenesis process is further reviewed below.

II. HGF/SF and its Receptor (MET) in Carcinogenesis Process

A. HGF/SF and its Receptor (MET)

1. Structures and Biosyntheses

Hepatocyte growth factor (HGF) and scatter factor (SF) were independently identified by their ability to induce the proliferation of primary hepatocytes and the dissociation/motility of epithelial cells ('scattering'), respectively (202-204). Molecular cloning of the cDNAs encoding HGF and SF demonstrated that they were the same molecule (205-207). Thus, they are now commonly referred to as HGF/SF. The *met* proto-oncogene was originally isolated as a transforming gene activated by chromosomal rearrangement in a human osteogenic sarcoma cell line (HOS) treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (208) and was later found to encode the receptor for HGF/SF (209).

The HGF/SF molecule shares structural homology and activation mechanism with the serine proteases of the blood-clotting cascade. It is synthesized and secreted as a single chain precursor (90 kDa pro-HGF/SF) that is subsequently cleaved at Arg^{494}-Val^{495} by specific proteases in the extracellular environment to form functional

heterodimer ($\alpha\beta$) linked by disulfide bonds (210). The 60-kDa α subunit contains four typical kringle domains, which are three-disulfide and triple-loop polypeptide highly conserved in the coagulation factors. The β subunit is closely related to the catalytic domain of serine-proteases; however, the serine residue of the active site is substituted by a tyrosine. The precise identity of the protease(s) cleaving the inactive monomer pro-HGF/SF to active heterodimeric HGF/SF *in vivo* is unclear. In injured tissue, a coagulation factor XII-like serine protease, named hepatocyte growth factor activator (HGFA), was shown to be induced and to activate pro-HGF/SF in a typical catalytic enzyme manner (211). Urokinase type-plasminogen activator (uPA) has also been shown to cleave pro-HGF/SF both *in vitro* and *in vivo*. This process is a stoichiometric reaction and may happen predominantly at the cell surface where the receptors for both HGF/SF and uPA are present (212, 213). In addition, there is evidence that coagulation factor XIIa and tPA can convert pro-HGF/SF to HGF/SF as well, at least *in vitro* (214, 215).

Besides the authentic full-length HGF/SF molecule, there are two naturally occurring splicing variants of HGF/SF: NK1 and NK2. NK1 contains the N-terminal domain and the first kringle domain (216), whereas NK2 contains the N-terminal domain and the first two kringle domains of HGF/SF (217, 218). NK1 and NK2 have unique biological properties compared with HGF/SF (see section IIB2).

The HGF/SF receptor MET is synthesized as a 170 kDa single chain precursor that undergoes co-translational glycosylation and proteolytic cleavage to form the mature protein, a 190 kDa heterodimer of disulfide-linked α chain (50 kDa) and β chain (145 kDa) (219). The single chain precursor is not exposed at the cell surface, whereas the dimeric mature protein is transported to the plasma membrane. The α subunit of the receptor is completely extracellular and heavily glycosylated. The β subunit spans the

plasma membrane. The intracellular part contains a divergent juxtamembrane region followed by a conserved tyrosine kinase domain and a C-terminal bidentate SH2 docking site that is responsible for receptor coupling to intracellular transducers (220, 221). Binding of HGF/SF to its receptor c-MET stimulates the tyrosine kinase activity of the receptor (209, 222).

2. *met* Gene Family

MET is the prototype of a novel class of heterodimeric receptor tyrosine kinases, which belong to the receptor tyrosine kinase (RTK) superfamily. It has at least two other members: RON and SEA (223, 224). The homology between MET, RON and SEA is concentrated in the kinase domain and in the bidendate SH2-docking site, which are directly involved in eliciting the biological responses distinctive to the MET family of RTKs. While the ligand for the SEA receptor remains elusive, the ligand for RON has been identified as the macrophage stimulatory protein (MSP) (223, 225), which shares structural and functional homology with HGF/SF. MSP was originally identified for its macrophage stimulating ability and was later found to promote multiple biological effects similar to those described for HGF/SF (226, 227).

In addition, a new gene family has been discovered recently that encodes transmembrane proteins homologous to the MET family (228). The prototype gene was identified on the human X chromosome and was named *sex*. Three other related proteins were isolated and named SEP, OCT and NOV, which show striking conservation with SEX in their primary sequences in the cytoplasmic domains. These proteins are putative receptors for unknown ligands, which possibly share structural homology with HGF/SF.

B. Biological Effects of HGF/SF

1. Biological Effects of Full Length HGF/SF

Since its discovery as a potent mitogen for primary hepatocytes, HGF/SF has been shown to have a variety of effects on different cell types. HGF/SF is produced mainly by mesenchymal cells (204, 229) and acts predominantly on MET-expressing epithelial cells in an endocrine and/or paracrine fashion (230). HGF/SF promotes a highly integrated biological program in epithelial cells, often referred to as 'invasive growth', which includes the dissociation of epithelial cell sheets, or cell scattering, increased motility and chemotaxis of dissociated cells, mitogenesis, invasion of extracellular matrix, and organization of invading cells into branching tubules (204, 229, 231). HGF/SF has also been reported to protect epithelial and carcinoma cells against apoptosis induced by DNA-damaging agents (232). In addition, HGF/SF can act on MET-expressing endothelial cells and induce endothelial cell migration, proliferation and capillary tube formation that occur during angiogenesis (the formation of new blood vessels) (233, 234).

MET-mediated biological effects of HGF are not restricted to epithelial and endothelial cells. Various mesenchymal cells express MET and/or HGF/SF as well. Thus, HGF/SF can act on mesenchymal cells through an autocrine and/or paracrine fashion. For example, the HGF/SF-MET pair is important for the migration of myogenic precursor cells into the limb bud (235, 236). HGF/SF stimulates the growth and release of erythroid hematopoietic precursors from bone marrow into blood circulation (237, 238). MET is expressed by both osteoclasts and oesteoblasts, and HGF/SF secreted by osteoclasts acts as a coupling factor to regulate the coordinated actions of these cells during bone remodeling (239, 240). HGF/SF is also an axonal chemoattractant and a neurotrophic factor for spinal motor neurons that express MET (241). Finally, HGF/SF

induces NIH3T3 fibroblasts transfected with *met*^{hu} proto-oncogene to display a motogenic-invasive rather than a proliferative program (242).

Gene knockout studies in mice further demonstrated a requirement of the HGF/SF-MET signaling pathway in normal development. Mouse embryos carrying null mutations in both *HGF/SF* alleles demonstrated impaired liver and placental development and died in midgestation (243, 244). *Met* null mutant mouse embryos had skeletal muscle defects of the limb and diaphragm (235).

In summary, HGF/SF is a multifunctional soluble factor. The HGF/SF-MET pair mediates diverse normal cellular processes such as growth, migration, morphogenesis, angiogenesis and anti-apoptosis. A growing number of receptor tyrosine kinases have also been shown to be able to mediate both growth and motility/scattering. This includes receptors for acidic fibroblast growth factor (aFGF), epithelial growth factor (EGF), c-ROS, c-NEU and the keratin growth factor (KGF) (245-247). However, the scattering effect observed for these factors appear to be specific to certain cell types, and unlike HGF/SF, they are not able to promote branching morphogenesis in three-dimensional matrices (247, 248). Such unique and pleiotropic properties of HGF/SF suggest that when induced in inappropriate contexts, HGF/SF-MET might participate in tumor formation and malignant progression.

However, it is also worth noting that in some tumor cell lines, HGF/SF inhibits, rather than promotes cell proliferation. HGF/SF has been found to inhibit the growth of hepatocellular carcinoma cells (HCC), though HGF/SF stimulates the growth of normal hepatocytes (249). Potent anti-proliferative activity of HGF/SF has also been observed for B6/F1 melanoma cells and KB squamous carcinoma cells (250). In addition, HGF/SF has been found to induce apoptosis in transformed liver epithelial cells (251).

2. Biological Effects of Naturally Splicing Variants of HGF/SF

Reports on the biological effects of the two naturally occurring splicing variants of HGF/SF, NK1 and NK2, have been controversial. NK1 was first generated by bioengineering in 1993, and was found to be an antagonist of HGF/SF in A549 human lung carcinoma cells. It competed for binding to the MET receptor, but was insufficient for stimulating phosphorylation of MET and failed to induce mitogenic activity even at high concentrations (252). However, in 1996, NK1 was found to occur naturally as well and was shown to be a partial agonist/antagonist in B5/589 human mammary epithelial cells. In this case, NK1 was found to bind to the MET receptor and stimulate the phosphorylation of MET. It induced modest mitogenic and scattering activities relative to HGF/SF. At 40-fold molar excess, NK1 inhibited HGF/SF-dependent DNA synthesis (216). NK2 was originally found to lack mitogenic activity and specifically inhibit HGF-induced mitogenesis in cultured B5/589 human mammary epithelial cells and melanocytes (217). However, NK2 was later reported to act as a partial agonist, able to induce scattering but not mitogenesis of certain cultured epithelial cells (253).

These data indicate that in addition to the full-length HGF/SF, the presence and functions of these two splicing variants might be important for the ultimate biological outcome for the cells as well. For example, a study by Itakura et al (254) showed that the NK2-MET autocrine pathway, instead of the regular HGF/SF-MET autocrine pathway, might be involved in the development and progression of lung carcinomas. SBC-5 small cell lung carcinoma cells expressed MET and NK2, but not HGF/SF. The addition of anti-HGF/SF antibodies to the cells specifically inhibited spreading and motility of SBC-5 cells without affecting growth, and the conditioned medium from SBC-5 cells induced scattering of other lineage lung carcinoma cells. Taken together, these studies indicate that the exact biological effects of HGF/SF and its variants might be cell

type specific and context dependent although many studies suggest that HGF/SF-MET might promote the development and progression of human tumors.

C. HGF/SF-MET Signaling

Upon HGF/SF stimulation, its receptor MET activates multiple intracellular signal transduction pathways, in line with HGF/SF's capability of evoking complex biological responses as described. The HGF-induced kinase activity of MET, the phosphorylation of the multifunctional docking sites of MET and the activation of downstream signal pathways are discussed in the following paragraphs.

1. HGF-induced Kinase Activity of MET

Activation of the MET protein occurs upon ligand-induced dimerization followed by transphosphorylation of the catalytic domain, which contains a conserved 'three tyrosine motif', including Y^{1230}, Y^{1234} and Y^{1235}. The Y^{1235} residue constitutes the major phosphorylation site, but phosphorylation of both Y^{1234} and Y^{1235} is essential for full activation of the enzyme. Upon phosphorylation of these residues, the enzymatic activity of the MET kinase is strongly up-regulated in an autocatalytic fashion (222, 255, 256). The juxtamembrane domain negatively regulates MET mediated activity (257). The inhibitory effects are mediated through two residues: Ser^{975} and Tyr^{1003}. The negative effect of Ser^{975} depends on its phosphorylation by protein kinase C (PKC) or Ca^{2+}/calmodulin dependent kinase (258, 259). The negative effect of Tyr^{1003} is less clear but may involve phosphorylation dependent recruitment of a cytosolic tyrosine phosphatase to the receptor (260, 261).

2. Multifunctional Docking Sites of MET

Upon activation of the kinase domain, two tyrosine residues (Y^{1349} and ^{Y1356}) at the C-terminal tail become phosphorylated and serve as multifunctional docking sites for multiple signal transducers and adaptors containing SH2 domains, including phosphotidylinositol 3-kinase (PI3K), phospholipase C gamma (PLCγ), pp60c-src,

Grb2/SOS, Shc, Shp2 and Stat3 (260, 262-266). The multifunctional docking sites (Y^{1349}VHVNATY^{1356}VNV) are made of tandemly arranged degenerate sequence YVH/NV, representing a variation from the common theme of the other tyrosine kinase receptors, where phosphorylation of different specific tyrosine residues determines which intracellular transducer will bind to the receptor and be activated (267, 268). The multifunctional docking sites are essential for HGF/SF-MET signaling, since mutation of the two tyrosine residues does not affect the receptor kinase activity but abolishes the biological functions of HGF/SF and the transforming activity of the hybrid protein TPR-MET (260, 263). Mutations of both residues in the mouse genome caused embryonic death, with placental, liver and limb muscle defects, mimicking the phenotype of the *met* null mutants, whereas mice carrying a mutation that disrupts the Grb2 binding site developed to term but also showed muscle defects (236).

3. Signal Transduction

Among the transducers, p85, PLCγ, pp60c-src, Shc, Shp2 and Stat3 interact directly with either version (Y^{1349}VHV or Y^{1356}VNV) of the multifunctional docking sites, whereas Grb2, which has a strong requirement for asparagine at the +2 position, specifically interacts with the sequence Y^{1356}VNV (263-265, 269, 270). Recently, it has been shown that the multifunctional docking sites are also recognized by the MET-binding domain (MBD), a novel phosphotyrosine-binding domain found in Gab1 (271). In addition, Grb2 can act as an adaptor for Gab1 in MET signaling by binding to the receptor via its SH2 domain and the MBD region of Gab1 via its SH3 domains (261, 272). Thus a high affinity Gab1/MET interaction is achieved through a cooperative double hook mechanism, on the one side through the indirect association via Grb2, and on the other side through the direct interaction via the multifunctional sites on the receptor.

Gab1 has the structure of a docking protein and contains 21 tyrosine residues that upon phosphorylation could function as docking sites for signaling molecules. Transducers like PI-3K, PLCγ, Shc, Shp2, Crk and CRKL have been shown to interact with Gab1 (271, 272). Among them, PI-3K, PLCγ, Shc, and Shp2 can also associate directly with MET, whereas Crk and CRKL do not bind to MET directly (272). The activation of these transducers then leads to the activation of further downstream activators or gene expression. For example, binding of the Grb/SOS complex to MET leads to the activation of Ras, which in turn activates the Raf1/MEK/ERK cascade that is involved in the regulation of cell growth and differentiation (40). Activation of PI-3K leads to the downstream activation of small GTPases (Rho, Rac and Cdc42), which are involved in cytoskeleton organization, cell motility and invasion (273, 274). PI-3K can also lead to activation of the PKB/Akt, which is involved in anti-apoptosis (275, 276).

Another event as a result of HGF/SF-MET signaling is the activation of proteases that mediate the degradation of the extracellular matrix-basement membrane, an important property for the invasion-metastasis process (277-279). HGF/SF-MET signaling increases the levels of uPA and its receptor uPAR, as well as receptor-bound uPA activity (280, 281). The induction of uPA expression by HGF/SF involves the Grb2/SOS/Ras/RhoA/Raf/MEK1/Erk2/AP-1 signaling pathway (282, 283). HGF/SF-MET signaling has also been shown to increase the expression of the matrix metalloproteinases, such as MMP1, MMP2, MMP9 and MT1-MMP (284-286).

Among the transducers implicated in HGF/SF-MET signaling, Ras and PI-3K appear to play a central role. This has been shown using signaling mutants of MET capable of selective activation of these two effectors. Coupling of the receptor to the Grb2/SOS/Ras pathway is both essential and sufficient for transformation, but not for invasion and metastasis, whereas activation of PI-3K alone is competent in eliciting

motility, but not transformation. The concomitant activation of Ras and PI-3K are necessary and sufficient for invasion and metastasis (270, 287, 288). These results indicate that MET mediated-biological effects can be dissociated based on the basis of their signaling requirements, which might be important for the particular biological functions of HGF/SF observed for a specific cell type (289).

D. Aberrations of HGF/SF-MET in Human Tumors

The *met* gene was originally identified in 1984 as a transforming gene activated by chromosomal rearrangement induced by a chemical carcinogen (MNNG) (208). The resulting oncogene is a hybrid between the 5' sequence of *tpr* and the 3' sequence of *met*. In TPR-MET, the extracellular domain of MET is replaced with TPR sequences, which provide two strong dimerization motifs (290). Dimerization causes constitutive activation of the MET receptor tyrosine kinase, which accounts for the transforming potential of TPR-MET (257). Such a hybrid gene has not been found in human tumors. Instead, the following types of aberrations of HGF/SF-MET have been observed: over-expression of MET, activating mutations of the *met* gene, and elevated serum HGF/SF levels and HGF activation.

1. Over-expression of MET

1.1. Over-expression of MET in Tumors of Epithelial Origins

Most commonly, MET is found to be over-expressed without alteration of its sequence. The first evidence for over-expression of the *met* proto-oncogene came from a cell line derived from a metastasis of a human gastric carcinoma, where the gene was amplified and over-expressed, and the tyrosine kinase constitutively activated (291). No alteration in the sequence was found in the cloned cDNA (292), suggesting that constitutive activation of the MET kinase may be caused by simple over-expression. Since then, MET has been found to be over-expressed in a significant number of human tumors of epithelial origins, including thyroid, pancreatic, colorectal, pulmonary, breast,

ovarian, endometrial, kidney, bladder, and prostatic cancers (293-303). Such over-expression was especially prominent in metastases.

For example, in thyroid tumors, over-expression of MET was observed in ~70% of carcinomas derived from the follicular epithelium and correlated with the aggressive phenotype. Southern blot analysis demonstrated that the *met* gene was not amplified nor rearranged (293). In ovarian neoplastic tissues, the level of MET expression was unchanged in benign ovarian tumors of various origins, but it was increased three to ten fold in about 30% of malignant ovarian carcinomas. In some cases, the MET protein was over-expressed over fifty fold without *met* gene amplification. In addition, a correlation was found between the over-expression of MET and the clinical aggressive behavior of ovarian tumors (300).

Of particular interest is the change in MET expression that occurs during the progression of colorectal tumors from adenomas to primitive carcinomas and liver metastases (296). Expression of the MET protein was increased from five to fifty fold in about 50% of the tumors (at any stages of progression) and in 70% of the liver metastases. The amplification of the *met* gene was found in only 10% of the primary tumors, but in eight of nine metastases examined. These data suggest that over-expression of MET provides a selective growth advantage to neoplastic colorectal cells at any stage of tumor progression, while gene amplification appears to give a further selective advantage for the acquisition of metastatic potential.

1.2. Over-expression of MET in Tumors of Mesenchymal Origins

MET was originally thought to affect only tumors derived from epithelial origins. However, a growing amount of evidence suggests that MET also participates in the formation and malignant progression of tumors of mesenchymal origins. As described above, *met* was originally identified as an oncogene activated upon chromosome translocation in a human osteosarcoma cell line. Moreover, the murine *met* homologue

was found to be amplified and over-expressed in spontaneously transformed fibroblasts *in vitro* (304). Later studies showed that MET was over-expressed in a variety of human tumors of mesenchymal origins, including fibrosarcomas, osteosarcomas, rhabdomyosarcomas, liposarcomas, myelomas, melanomas, and gliomas (305-310).

For example, a study by Rong et al (305) showed that human cell lines from various sarcomas expressed high levels of active MET. HGF/SF was also detected in human sarcoma cell lines but at a reduced level when compared to primary fibroblasts. Moreover, paraffin-embedded sections of primary tumors from human osteosarcomas, chondrosarcomas, rhabdomyosarcoma, leiomyosarcomas synovial sarcomas, and melanomas stained intensely for both MET and/or HGF/SF and displayed extensive heterogeneity with regard to both paracrine and autocrine stimulations.

In human bone tumors, the MET receptor was not detectable in the majority of the bone tumors, but was over-expressed in 60% of the osteosarcomas examined. In some cases, HGF/SF and MET were co-expressed, and the MET protein was constitutively activated (306).

Such co-expression of HGF/SF and MET has also been observed in human rhabdomyosarcomas, Kaposi's sarcomas, myelomas and glioblastomas (307, 308, 311, 312), suggesting that autocrine HGF/SF-MET signaling plays an important role in the development and dissemination of human sarcomas. Autocrine HGF/SF-MET signaling has been found in various carcinomas as well, including lung, pancreatic, and breast cancers (294, 313, 314). Thus autocrine HGF/SF-MET signaling might play a role in the development and dissemination of this class of tumors as well.

However, the role of HGF/SF-MET signaling in human malignancy is not necessarily limited to situations in which both of these molecules are expressed in an autocrine fashion. Rather, it is likely that HGF/SF is supplied to the MET expressing tumors via paracrine and/or endocrine fashion since HGF/SF is normally expressed and

secreted by the stroma cells of the tumors (315), and the serum HGF/SF level is often increased in cancer patients (see section IID3). In addition, MET-expressing tumors may play an active role in the recruitment of HGF/SF since tumor cells have been shown to produce soluble factors that induce HGF/SF expression (316-318).

2. Activating Mutations in the *met* Gene

A genetic connection between *met* and hereditary papillary renal carcinoma (HPRC) has established a direct role for this receptor in human cancer (319). HPRC is a form of inherited kidney cancer characterized by a predisposition to develop multiple, bilateral renal tumors. By using comparative genomic hybridization and linkage analysis, the *HPRC* gene was localized to a region of chromosome 7 where the *met* gene had been previously mapped. Sequencing of the *met* coding region from affected members of HPRC families as well as from a subset of tumor samples of patients with sporadic papillary renal carcinoma identified nine mutations. All mutations were missense and were localized within the tyrosine kinase domain of the MET receptor, which can be subdivided into amino- and carboxyl-terminal lobes separated by a large cleft, referred to as the activation loop. The MET receptors containing such mutations display different abilities to induce transformation in NIH3T3 cells (320, 321). Only mutations that alter residues in the kinase activation loop efficiently transformed NIH3T3 cells. Further study showed that these mutated MET receptors can be divided into two groups based on their biological properties (322). One group, including the M1250T and D1228H mutants, displayed increased tyrosine kinase activity, stimulated the Ras pathway efficiently, and transformed recipient cells in focus-formation assays. The other group, including the L1195V and Y1230C mutants, were almost devoid of *in vitro* transforming potential but were effective in inducing protection from apotosis, sustaining anchorage-independent growth, promoting invasion and interacting more efficiently with PI3K. Thus, mutations

of the *met* gene in HPRC can be responsible for transformation through different mechanisms.

A recent study by Di Renzo et al (323) showed that mutations of the *met* gene might not be limited to HPRC. In lymph node metastases of head and neck squamous cell carcinoma (HNSCC), they identified two somatic mutations in the *met* proto-oncogene. One is the Y1230C mutation, known as a *met* germline mutation that predisposes to HPRC. The other is the Y1235D mutation that is novel and changes a critical tyrosine residue, known to regulate MET kinase activity. The mutated MET receptors were constitutively active and conferred an invasive phenotype to transfected cells. Most importantly, cells carrying these mutations were selected during metastatic spread. Transcripts of the mutated alleles were highly represented in metastases, but barely detectable in primary tumors.

3. Elevated Serum HGF/SF Levels and HGF/SF Activation

Besides the various aberrations observed in the receptor MET, human tumors also exhibit abnormalities in their HGF/SF expression. Increased serum concentration of HGF/SF has been observed in patients with breast cancer, colorectal carcinoma, gastric carcinoma, liver cancer metastases, bladder cancer and acute myelocytic leukemia (324-331). For example, the serum HGF/SF concentration from patients with gastric carcinomas significantly increased with increasing pathologic tumor grades. Also, there was a significantly higher concentration of HGF/SF in patients with nodal and/or liver metastases compared to patients without metastases. In multivariate analysis, the serum HGF/SF concentration was found to be an important independent factor in predicting overall survival (328). These studies suggest that serum HGF/SF level may be a useful biomarker for the diagnosis and prognosis of the cancer patients. In addition, the HGF/SF level was found to be elevated in urine from patients with bladder carcinomas compared to normal control subjects (318). A biologically significant amount

of HGF/SF was also found to be present in the pleural effusion fluid of patients with primary lung cancers or with metastases that had spreaded to the pleura (332, 333).

The origins of the elevated serum and/or body fluid HGF/SF level remain underdetermined. Presumably, it is produced either by the tumors cells themselves, since they tend to possess an autocrine pathway, or by the stroma cells surrounding the tumor, since tumor cells can secret soluble factors to induce HGF/SF expression (see section IID1.2). This is supported by a study of gastric cancer, which showed that the concentration of HGF/SF in the tumor tissues was significantly higher than that in the normal gastric mucosa, and that surgical removal of the tumor significantly reduced the serum HGF/SF concentration (334).

Another avenue to achieve high levels of active HGF/SF is through increased HGF/SF activation. As discussed in section IIA1, the activation of HGF/SF in the extracellular milieu is a critical limiting step in the HGF/SF induced signaling pathway mediated through MET receptor tyrosine kinase. A recent study by Kataoka et al (335) showed that HGF/SF activation was increased in colorectal carcinoma tissues and the balance between HGF/SF activator (HGFA) and HGFA inhibitor 1 (HAI-1) could play an important role in the regulation of HGF/SF activity. Increased expression of HGFA, a factor XII-like serine protease, was observed in carcinoma cells compared with adjacent normal or adenoma cells. In contrast, the expression of HAI-1 decreased significantly in the carcinoma cells. The altered balance between HGFA and HAI-1 resulted in an increased activation of HGF/SF in colorectal carcinomas.

In summary, the frequent observation of various alterations in the HGF/SF-MET pathway strongly implicates their important roles for the carcinogenesis process. Further evidence regarding the causal role of the HGF/SF-MET pathway in tumor formation and malignant progression of human sarcomas will be reviewed because of the fibroblastic nature of the MSU-1 lineage that were used in my study.

E. Further Evidence that HGF/SF-MET May Play a Causal Role in Tumor Formation and Malignant Progression of Human Sarcomas

1. Transformation by Inappropriate HGF/SF-MET Autocrine Loop

1.1. Studies in Mouse Cells

NIH3T3 is a murine embryonic fibroblast cell line that naturally produces murine HGF/SF (HGF/SF^{mu}), but little or no MET^{mu}. As mentioned above, spontaneously transformed NIH3T3 cells often exhibited over-expression of endogenous MET^{mu} (304). Furthermore, genetic transfer of the *met*^{mu} proto-oncogene into NIH3T3 cells caused morphologic transformation *in vitro* and tumorigenicity *in vivo* (336, 337). However, human *met* proto-oncogene (*met*^{hu}) did not induce morphological transformation or tumorigenicity of NIH3T3 cells, unless *met*^{hu} was co-expressed with human *HGF/SF* (*HGF/SF*^{hu}) (337). Their explanation for the results was that HGF/SF^{mu} might have low affinity for MET^{hu}, which was supported by the observation that HGF/SF^{mu} cannot induce the scattering of human cells *in vitro* (338). Thus, an aurocine stimulatory HGF/SF-MET loop is important for the transformation of NIH3T3 cells. Also, these data indicate that over-expression of unaltered MET receptor does not induce the transformation of cells which do not themselves produce HGF/SF, or do not have access to sufficient quantities of HGF/SF via paracrine or endocrine avenues. Such observations are supported by studies in C127 mouse cells as well.

The C127 cell line is an immortalized cell line established from the stroma of a mouse mammary tumor and expresses negligible amounts of HGF/SF^{mu} and MET^{mu}. C127 cells engineered to over-express HGF/SF^{hu} (which effectively activate MET^{mu}) or MET^{mu} alone were neither transformed nor tumorigenic, whereas cells engineered to express both HGF/SF^{hu} and MET^{mu} were morphologically transformed *in vitro* and highly tumorigenic and metastatic in athymic mice (339).

The above studies supported the role of MET in the transformation of mouse fibroblasts. However, since animal cells are normally much easier to be transformed than human cells (340, 341), further evidence from studies carried out in human cells is needed.

1.2. Studies in Human Cells

SK-LMS-1 is an immortal cell line derived from a low-malignancy human leiomyosarcoma. The cells express a significant amount of MET, but only a low level of HGF/SF (305). SK-LMS-1 cells engineered to over-express HGF/SF^{hu} exhibit significantly enhanced tumorigenicity in athymic mice with a two to three fold increase in tumor incidence and two to three fold decrease in tumor latency compared with the parental cells (281).

9L is a glioma cell line that expresses MET but not HGF/SF. Gene transfer of *HGF/SF* into this cell line led to an increase of the tumorigenicity and angiogenesis *in vivo* (intracranial implantation) (342). Enhanced tumorigenicity and growth were also observed following ectopic HGF/SF expression in U373, a human glioblastoma cell line, which expresses MET but not HGF/SF (343).

2. Diverse Tumorigenesis in Transgenic Mice Over-expressing HGF/SF or TPR-MET

Transgenic mouse models have offered additional avenues to test the oncogenic potentials of HGF/SF and MET *in vivo*. Since HGF/SF has been shown to have some controversial roles in different cell types, creation of transgenic mice would provide unique information since HGF/SF can be inappropriately targeted to a variety of tissues. *HGF/SF* transgenic mice developed a remarkably broad range of histologically distinct tumors of both mesenchymal and epithelial origins. Many neoplasms arose from tissues exhibiting abnormal development, including the mammary gland, skeletal muscle and melanocytes, suggesting a functional link between mechanisms regulating

morphogenesis and those promoting tumorigenesis. Most neoplasms, especially melanomas, demonstrated over-expression of both the HGF/SF transgene and endogenous MET and had enhanced MET kinase activity, strongly suggesting that autocrine signaling broadly promotes tumorigenesis (344). On the other hand, transgenic expression of *tpr-met* oncogene (which encodes a constitutively active form of MET) led predominantly to the development of mammary tumors, although there were also several mesenchymal types of malignancies (osteosarcomas, spindle cell sarcomas, lymphomas, etc.) in the transgenic mice (345). These studies suggest that subversion of normal mesenchymal-epithelial paracrine regulation through the forced misdirection of HGF/SF or TPR-MET expression induces aberrant morphogenesis and subsequent malignant transformation of cells of diverse origins.

3. Reduced Tumorigenicity and Metastasis by Down-regulating or Interfering with HGF/SF-MET Signaling

More direct evidence of the oncogenic role of HGF/SF-MET comes from studies using strategies to down-regulate or interfere with the endogenous HGF/SF-MET signaling. For example, several studies of glioblastoma, the most common malignant glial neoplasm, showed that human gliomas express both HGF/SF and MET, and their expression levels are associated with malignant progression (346-348). A study by Abounader et al (349) employed chimeric constructs with U1 small nuclear RNA (U1snRNA) and ribozymes to specifically target *HGF/SF* or *met* mRNA in U87 human glioblastoma cells, which possess an autocrine HGF/SF-MET loop. A significant reduction of endogenous HGF or MET level was observed at both mRNA and protein levels. In some cases, HGF or MET level was down-regulated to as low as 2% of the parental or control cells. Inhibition of HGF/SF or MET expression in U87 cells further led to a reduction of HGF/SF-MET dependent signal transduction, decreased colony

formation in soft agar, and substantial inhibition of tumorigenicity and tumor growth *in vivo*.

Though not related to sarcomas, another study by Firon et al (350) is worth mentioning. They used dominant-negative forms of MET to address the importance of MET for the tumorigenesis and malignant progression of DA3, a poorly differentiated metastatic murine mammary adenocarcinoma cell line. Two dominant-negative forms (DN) of MET were generated: one was truncated at the kinase domain; the other was produced by the substitution of three tyrosine residues in the multifunctional docking sites. DA3 cells transfected with DN forms of MET exhibited reduced MET phosphorylation following exposure to HGF/SF and greatly reduced tumorigenicity and spontaneous metastasis.

Naturally secreted and artificially engineered variant forms of HGF/SF also provide useful tools to antagonize the biological effects of HGF/SF. Gene transfer of *NK2*, a natural splicing variant of *HGF*, into the U87 human glioma cell line possessing an HGF/SF-MET autocrine loop dramatically reduced the formation of colonies in soft agar and the growth of the intracranial tumor xenografts (351). Otsuka et al (352) demonstrated that *NK2* transgenic mice were healthy and failed to display any hyperplastic lesions or tumorigenesis characteristics of *HGF/SF* transgenic mice as described in section IIE2. Instead, when co-expressed in *NK2-HGF* bi-transgenic mice, NK2 antagonized the pathological consequences of HGF and suppressed the subcutaneous growth of transplanted MET-containing melanoma cells. Unlike NK2, NK4 is an artificially engineered variant form of HGF and has not been found to occur naturally. It contains the N-terminal domain and subsequent four kringle domains of HGF and has been shown to be a complete antagonist of HGF (353-355). Infusion of NK4 inhibited the tumor growth and invasion of subcutaneously implanted GB-d1 gallbladder carcinoma cells (353). Administration of NK4 suppressed primary tumor

growth and metastasis of Lewis lung carcinoma and Jyg-MC(A) mammary carcinoma cells as well (355).

A recent study by Cao et al (356) employed neutralizing antibody strategy to interfere with the biological effects of HGF/SF. Pooled mAbs raised against the native form of HGF/SF were able to neutralize the *in vitro* biological activities of HGF/SF. *In vivo*, the neutralizing mAbs inhibited the subcutaneous growth of C127 cells that had been engineered to express MET^{mu} and HGF/SF^{hu} in an autocrine fashion, and the growth of human glioblastoma multiforme xenografts from U118 cells which co-expresses HGF/SF and MET.

F. Summary

The studies reviewed in section IIE (especially section IIE3) strongly indicate a causal role of HGF/SF-MET in tumor formation and malignant progression of human sarcomas. In most cases, MET was only found to be over-expressed without amplification or sequence alteration. The role of high levels of endogenous MET in the malignant transformation of human fibroblasts has not been examined directly. Chapter II consists of a manuscript that will be submitted to the journal *Cancer Research*. It describes the studies to test the role of endogenous HGF and MET expression in human fibrosarcomas by down-regulating HGF and/or MET expression using chimeric U1 small nuclear RNA (U1snRNA)/ribozyme constructs, which specifically target *HGF/SF* or *met* mRNA.

Conventionally, only mutated genes (such as *p53* and *ras*) have been considered as candidate cancer-related genes. Recent advances in various techniques such as differential display (DD), representative difference analysis (RDA), serial analysis of gene expression (SAGE), and DNA microarray have identified many more genes that are altered in expression in cancer cells rather than are mutated (357-360). Nonmutated genes with stably altered expression patterns may be a key component of the cancer

genetics puzzle and may ultimately be traced back to mutations in upstream genes, such as those encoding transcription factors (28). The second part of my study is to examine the mechanisms responsible for MET over-expression in human sarcomas. The role of transcription factor Sp1 in the up-regulation of MET expression was tested. This is described in Chapter III, which consists of another manuscript that has been submitted to the journal *Cancer Research*.

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CHAPTER II

INHIBITION of HUMAN FIBROSARCOMA TUMORIGENICITY BY U1 SMALL NUCLEAR RNA/RIBOZYMES TARGETING EXPRESSION OF HEPATOCYTE GROWTH FACTOR AND ITS RECEPTOR MET

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ABSTRACT

Hepatocyte growth factor or scatter factor (HGF/SF) and its receptor (MET) have been associated with tumor formation and malignant progression of human fibrosarcomas. However, the role of endogenous HGF or MET expression in human fibrosarcomas has not been examined directly. Our previous study demonstrated that the MET protein is over-expressed in six out of six human fibrosarcoma cell lines derived from tumors formed in athymic mice by carcinogen-transformed MSU-1.1 cells and four out of five fibrosarcoma cell lines derived from patients' tumors. In the present study, we further determined that the HGF mRNA was co-expressed at various levels in the above 11 fibrosarcoma cell lines. The MET protein was constitutively phosphorylated at moderate to high levels in all 11 fibrosarcoma cell lines, and the phosphorylation of the MET protein could be suppressed by neutralizing HGF antibody in the fibrosarcoma cell line tested. Such findings suggest that HGF-MET autocrine activation plays an important role in tumor formation and growth of human fibrosarcomas. Therefore, we tested the hypothesis that human fibrosarcomas can be HGF-MET dependent and that reduction of endogenous HGF and/or MET expression can lead to inhibition of tumor formation and growth. Two human fibrosarcoma cell lines derived from tumors formed in athymic mice by γ-radiation-transformed MSU-1.1 cells were chosen for the study. One, γ2-3A/SB1, expresses a high level of MET and a low level of *hgf*, whereas the other cell line, γ4-2A/SFT2, expresses both MET and *hgf* at moderate levels. A chimeric transgene consisting of U1 small nuclear RNA and a hammerhead ribozyme targeting *met* was used to specifically down-regulate the amount of MET protein in these two cell

lines. In addition, U1snRNA/ribozymes targeting both *hgf* and *met* were used for γ4-2A/SFT2 cells. For the γ2-3A/SB1 cell line, reduction of MET expression greatly inhibited tumor formation and growth. For the γ4-2A/SFT2 cell line, reduction of MET alone inhibited tumor growth only slightly, whereas reduction of both MET and HGF expression greatly inhibited tumor formation and tumor growth. These results directly demonstrate strong dependence on endogenous MET and/or HGF expression for both tumor formation and growth of human fibrosarcomas.

INTRODUCTION

Hepatocyte growth factor (HGF), also known as scatter factor (SF), is a multifunctional protein that can induce a variety of biological effects, such as cell scattering and increased motility, mitogenesis, invasion of extracellular matrix and formation of branching tubule structures, and induction of new blood vessel formation (angiogenesis) etc. (1-4). HGF is expressed and secreted mainly by mesenchymal cells (1). The receptor of HGF (known as MET) is a heterodimeric receptor tyrosine kinase, which is expressed mainly in epithelial and endothelial cells (5, 6). Thus, HGF and MET act mainly in a paracrine/endocrine pattern, which is crucial for the developmental mesenchymal-epithelial interactions and is believed to be essential for the normal development of various organs (7-9). When expressed inappropriately, HGF-MET has been clearly implicated in oncogenesis and malignant progression of human epithelial cancers (see Ref. 10 for review).

HGF and MET were long thought to act only in a paracrine/endocrine pattern, but recently studies showed that MET is also expressed in some mesenchymal cells and reacts with HGF in an autocrine/paracrine pattern (11, 12). In addition, MET has been shown to be expressed at high levels in a variety of human tumors of mesenchymal origins, including human fibrosarcomas (11, 13-17). Establishment of a human HGF-MET autocrine loop in murine fibroblasts NIH3T3 or C127 cells induced morphological transformation *in vitro* and tumorigenicity *in vivo* (18, 19). Gene transfer of *hgf* into a low malignancy human leiomyosarcoma cell line SK-LMS-1 that expresses MET but has low levels of HGF, enhanced tumorigenicity and invasion/metastasis (20). Although these gain-of-function findings are consistent with a role of HGF-MET signaling in the malignant progression of human fibrosarcomas and other mesenchymal tumors, the role of *endogenous* high levels of MET expression in human fibrosarcomas has not been

examined directly. In addition, since rodent cells are normally much easier to be transformed than human cells (21, 22), further studies using human fibroblasts are needed.

To study the molecular mechanism underlying neoplastic transformation of human cells, McCormick and Maher and their colleagues developed a human fibroblastic cell lineage, designated MSU-1 (23). This lineage began with a foreskin-derived finite life span normal human fibroblast cell line, designated LG1, which spontaneously acquired an infinite life span following v-*myc* transfection, giving rise to a diploid, infinite-life-span cell strain, designated MSU-1.0. A near diploid, karyotypically stable cell strain with 45 chromosomes including two marker chromosomes, designated MSU-1.1, was further derived from the MSU-1.0 cells (24). Various carcinogen treatments of MSU-1.1 cells, followed by focus selection, gave rise to cell strains that form fibrosarcomas in athymic mice (25-27). Cells from representative tumors were put into culture, and the cell lines derived from them were used for further studies. Since the cell strains/lines in the system are isogenic, direct comparisons can be made of the levels of expression between malignant cell lines and their non-tumorigenic precursors. In contrast, when human cancer-derived cell lines are used for studies, very seldom are their normal precursors available to be used for comparison. In the previous study (see Chapter III), using the MSU-1 lineage, several foreskin-derived normal human fibroblast cell lines and several fibrosarcoma cell lines derived from patients' tumors, we demonstrated that MET over-expression is a common feature of human fibrosarcoma cell lines, including those transformed in culture by various carcinogens and those derived from patients' tumors. In the present study, we further demonstrated that HGF is co-expressed at various levels, and the MET protein is constitutively phosphorylated in theses fibrosarcoma cell lines. Using chimeric U1 snRNA/ribozyme strategy that has been described recently as a powerful tool to down-regulate specific gene expression

(28, 29), we also tested whether *endogenous* MET and/or HGF expression plays an important role for the malignant transformation of human fibroblasts.

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MATERIALS AND METHODS

Cell Lines and Cell Culture

The origin of the human fibroblast cell lines/strains used for the study has been described previously (see Chapter III). Cells were routinely cultured in complete medium at 37°C in a humidified incubator containing 5% CO_2 in air. The complete medium contains Eagle's minimum essential medium modified by addition of L-aspartic acid (0.2 mM), L-serine (0.2 mM) and pyruvate (1 mM), and supplemented with 10% supplemented calf serum (SCS) (Hyclone Laboratory, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml).

Western Blot Analysis

MET Protein. Cell lysates were prepared using single-detergent lysis buffer composed of 50 mM Tris-HCl, pH7.2, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM Na_3VO_4, 200 mM benzamidine, 1mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 25 μg/ml leupeptin. The protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL) with serum bovine albumin as the standard. Cell lysates containing 50 μg of protein were mixed with 5X SDS-PAGE sample buffer (0.125M Tris-HCl, pH6.8, 25% glycerol, 5% SDS, 0.0125% bromophenol blue, and 25% β-mercaptoethanol as the reducing reagent). The samples were separated on a 7.5% SDS-PAGE gel and then transferred onto an Immobilion-P membrane (Millipore, Bedford, MA). The blot was cut into two parts and then probed separately with a monoclonal antibody against MET (Catalog # 05-238, Upstate Biotechnology, Lake Placid, NY) or a monoclonal antibody against actin (Catalog # A5441, Sigma, St. Louis, MO), which was used as loading control.

SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL) was used according to manufacturers' instructions to detect the signal. The bands were quantified using Quantity One software (BioRad Laboratories, Hercules, CA).

Phospho-MET Protein. The same method was used to detect phospho-MET protein except that a primary antibody against phospho-MET (Y1234, Y1235) (Catalog # 07-211, Upstate Biotechnology, Lake Placid, NY) was used according to manufacturer's instructions. For the assay of autocrine MET phosphorylation, the cells were treated with neutralizing HGF antibody (a gift from Drs. Craig Webb and George F. Vande Woude at Van Andel Research Institute, Grand Rapid, MI) in serum-free medium for 18 hrs before cell lysates were collected.

Northern Blot Hybridization

Total RNA was extracted using RNAzolB (Tel-Test, Friendswood, TX). 25 μg of total RNA was subjected to electrophoresis on a 1% denaturing formaldehyde agarose gel, downward transferred to Hybond-N membrane (Amersham, Arlington Heights, IL) in 2X SSC (standard saline citrate buffer) (0.15 M NaCl, 0.015 M sodium citrate, pH7.0), and fixed onto the membrane using UV crosslinking (UV Stratalinker 2400, Stratagene, La Jolla, CA). DNA probe for the coding region of HGF mRNA (1.3 kb) was radiolabeled by the random primed labeling method (30). Northern hybridization was performed at 42°C overnight in Northern hybridization solution containing 50% formamide, 5X SSC, 5X Denhardts solution, 25 mM K_3PO_4 and 50 μg/ml salmon sperm DNA. The blots were washed twice at 42°C with 1X SSC/0.1% SDS and twice at 55°C with 0.25X SSC/0.1% SDS. The signals were captured by Molecular Imager BI PhosphoScreen (BioRad Laboratories, Hercules, CA) and were then quantified using Multi-analyst software

(BioRad Laboratories, Hercules, CA). The blot was stripped using boiling 0.1% SDS solution and re-probed with a probe for 18S rRNA, which was used a loading control.

Plasmid Constructs

The construction of the parental vector pZeoU1EcoSpe (referred to as pZeoU1 in this study) and chimeric pZeoU1EcoSpe/hammerhead ribozymes targeting *HGF* mRNA at position 701 and *met* mRNA at position 560 (referred to as pZeoU1/HGF701 and pZeoU1/met560) was described previously (28, 29). The latter construct targeting *met* mRNA was also cloned into a blasticidin cassette (pCMV/Bsd) (Invitrogen Corp., Carlsbad, CA) (referred to as pBsdU1/met560) to change the drug selection marker for dual transfection studies.

Stable Transfection and Screening of Human Fibrosarcoma Cells

Cells in exponential growth were plated in 100 mm diameter dishes at $5X10^3$-$1X10^4$ cells/dish. After 36 hr, 1 μg of plasmid DNA (pZeoU1 or pZeoU1/met560) were transfected using LipofectAMINE (Life Technologies, Gibco BRL, Grand Island, NY) according to manufacturer's instructions. Transfected cells were selectively grown in culture medium containing 400-1000 μg/ml zeocin (Invitrogen Corp., Carlsbad, CA). For the double transfection with pBsdU1/met560 and pZeoU1/HGF701, 1μg of each plasmid DNA was transfected simultaneously, and transfected cells were selectively grown in culture medium containing 1000 μg/ml zeocin and 4 μg/ml blasticidin (Invitrogen Corp., Carlsbad, CA). Drug resistant clones were randomly selected and screened for reduction of MET protein levels by Western blot analysis. For the double transfectants, the clones with reduced MET levels were further screened for reduction of *HGF* mRNA levels by Northern blot analysis.

Tumorigenicity Assay *in vivo*

BALB/c athymic mice 5-6 weeks of age were injected subcutaneously in the right and left rear flank regions with $1X10^6$ cells in 0.2 ml of serum-free Eagle's medium. For each cell strain tested, three mice were injected (six sites total). Tumor dimensions were measured twice weekly using a vernier caliper. The size of the tumors was calculated using the formula for the volume of a hemi-ellipsoid, the geometric figure most nearly approximating the shape of the tumor: Volume = 0.5236 × length × width × height.

Growth Rate *in vitro*

The cells were plated at $1X10^4$ cells per 60 mm diameter dish and harvested in triplicate plates on day 1, 3, 5, and 7. The total number of cells were counted using Coulter Particle Counter. The exponential growth phase of the growth curve was then used to calculate the population doubling time. Results from three independent experiments were presented.

RESULTS

Over-expression and Constitutive Phosphorylation of the MET Protein in Human Fibrosarcoma Cell Lines

Our previous study (see Chapter III) demonstrates that the MET protein is over-expressed in six out of six malignant cells lines derived from fibrosarcomas formed in athymic mice by carcinogen-transformed MSU-1.1 cells and four out of five human fibrosarcoma cell lines derived from patients' tumors, compared to the levels found in several foreskin-derived normal human fibroblast cell lines and in the two infinite-life-span cell strains, MSU-1.0 and MSU-1.1.

To determine whether the high levels of MET expression in the above human fibrosarcoma cell lines are active, we examined the basal phosphorylation levels of MET protein using an anti-phospho-MET antibody that recognizes Y^{1234} and Y^{1235} residues in the kinase domain of MET β-chain p145 (Fig. 1). The specificity of this antibody for phospho-MET was confirmed by the absence of signals upon treating the blots with Yersinia tyrosine phosphatase and then probing with the antibody (data not shown). Phosphorylation of the MET protein was very low or undetectable in four out of the five normal human fibroblast cell lines (Fig. 1A, lane 1, Fig. 1B, lane 1-5) as well as in the non-tumorigenic cell strains MSU-1.0 and MSU-1.1 (Fig. 1A, lane 2-3). In contrast, moderate to high levels of phosphorylation of the MET protein were observed in all 11 fibrosarcoma cell lines tested, including six malignant cell lines derived from fibrosarcomas formed in athymic mice by carcinogen-transformed MSU-1.1 cells (Fig. 1A, lanes 4-9) and four out of five human fibrosarcoma cell lines derived from patients' tumors (Fig. 1B, lanes 6-10). Thus MET over-expression and constitutive activation is a

common feature of human fibrosarcoma cell lines, including those transformed in culture by various carcinogens and those derived from patients' tumors.

Expression of HGF in Human Fibrosarcoma Cell Lines

Since HGF is the known ligand that binds to the receptor, MET, and induces the phosphorylation of the Y^{1234} and Y^{1235} residues of the MET protein, we examined the expression of HGF mRNA in above cell lines by Northern blot analysis. As shown in Figure 2, besides two minor transcripts at 7 kb and 4 kb, a 1.5 kb transcript was the major *hgf* transcript observed for all the cell lines tested, including two normal human fibroblast cell lines, the infinite life span cell strain MSU-1.1, and the 11 human fiborsarcoma cell lines, including the six malignant cell lines transformed in culture from MSU-1.1 cells and the five human fibrosarcoma cell lines derived from patients' tumors (Fig. 2). Most of the human fibrosarcoma cell lines tested expressed the same high levels of *hgf* as the normal human fibroblasts, although a few fibrosarcoma cell lines expressed *hgf* at relatively lower amounts than normal fibroblasts, including the γ2-3A/SB1 cell line, which was chosen for further study below.

Activation of the MET Receptor by HGF-MET Autocrine Loop in Human Fibrosarcoma Cell Lines

The co-expression of the HGF-MET pair strongly suggests that an HGF-MET autocrine loop is responsible for the constitutive phosphorylation of the MET protein observed for human fibrosarcoma cell lines. Using neutralizing HGF antibody, we tested this in human fibrosarcoma cell line γ2-3A/SB1, which had shown high levels of MET phosphorylation. As shown in Figure 3, the high level of phosphorylation of the MET protein was suppressed upon neutralizing HGF antibody treatments, suggesting the

presence of an HGF-MET autocrine loop in this fibrosarcoma cell line. In addition, we noticed that one fibrosarcoma cell line, SHAC, which showed a low level of MET protein in the previous study[5], showed high level of phosphorylation of the MET protein as well (Fig. 1B, lane 6). These findings suggest that in human fibrosarcomas, autocrine stimulation rather than over-expression activates the MET receptor.

Reduction of MET and/or HGF Expression in Human Fibrosarcoma Cell Lines by U1snRNA/ribozymes Gene Transfer

To test whether there is dependence on endogenous MET and/or HGF expression for human fibrosarcoma tumorigenicity, chimeric U1snRNA/hammerhead ribozyme constructs (Fig. 4) were used to specifically down-regulate *met* and/or *hgf* expression in human fibrosarcoma cell lines. Two cell lines were chosen to carry out the study: γ2-3A/SB1 and γ4-2A/SFT2, which are derived from fibrosarcomas formed in athymic mice by γ-radiation transformed MSU-1.1 cells. As shown in the previous study[5] and in Figure 2 of the present study, the former cell line expressed high level of MET and low level of *hgf*, whereas the latter cell line expressed both MET and *hgf* at moderate levels.

These two cell lines were stably transfected with control plasmid (pZeoU1) or U1snRNA/ribozyme targeting *met* mRNA (pZeoU1/met560). In addition, U1snRNA/ribozymes targeting both *met* and *hgf* mRNA (pBsdU1/met560 and pZeoU1/HGF701) were stably transfected into the γ4-2A/SFT2 cell line. Clonally derived cell strains were then screened by Western blot analysis for reduction of MET protein expression and/or by Northern blot analysis for reduction of HGF mRNA expression. Cell strains with at least 50% decrease in MET and/or *hgf* expression were designated as positive. The positive cell strains selected for subsequent experiments and their

relative levels of MET and *hgf* as compared to the parental and vector control cell strains are summarized in Figure 5 and Table 1. The U1snRNA ribozyme constructs were effective in down-regulating the MET and *hgf* levels, as in some case the MET level was down-regulated to as low as 3% that of the parental and vector control cell strains.

Inhibition of Human Fibrosarcoma Tumorigenicity *in vivo* after Reduction of MET and/or HGF Levels by U1snRNA/ribozymes

The role of HGF and MET on tumor formation and growth was examined by subcutaneously injecting non-transfected parental cell lines, vector-transfected control cell strains and cell strains with reduced MET and/or *hgf* levels into athymic mice and monitoring the animals for growth of tumors. As summarized in Table 2, for the γ2-3A/SB1 cell line, the parental and vector control cell strains formed tumors at 6/6 or 5/6 sites injected and the average latency for the tumors to reach 200 mm^3 was 7-8 week. The cell strains with reduced MET levels formed tumors at a lower frequency (varying from 4/6 to 0/6 sites injected) and a longer latency (varying from 12-13 weeks to indefinitely). For the γ4-2A/SFT2 cell line, the parental and vector control cell strains formed tumors at 6/6 or 4/4 sites injected and the average latency for tumors to reach 200 mm^3 was 3-5 weeks. For the cell strains with reduced MET levels, the frequency of tumor formation did not change, but they formed tumors after a longer latency (6-8 weeks). The cell strains with reduced levels of both MET and *hgf* formed tumors at a much longer latency (varying from 12 weeks to indefinitely).

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No Significant Effects on Growth Rates *in vitro* after Reduction of MET and/or HGF Levels by U1snRNA/ribozymes

If the positive transfectants used in the above studies had a much slower growth rate than the parental and vector control cell strains, this could partially explain the extended latency for tumor formation and growth. Therefore, we determined the population doubling time in culture of the non-transfected parental cell lines, the vector-transfected control cell strains, and the cell strains with reduced MET and/or *hgf* levels by allowing the cells to grow exponentially and counting the number of cells at several time points. As shown in Table 3, for both cell lines, there was no significant difference in the population doubling time of the parental cell lines, the vector control cell strains, or the cell strains with reduced MET and/or *hgf* levels. Thus the biological effects observed could not be accounted for by a slower growth rate of the positive transfectants.

Fig. 1. Analysis of MET phosphorylation levels (A and B) in human fibroblasts by Western blotting. Actin was used as loading control. Cell lines/strains in panel A are from MSU-1 lineage (lane 1: LG1, lane 2: MSU-1.0, lane 3: MSU-1.1, lanes 4-9: malignant cell lines derived from fibrosarcomas formed in athymic mice by MSU-1.1 cells transformed in culture by various carcinogens). Cell lines in panels B are several normal fibroblast cell lines (lanes 1-5) and several human fibrosarcoma cell lines derived from patients' tumors (lanes 6-10).

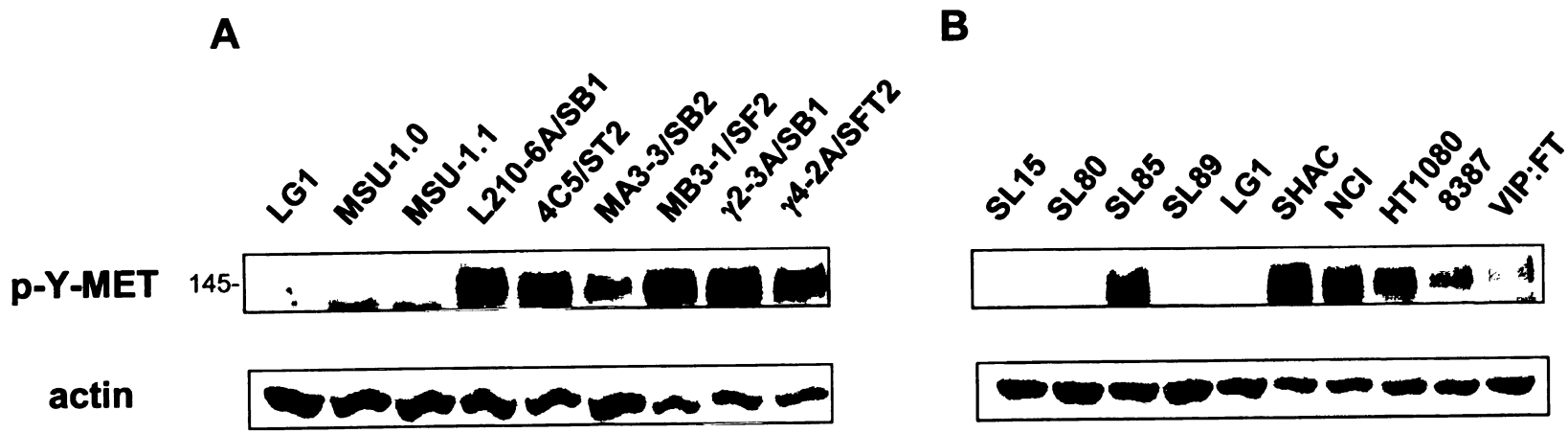
A
LG1
MSU-1.0
MSU-1.1
L210-6A/SB1
4C5/ST2
MA3-3/SB2
MB3-1/SF2
γ2-3A/SB1
γ4-2A/SFT2
p-Y-MET
145-
actin
B
SL15
SL80
SL85
SL89
LG1
SHAC
NCI
HT1080
8387
VIP:FT

Fig. 1

Fig. 2. Analysis of HGF mRNA expression in human fibroblasts by Northern hybridization. A 1.3 kb HGF cDNA probe was prepared by random labeling. The same blot was stripped and re-probed with 18S rRNA, which was used as a loading control (lanes 1 and 2: normal fibroblast cell lines; lane 3: MSU-1.1; lanes 4-9: malignant cell lines derived from fibrosarcomas formed in athymic mice by MSU-1.1 cells transformed in culture by various carcinogens; lanes 10-14: human fibrosarcoma cell lines derived from patients' tumors).

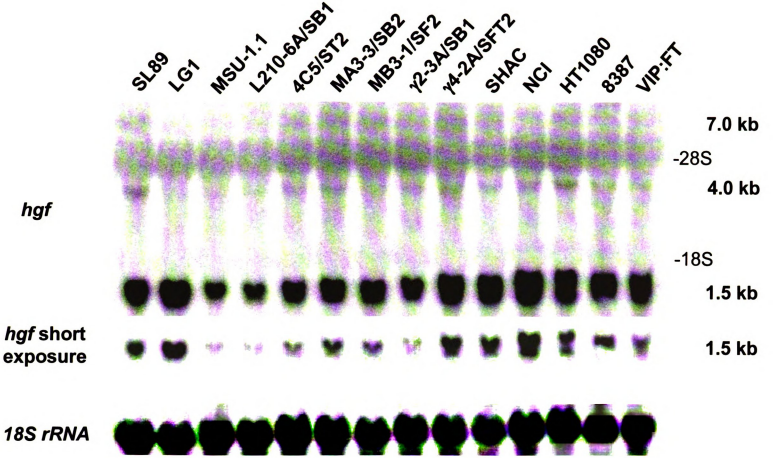
SL89
LG1
MSU-1.1
L210-6A/SB1
4C5/ST2
MA3-3/SB2
MB3-1/SF2
γ2-3A/SB1
γ4-2A/SFT2
SHAC
NCI
HT1080
8387
VIP:FT
7.0 kb
-28S
4.0 kb
hgf
-18S
1.5 kb
hgf short exposure
1.5 kb
18S rRNA

Fig. 2

Fig.

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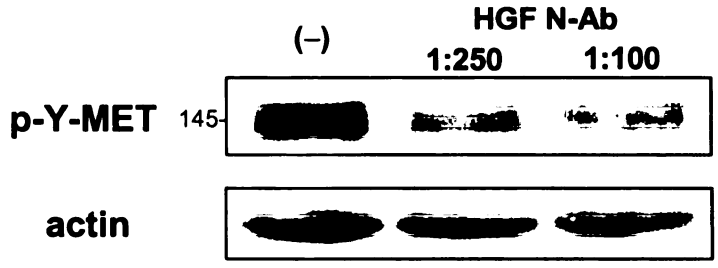

Fig. 3. Evidence of an autocrine HGF-MET loop in human fibrosarcoma cell line γ2-3A/SB1. Cells were treated without or with various concentrations of HGF neutralizing antibody (N-Ab) in serum-free medium for 18 hr before being harvested. Cell lysates were analyzed for MET phosphorylation by Western blotting. Actin was used as loading control.

Fig. 4. Schematic representation and sequence of chimeric U1snRNA/ribozyme constructs used to down-regulate *met* and/or *hgf* expression in human fibrosarcoma cell lines γ2-3A/SB1 and γ4-2A/SFT2.

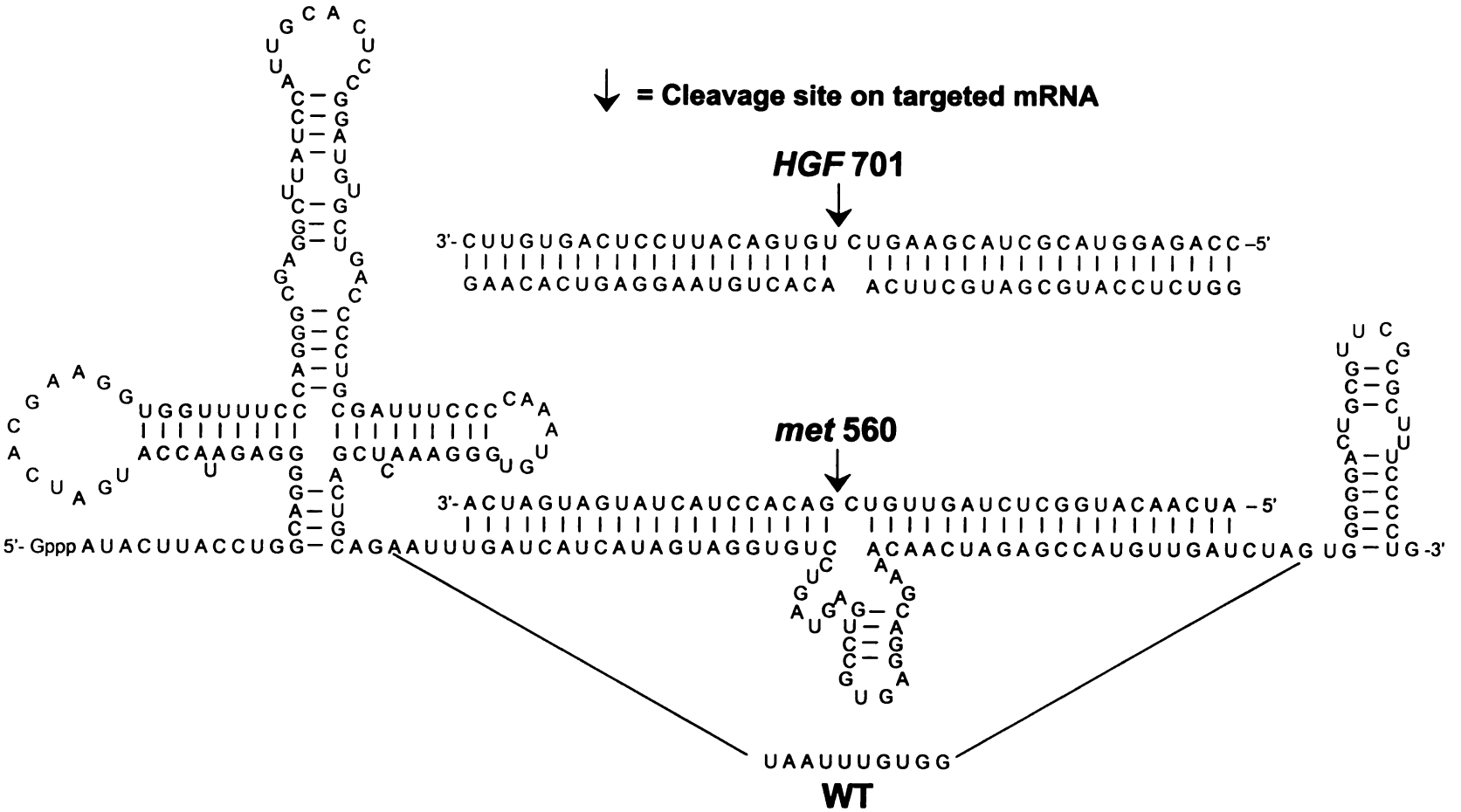
= Cleavage site on targeted mRNA
HGF 701
3'-CUUGUGACUCCUUACAGUGUCUGAAGCAUCGCAUGGAGACC-5'
GAACACUGAGGAAUGUCACA ACUUCGUAGCGUACCUCUGG
met 560
3'-ACUAGUAGUAUCAUCCACAGCUGUUGAUCUCGGUACAACUA-5'
5'-GpppAUACUUACCUGG-CAGAAUUUGAUCAUCAUAGUAGGUGUC ACAACUAGAGCCAUGUUGAUCUAGUG-UG-3'
UAAUUUGUGG
WT

Fig. 4

Fig. 5. Reduction of MET and/or *hgf* expression in selected human fibrosarcoma cell lines. A. Western blot analysis of MET expression in the γ2-3A/SB1 cell line transfected with chimeric U1snRNA/ribozyme targeting *met* or with control plasmid; B. Western blot analysis of MET expression in the γ4-2A/SFT2 cell line transfected with chimeric U1snRNA/ribozyme targeting *met* or with control plasmid; C. Western blot analysis of MET and Northern blot analysis of *hgf* expression in the γ4-2A/SFT2 cell line transfected with chimeric U1snRNA/ribozymes targeting *met* and *hgf*. P: parental; VC, vector control; met560: U1snRNA/ribozyme targeting *met*; met560/HGF701: U1snRNA/ribozymes targeting *met* and *HGF*.

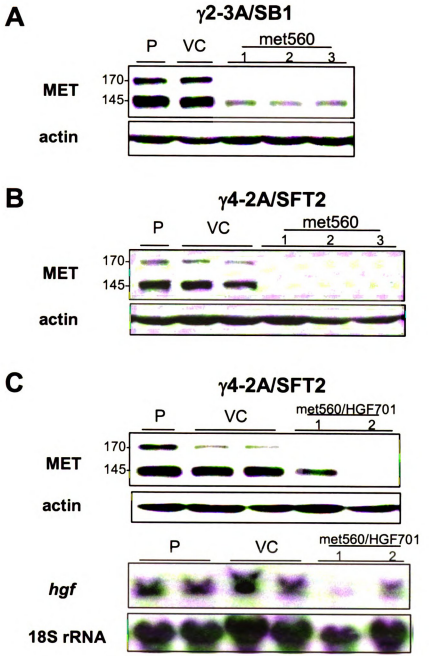
A
γ2-3A/SB1
P
VC
met560
1
2
3
MET
170
145
actin
B
γ4-2A/SFT2
P
VC
met560
1
2
3
MET
170
145
actin
C
γ4-2A/SFT2
P
VC
met560/HGF701
1
2
MET
170
145
actin
P
VC
met560/HGF701
1
2
hgf
18S rRNA

Fig. 5

Table II- 1 Inhibition of MET and/or HGF expression in human fibrosarcoma cell lines by U1snRNA/ribozymes targeting *met* and/or *HGF*

Cell lines	MET protein levels (% of parental and control)	HGF mRNA levels (% of parental and control)
γ2-3A/SB1-met560		
1	33	N/A
2	27	N/A
3	30	N/A
γ4-2A/SFT2-met560		
1	3	N/A
2	14	N/A
3	40	N/A
γ4-2A/SFT2-met560/HGF701		
1	41	13
2	17	49

Table II- 2 Inhibition of *in vivo* tumorigenicity and tumor growth of human fibrosarcoma cell lines by U1snRNA/ribozymes targeting *met* and/or *HGF*

Cell lines/strains	No. of sites with tumor /No. of sites injected	Tumor forming latency (weeks)[a]
γ2-3A/SB1		
Parental	6/6	7-8
VC	5/6	7-8
met560-1	4/6	12-13
met560-2	1/6	>17
met560-3	0/6	–
γ4-2A/SFT2		
Parental	6/6	3-4
VC1	4/4	4-5
VC2	6/6	3-4
met560-1	6/6	7
met560-2	5/6	6
met560-3	4/6	8
met560/HGF701-1	3/4	12
met560/HGF701-2	0/6	–

[a] Time required for the tumor to reach a volume of 200 mm^3.

Table II- 3 Growth properties of the parental, vector control and positive cell strains with reduced MET and/or HGF levels

Cell lines/strains	**Population DT**[a] mean (SD)[b] (h)	**Cell lines/strains**	**Population DT** mean (SD) (h)
γ2-3A/SB1		**γ4-2A/SFT2**	
Parental	20.1 (0.8)	Parental	19.2 (0.3)
VC	20.9 (0.3)	VC1	19.2 (0.5)
met560-1	21.2 (0.1)	VC2	20.1 (0.2)
met560-2	22.0 (0.7)	met560-1	22.4 (0.7)
met560-3	20.8 (0.2)	met560-2	19.4 (0.1)
		met560-3	21.3 (0.7)
		met560/HGF701-1	20.9 (0.3)
		met560/HGF701-2	23.3 (0.8)

[a] DT: doubling time.

[b] SD: standard deviation.

DISCUSSION

There are many human cancer-derived cell lines available for study, such as the five human fibrosarcoma cell lines utilized in the present study, including the HT1080 cell line. However, in almost all cases, the normal cells that gave rise to the tumor cells are not available. The unique value of the MSU-1 lineage is that in the lineage we have a family of cells clonally derived from another ranging from normal to tumor-derived cells. Since the cell strains/lines in the system are isogenic, direct comparisons can be made of the levels of expression between malignant cell lines and their non-tumorigenic precursors. Using the MSU-1 lineage as an important tool, as well as using several human fibrosarcoma cell lines derived from patients' tumors and several normal human fibroblast cell lines, we demonstrate that MET over-expression occurs frequently in human fibrosarcomas, including those transformed in culture and those derived from patients' tumors. The data also show that this over-expression is not associated with the transformation induced by a specific carcinogen. In addition, our previous study (see Chapter III) demonstrates that such high levels of MET expression cannot be attributed to gene amplification, but rather result from up-regulation by high levels of transcription factor Sp1.

Besides over-expression of the MET protein, our results show that human fibrosarcoma cell lines often co-express the ligand, HGF, and show constitutive phosphorylation of the MET protein. In addition, an HGF-MET autocrine loop was shown to be responsible for MET activation. The co-expression of HGF and MET and/or the constitutive activation of MET receptor has been reported for various human sarcomas, such as fibrosarcomas (11), osteosarcomas (13), rhabdomyosarcomas (14), Kaposi's sarcomas (31), myelomas (15) and glioblastomas (32). Consistent with the

literature reports, our findings suggest that autocrine HGF-MET signaling plays an important role for the development and dissemination of human fibrosarcomas.

Several lines of evidence support a role of HGF-MET signaling in tumor formation and malignant progression of human tumors, including human fibrosarcomas: 1) the high expression levels of MET and/or HGF in a variety of tumors and their correlation with malignancy and poor prognosis (10, 33); 2) the enhanced tumorigenicity and/or invasion following creation of an HGF-MET autocrine loop in tumor cells (18, 19, 34); 3) the genetic link between activating *met* mutations and hereditary papillary renal carcinoma (HPRC) (35) and the ability of the activating *met* mutants to promote tumor formation and cause invasion/metastasis (36); 4) diverse tumorigenesis observed in transgenic mice that were engineered to over-express HGF or TPR-MET, a truncated activated form of MET (37, 38). To date, however, the role of endogenous HGF or MET expression in the tumorigenesis of human fibrosarcomas has not been examined directly.

We tested this hypothesis by down-regulating endogenous MET and/or HGF expression using a U1 small nuclear RNA/ribozyme strategy in two human fiborsarcoma cell lines: one cell line (γ2-3A/SB1) expresses high level of MET and low level of HGF, whereas the other cell line (γ4-2A/SFT2) expresses both MET and HGF at moderate levels. Down-regulating MET expression in the former cell line greatly inhibited tumor formation and growth. For the latter cell line, down-regulating MET expression alone only slightly inhibited tumor growth, whereas reduction of both MET and HGF expression greatly inhibited tumor formation and growth. These results directly demonstrate dependence on MET and HGF expression for the tumorigenesis of human fibrosarcomas. Such inhibition of fibrosarcoma growth is likely to involve multiple mechanisms. First, reduction of MET and/or HGF can lead to inhibition of HGF-MET

autocrine loop, which in turn, can lead to inhibition of various downstream responses, such as induction of angiogenic factors (e.g. VEGF) (39), cell migration and invasion (20, 40), anti-apoptosis (41), etc. Since the *in vitro* growth properties of the cell strains with reduced MET and/or HGF levels are not significantly altered from those of the parental or vector control cell strains, it is likely that in human fibroblasts, HGF does not have a strong mitogenic effect. This is consistent with a previous report by Giordano et al (40) and our observations (unpublished studies). Thus, the inhibition of tumor growth *in vivo* is not likely to be the result of a change in the growth rate of cells per se, but rather of the inhibition of the various downstream responses described above. Secondly, other tumor-promoting avenues might also be inhibited. HGF is a strong angiogenic factor (5, 42). For the γ4-2A/SFT2 cell line, the greater inhibition of tumor growth upon reduction of both MET and HGF expression, compared to that upon reduction of MET only, suggests that HGF secreted by this cell line may act on nearby MET-expressing endothelial cells of blood vessels in a paracrine/endocrine pattern and result in angiogenesis, a critical factor for tumor growth after the tumor reaches a certain size.

In summary, we have shown that MET over-expression and constitutive activation is a common feature of human fibrosarcomas. By down-regulating MET and/or HGF expression using a U1 snRNA/ribozyme strategy, we demonstrated that there is strong dependence on endogenous MET and/or HGF expression for human fibrosarcoma tumorigenicity. This study clearly demonstrates a causal role of HGF-MET for the malignant transformation of human fibroblasts. It also strongly suggests that ribozymes could be of special therapeutical value in the treatment of cancer.

ACKNOWLEDGEMENTS

We thank Drs. Craig Webb and George Vande Woude at Van Andel Research Institute, Grand Rapid, MI for providing us with the HGF neutralizing antibody. We also thank Angela Hendrick for her technical assistance on screening transfectants.

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CHAPTER III

UP-REGULATION of MET (HGF RECEPTOR) BY TRANSCRIPTION FACTOR SP1 IN HUMAN FIBROSARCOMAS

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ABSTRACT

The hepatocyte growth factor receptor (MET) has been found to be expressed at high levels in a variety of human tumors, including human fibrosarcomas. Many studies indicate that this over-expression plays a causal role in tumor formation and/or malignant progression. To test the hypothesis that high levels of MET expression in human fibrosarcomas result from up-regulation by the transcription factor Sp1, malignant cell lines derived from tumors formed in athymic mice by carcinogen-transformed human fibroblastic MSU-1.1 cells and human fibrosarcoma cell lines derived from patients' tumors were examined for the levels of expression of MET and Sp1. Ten out of the eleven human fibrosarcoma cell lines tested showed high levels of MET expression, and six out of the ten fibrosarcoma cell lines with high levels of MET showed coordinately high levels of Sp1, compared to the levels found in several foreskin-derived normal human fibroblasts and the MSU-1.1 cell strain. The Sp1 expressed at high levels in those cell lines was functional as shown by its strong binding to oligonucleotides spanning the -156/-119 region of the *met* promoter. Two of the human fibrosarcoma cell lines, γ2-3A/SB1 and HT1080, and two of the normal fibroblast cell lines, LG1 and SL89, were compared further. The γ2-3A/SB1 and HT1080 cells exhibited high levels of MET and Sp1, whereas the LG1 and SL89 cells exhibited low levels of both proteins. Deletion analysis and site-directed mutagenesis of the *met* promoter revealed that the tandem Sp1 sites in the proximal promoter region (-223 to +60) are important for the transcription of the *met* gene. When the four cell lines were transfected with a *met* promoter-luciferase chimeric construct, the two fibrosarcoma cell lines, γ2-3A/SB1 and HT1080, exhibited *met* promoter activity at a much higher level than did the two normal fibroblast cell lines, LG1 and SL89. Transfection of the LG1 cells with an expression

vector carrying Sp1 cDNA resulted in a dose-dependent increase in the *met* promoter activity. What is more, transfection of the HT1080 cells with an Sp1 decoy to interfere with and inhibit Sp1 binding to DNA led to a dramatic reduction in MET expression. Taken together, these data demonstrate that in a majority of the human fibrosarcoma cell lines tested, the over-expression of MET results from high levels of transcription factor Sp1. These results suggest that when highly expressed, Sp1 functions as an oncoprotein.

INTRODUCTION

Hepatocyte growth factor, also known as scatter factor, is a multifunctional growth factor, which can induce mitogenesis, motogenesis, morphogenesis and angiogenesis as well as other biological effects (1-5). The receptor for HGF is MET, which belongs to a novel class of receptor tyrosine kinases consisting of a heterodimer of disulfide-linked α chain (50 kDa) and β chain (145 kDa) (6-7). High levels of MET have been found in a variety of human tumors, including human fibrosarcomas (8-13). Gene transfer and ribozyme inhibition studies of HGF and its receptor, MET, indicate that the HGF-MET pathway plays a causal role in tumor formation and malignant progression (14-18).

Although high levels of MET are frequently found in human tumors, gene amplification occurs in only a small portion of those cases (9, 19), suggesting a role for aberrant transcriptional regulation of the *met* gene causing over-expression. It is known that sequence-specific DNA binding proteins play a crucial role in transcription control (20). Recently, Liu (21) characterized the human *met* promoter as a promoter that lacks a TATA or CAAT box but has GC-rich regions. Functional analysis identified the -223 to -68 region of the promoter, which contains multiple Sp1 sites, as the minimum promoter region for basal transcription of the human *met* gene. Seol et al. (22) found that such features are highly conserved in the murine *met* promoter as well.

Transcription factor Sp1 is a member of the C_2H_2-type zinc finger family. It binds to GC-boxes and regulates the transcription of a wide variety of genes, such as housekeeping, tissue-specific and cell cycle-regulated genes (see Ref. 23 for review). Although Sp1 has been widely studied since it was first identified in 1987 (24), very few studies have examined whether it plays a role in tumor growth and malignant

progression. The first report, which showed simultaneous high-expression of *Sp1 and EGFR* in human gastric carcinomas, was published in 1992 (25). Very recently, Zannetti et al (26) demonstrated highly correlated up-regulation of Sp1 DNA-binding activity and the level of expression of uPAR in human breast carcinomas, and Shi et al (27) reported coordinated high levels of Sp1 and VEGF in human pancreatic adenocarcinomas.

To study the molecular mechanisms underlying neoplastic transformation of human cells, McCormick and Maher and their colleagues developed a human fibroblastic cell lineage, designated MSU-1 (28). This lineage began with a neonatal foreskin-derived finite life span normal human fibroblast cell line, designated LG1, which was subsequently step-by-step transformed in culture: first into a diploid infinite life span cell strain, designated MSU-1.0, then into a near-diploid, karyotypically stable cell strain that has 45 chromosomes including two marker chromosomes, designated MSU-1.1 (29). The MSU-1.1 cells were subsequently transformed into cells that are fully malignant, as demonstrated by their ability to form fibrosarcomas in athymic mice (30-32). Cells from representative tumors were put into culture, and the cell lines derived from them were used for further studies. Several such tumor-derived cell lines obtained with MSU-1.1 cells transformed in culture, as well as several foreskin-derived normal human fibroblast cell lines, and several human cell lines derived from patients' fibrosarcomas were used for the studies reported here (see Table 1). Using these cell lines, we determined that MET over-expression is a common feature of human fibrosarcomas and showed that in a majority of the human fibrosarcoma cell lines tested, high levels of MET expression result from up-regulation by the transcription factor Sp1.

MATERIALS AND METHODS

Cell Lines and Cell Culture

The human fibroblast cell lines used for the study are listed and characterized in Table 1. Cells were routinely cultured in complete medium at 37°C in a humidified incubator containing 5% CO_2 in air. The complete medium consisted of Eagle's minimum essential medium modified by addition of L-aspartic acid (0.2 mM), L-serine (0.2 mM) and pyruvate (1 mM), and supplemented with 10% supplemented calf serum (SCS) (Hyclone Laboratory, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml) and hydrocortisone (1 μg/ml).

Western Blot Analysis

MET Protein. Cell lysates were prepared using single-detergent lysis buffer composed of 50 mM Tris-HCl, pH7.2, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM Na_3VO_4, 200 mM benzamidine, 1mM PMSF, 25 μg/ml aprotinin, and 25 μg/ml leupeptin. The protein concentration was determined using a Bicinchoninic Acid (BCA) Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL) with serum bovine albumin as the standard. Cell lysates containing 50 μg of protein were mixed with 5X SDS-PAGE sample buffer (0.125M Tris-HCl, pH6.8, 25% glycerol, 5% SDS, 0.0125% bromophenol blue, and 25% β-mercaptoethanol as the reducing reagent). The samples were separated on a 7.5% SDS-PAGE gel and then transferred onto an Immobilion-P membrane (Millipore, Bedford, MA). The blot was probed with a monoclonal antibody against MET (Catalog # 05-238, Upstate Biotechnology, Lake Placid, NY). SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical, Rockford, IL) was used

according to manufacturers' instructions to detect the signal. The bands were quantified using the Quantity One software (BioRad Laboratories, Hercules, CA).

Sp1 Protein. The same method was used to detect Sp1 protein except that a triple-detergent lysis buffer (50 mM Tris-HCl, pH7.2, 150 mM NaCl, 50 mM NaF, 1% NP-40, 0.1% SDS, and 0.1% sodium deoxycholate, 1 mM Na_3VO_4, 200 mM benzamidine, 1mM PMSF, 25 μg/ml aprotinin, and 25 μg/ml leupeptin) was used to collect whole cell lysates. The primary antibody for detection of Sp1 protein was a monoclonal antibody (Catalog # SC-420) from Santa Cruz Biotechnology (Santa Cruz, CA).

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were prepared following methods described by Dignam et al. (33) with modifications. Cells were washed with cold PBS and collected by centrifugation. The cell pellets were resuspended in cold hypotonic buffer (10 mM HEPES, pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DTT), homogenized in a Dounce homogenizer using a type B pestle, and centrifuged at 3,300 g for 15 min. The packed nuclei were resuspended in low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.02 M KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT), extracted with high-salt buffer (the same as low-salt buffer, but with KCl increased to 1.2 M), and centrifuged at 25,000 g for 30 min. The supernatant was dialyzed against dialysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT), cleared by centrifugation at 25,000 g for 20 min, and stored at –80°C.

Double stranded oligonucleotides, spanning the -156/-119 region of the *met* promoter, i.e. 5' ACCTTGTCGTGGGCGGGGCAGAGGCGGGAGGAAACG 3' were end-labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP. Nuclear extract (15 μg protein)

was pre-incubated in a DNA binding buffer (4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 0.05 mg/ml poly(dI-dC).poly(dI-dC)) at room temperature for 10 min, and then incubated with 1 μl (100,000cpm) of the ^{32}P end-labeled oligonucleotides for an additional 20 min. In some experiments, 1μl of anti-Sp1 or anti-Sp3 antibody (Catalog # SC-420 X and SC-644 X, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) was added, and the reaction was incubated on ice for 1 hr. The free and bound labeled oligonucleotides were separated on a 4% non-denaturing polyacrylamide gel followed by autoradiography using a Molecular Imager BI PhosphoScreen (BioRad Laboratories, Hercules, CA).

Construction of Plasmids

The human *met* promoter-luciferase plasmids were constructed by cloning various lengths of the 5' flanking region of human *met* gene into the polylinker region of the promoterless vector pGL3-basic containing firefly luciferase (Promega, Madison, WI). The DNA fragments containing various lengths of the *met* promoter were generated by PCR amplification as described previously (21).

Mutations were introduced into the consensus Sp1 binding sites (changing GGGCGG to GTTCAT) in the above *met* promoter-luciferase constructs using QuikChange™ XL Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The sequences of the mutated plasmid DNA were verified by automated nucleotide sequencing using the Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech, Piscataway, NJ) and the Cy5 labeled GL primer 2, 5' CTTTATGTTTTTGGCGTCTTCC 3' (See Catalog of Promega, Madison, WI for the location of the sequencing primer). The primers used to sequentially mutate the tandem Sp1 sites 2 and 3 in the -146/-129 region of the *met* promoter were: 5'

CCTTGTCGTGTTCGGGGCAGAGGCTTGAGGAAACGC 3' (sense), 5' GCGTTTCCT CAAGCCTCTGCCCCGAACACGACAAGG 3' (antisense); 5' CCTTGTCGTGTTCATG-GCAGATACTTGAGGAAACGC 3' (sense), 5' GCGTTTCCTCAAGTATCTGCCATGA-ACACGACAAGG 3' (antisense). The primers used to sequentially mutate the Sp1 site 1 in the - 79/-74 region of the *met* promoter were: 5' CGGCAGGAAGTTCGGGGGCCG-ATTTCC 3' (sense), 5' GGAAATCGGCCCCCGAACTTCCTGCCG 3' (antisense); 5' CGGCAGGAAGTTCATGGGCCGATTTCC 3' (sense), 5'GGAAATCGGCCCATGAA-CTTCCTGCCG 3' (antisense).

Transient Transfections

The cells were cultured in 6-well plates for 36 hr and then transiently transfected with the *met* promoter-luciferase chimeric constructs using LipofectAMINE (Life Technologies, Gibco BRL, Grand Island, NY). In each experiment, 1 X 10^5 cells were co-tranfected with 1.5 pmole of DNA from each *met* promoter-luciferase construct and 0.1 μg or 0.01 μg of the CMV promoter-*Renilla* luciferase plamid DNA (control luciferase for transfection efficiency) (Promega, Madison, WI). In certain designated experiments, various amounts of plasmid DNA containing CMV-Sp1 cDNA were additionally added. The cells were incubated with the DNA/liposome/serum-free medium for 7 hr and then incubated for an additional 48 hr or for various periods of time in complete medium containing 10% supplemented calf serum before being harvested.

Luciferase Activity Assays

After the transient transfection, the cells were washed with PBS twice and collected in 100 μl 1X Passive Lysis Buffer (Promega, Madison, WI) using a rubber policeman. The suspension was subjected to one freeze-thaw cycle and centrifuged at

12,000 g for 4 min. The supernatant was frozen rapidly in liquid nitrogen, and stored at –80°C. The luciferase activity of the lysates was measured using the Dual Luciferase Assay System (Promega, Madison, WI). All experiments were carried out in triplicates to ensure reproducibility. Relative luciferase activity (RLA) was reported after normalization for the control *Renilla* luciferase activity and the protein concentration of the lysates.

Transcription Factor Decoy Inhibition Assays

Complementary single stranded oligonucletides were synthesized as phosphorothioates and annealed by heating to 95°C for 10 min followed by slow cooling to room temperature. The decoy sequence for Sp1 was (5' ATTCGATCGGGGCGGGGCGAGC 3') and the mismatch control sequence was (5'ATTCGATCGGTTAAGTGCGAGC 3'). The decoy or the mismatch control oligonucleotides were delivered into the cells using LipofectAMINE (Life Technologies, Gibco BRL, Grand Island, NY). Cell lysates were collected at various time points after the treatments. Cell lysates containing 50 μg of protein were then analyzed for MET protein expression by Western blot analysis as described above.

Statistical Analysis

The levels of MET and Sp1 expression were compared using Pearson's coefficient of correlation and ANOVA procedures for simple regression and correlation.

RESULTS

Over-expression of MET in Human Fibrosarcoma Cell Lines

To test whether over-expression of MET is a common feature of human fibrosarcomas, we compared the level of MET expression in several normal human fibroblast cell lines and two infinite life span non-tumorigenic fibroblast cell strains with that found in six malignant cell lines derived from fibrosarcomas formed in athymic mice by various carcinogen-transformed MSU-1.1 cells, as well as in five human fibrosarcoma cell lines derived from patients' tumors (Fig. 1A and 1B) (see Table 1 for their origin). Low levels of MET protein, including the mature β-chain p145 and its precursor p170, were detected in the five normal human fibroblast cell lines tested (Fig. 1A, lane 1; Fig. 1B, lanes 1-5). For the MSU-1 lineage, the MET level was increased stepwise, with a slight increase in the infinite life span, partially growth factor independent cell strain MSU-1.1, and a further increase in the six malignant cell lines derived from carcinogen-transformed MSU-1.1 cells, compared to that found in the finite life span founder cell line, LG1, and the first derivative cell strain, MSU-1.0 (Fig. 1A). As shown in Figure 1B, high levels of MET expression were observed in four out of the five human fibrosarcoma cell lines derived from patients' tumors, compared to the average MET level in the five normal human fibroblast cell lines tested. These results indicate that MET is frequently over-expressed in human fibrosarcoma cell lines, not only in those derived from patients' tumors, but also in those derived from fibrosarcomas formed by human cells transformed in culture by carcinogen treatments.

To see if increased expression of MET in these cell lines resulted from gene amplification, we analyzed the copy number of the *met* gene in these cell lines by Southern blot analysis using a probe against nucleotides 1818-3142 of the *met* cDNA.

Gene amplification was observed for only two out of the eleven human fibrosarcoma cell lines tested, viz, L210-6A/SB1 and VIP:FT, and the amplification was only about two-fold above that seen in the normal human fibroblast cell lines and in the other nine fibrosarcoma cell lines (data not shown).

Over-expression of Sp1 in Human Fibrosarcoma Cell Lines

Having ruled out gene amplification as the explanation for over-expression of MET, we then examined them for aberrant transcriptional regulation. The human *met* promoter has been characterized as a TATA-less promoter and shown to have multiple binding sites for regulatory transcription factor Sp1 (21). Figure 1C and 1D show the results of Western blot analysis of Sp1 expression levels in the above cell lines. Two bands (p95 and p106), which are the un-phosphorylated and phosphorylated forms of Sp1, respectively, were detected for the Sp1 protein. The level of Sp1 in the five normal fibroblast cell lines and in the infinite life span, non-transformed cell strain MSU-1.0 was virtually undetectable or very low (Fig. 1C, lanes 1-2; Fig. 1D, lanes 1-5). The MSU-1.1 cells showed a moderate increase in the level of Sp1 (Fig. 1C, lane 3) above that in the precursor strain, MSU-1.0. Out of the six malignant cell lines derived from MSU-1.1 that showed over-expression of MET in Figure 1A, four cell lines also showed over-expression of Sp1 (Fig. 1C). Similarly, out of the four fibrosarcoma cell lines derived from patients' tumors that showed high levels of MET in Figure 1B, two also showed high levels of Sp1 expression, compared to the average Sp1 level in the five normal human fibroblast cell lines tested (Fig. 1D). In summary, all the normal human fibroblast cell lines and the infinite life span cell strain MSU-1.0 showed low levels of both MET and Sp1; the infinite life span and partially growth factor independent cell strain MSU-1.1 showed a slightly higher level of MET and Sp1, whereas six out of the ten human fibrosarcoma cell lines with high levels of MET showed high levels of Sp1 as well.

To further confirm whether there is a direct correlation between Sp1 level and MET expression, we quantified the expression levels of Sp1 and MET using the BioRad Quantity One program, and compared the values to that of the LG1 cell line, one of the five normal human fibroblasts cell lines tested and also the founder cell line of the MSU-1 lineage. The data are summarized in Table 2. The levels of Sp1 and MET were compared using Pearson's coefficient of correlation and ANOVA procedures for simple regression and correlation. For the five normal fibroblast cell lines, infinite life span cell strains MSU-1.0 and MSU-1.1, and the six human fibrosarcoma cell lines that showed high levels of MET and Sp1, there is a positive and significant correlation between MET and Sp1 levels ($r = 0.80$; $P<0.001$).

Strong Sp1 DNA Binding Activities to the -156/-119 Region of the *met* Promoter in Human Fibrosarcoma Cell Lines

To determine whether the high levels of Sp1 protein observed in the above human fibrosarcoma cell lines are functional, electrophoretic mobility shift assays were carried out using nuclear extract of those cell lines. The probe used for the study spans the -156/-119 region of the *met* promoter, i.e. 5' ACCTTGTCGTGGGCGGGGCA-GAGGCGGGAGGAAACG 3', which contains two putative Sp1 binding sites. Because Sp3, another Sp-family protein, has the same consensus binding sequence as Sp1, antibodies against Sp1 or Sp3 were used to super-shift the DNA-protein complexes, and in that way identify the factor involved in the binding. The nuclear extracts of all six of the human fibrosarcoma cell lines exhibiting high level of both MET and Sp1 showed strong Sp1 binding to the -156/-119 region of the *met* promoter, whereas the nuclear extracts of the normal human fibroblast cell lines showed undetectable or very weak Sp1 binding. Examples are shown in Figure 2. As shown in the first lane of the assayed fibrosarcoma cell lines, HT1080 and VIP:FT, a major DNA-protein complex (t) and a

faster moving minor complex (b) were observed. The results of the super-shift assays by anti-Sp1 or anti-Sp3 antibodies, shown in the second and third lanes of each assayed sample, indicated that the majority of the t complex consisted of Sp1-DNA. Therefore the Sp1 protein shown to be expressed at high levels in the human fibrosarcoma cell lines is functional and can result in strong binding to the *met* promoter DNA.

Sp1 Binding Sites are Important for the *met* Gene Expression in Human Fibroblasts

To delineate the sequences essential for the *met* gene expression, we constructed a series of human *met* promoter-firefly luciferase chimeric constructs in which various lengths of the 5'-flanking region of the human *met* gene were cloned into the promoter-less firefly luciferase vector (Fig. 3A). These constructs and the control CMV promoter-*Renilla* luciferase plasmid were transiently transfected into human fibrosarcoma cell line, HT1080, which had shown highly correlated MET and Sp1 levels. After normalization for the control *Renilla* luciferse activity, the relative luciferase activities (RLA) of each *met* promoter-luciferase construct are presented in Figure 3B. The 2.6met-luc construct, which contains the –2,615 to +60 region of the *met* promoter, had obvious promoter activity (~ 30 RLA) compared with the promoter-less vector. Deletion of the -2,615 to -223 region of the *met* promoter (from 2.6met-luc to 0.2met-luc) affected the promoter activity only slightly. However, further deletion of the -223 to -68 region (from 0.2met-luc to 0.1met-luc) dramatically reduced the promoter activity. Thus the proximal promoter region (-223 to -68) is essential for the constitutive *met* promoter activity.

Since this region contains multiple Sp1 sites, we further generated a series of 0.2met-luc constructs with mutations in the Sp1 sites (Fig. 3C) to determine the role of Sp1 sites for *met* transcription. Mutation of the Sp1 site 1 in the -79/-74 region resulted

in a slight increase of the promoter activity, whereas mutation of the two tandem Sp1 sites (2 an 3) in the -146/-129 region resulted in an ~ 65% reduction of the promoter activity. When all three Sp1 sites were mutated, there was an ~ 40% reduction of the promoter activity (Fig. 3C). As a further confirmation of the results obtained using the 0.2met-luc as the mutagenesis template, we also generated a series of 2.6met-luc constructs with mutations in various Sp1 sites. Similar results were obtained (Fig. 3C). These results demonstrate that the two tandem Sp1 sites in the -146/-129 region are important positive elements for *met* transcription in human fibroblasts, whereas the Sp1 site in the -79/-74 region is most likely a weak negative element.

Comparison of the *met* Promoter Activity in Cell Lines with Different Sp1 Levels

To test whether cells with high level of Sp1 would have high *met* promoter activity, we examined the *met* promoter activities in four cell lines with different Sp1 levels, i.e., two normal human fibroblast cell lines (LG1 and SL89) with low levels of Sp1 and MET, and two human fibrosarcoma cell lines with high levels of Sp1 and MET (γ2-3A/SB1 and HT1080, which are derived from carcinogen transformation in culture and from patient's tumor, respectively). For this experiment, it was important to choose the CMV promoter-*Renilla* luciferase but not other *Renilla* luciferse constructs (such as thymidine kinase promoter- or SV40 promoter-*Renilla* luciferase) as the control plasmid because both thymidine kinase promoter and SV40 promoter contain multiple Sp1 sites and are highly modulatable by Sp1 levels (34, 35), whereas the CMV promoter does not contain multiple Sp1 sites, and our test showed that it was not responsive to exogenous Sp1 introduced by an expression vector carrying Sp1 cDNA (data not shown).

The relative time-wise post transfection *met* promoter-luciferase activities (with the activity of the LG1 cell line at 0 hr time point as 1.0) are summarized in Figure 4 after normalization for the control CMV promoter-*Renilla* luciferse activities and the protein

concentration of the lysates. For the fibrosarcoma cell line γ2-3A/SB1, the *met* promoter activity went up gradually and the level increased ~ 17 fold at 48 hr post transfection. For the fibrosarcoma cell line HT1080, the *met* promoter activity went up shortly after the transfection, reaching peak value (~ 16 fold) at about 24 hr post transfection, then gradually went down towards the basal level. For the normal fibroblast cell lines LG1 and SL89, the promoter activities remained at about the same low level throughout 48 hr post transfection.

To determine whether expression level of Sp1 could modulate the *met* promoter activity, we co-transfected the LG1 cell line, which has a low constitutive level of Sp1 protein, with the 2.6met-luc construct and increasing concentrations of pCMV-Sp1 expression vector. As shown in Figure 5, without exogenous Sp1 stimulation, the promoter activity remained at about the same low level throughout 48 hr, and then increased ~ 3 fold at 72 hr post transfection. When LG1 cells were co-transfected with 0.4 μg or 0.8 μg pCMV-Sp1, there was a gradually dose-dependent increase of the promoter activity throughout 72 hr post transfection. At 72 hr, 0.4 μg pCMV-Sp1 resulted in ~ 7 fold increase of the promoter activity, and 0.8 μg pCMV-Sp1 resulted in ~ 11 fold induction of the promoter activity. Thus, the *met* promoter activity is highly modulatable by Sp1, and Sp1 acts as a strong activator for *met* transcription in human fibroblasts.

Inhibition of Sp1 Binding to the *met* Promoter Reduced MET Expression

Finally, we used a transcription factor decoy strategy (36-37) to test whether interfering with and inhibiting the Sp1 binding to the *met* promoter would lead to a decrease of MET expression in the human fibrosarcoma cell line HT1080. A double-stranded decoy containing the consensus Sp1 binding site and a mismatched control with a mutated Sp1 binding sequence were synthesized as phosphorothioate

oligonucleotides, which are highly resistant to nucleases (38). The ability of the decoy, but not the mismatched control, to compete for binding to transcription factor Sp1 was tested using the electrophoretic mobility shift assay (data not shown). The decoy or the mismatched control oligonucleotides were transiently delivered into the cells, and the amount of the MET protein present in the cell lysates post treatment was evaluated by Western blot analysis. As shown in Figure 6, at 12 hours after the treatment with the decoy, the amount of the 170 kDa MET precursor was dramatically reduced (~ 80-90%) and the 145 kDa mature MET β chain protein was moderately decreased (~ 50%) as well. This inhibition lasted for at least 24 hr. For the mismatched control treatment, there was no obvious change in the MET level. The level of an unrelated control protein (actin) remained constant in spite of the presence of the transcription factor decoy or the mismatched control oligonucleotides.

Table III- 1 Human fibroblast cell strains/lines used in this study

Cell strains/lines	***Origin***
LG1	Foreskin-derived normal human fibroblast cell line, used to derive the MSU-1 lineage (29)
MSU-1.0	Infinite life span, diploid cell strain derived from LG1 cells following v-*myc* transfection (29)
MSU-1.1	Infinite life span, near diploid, chromosomally stable and partially growth factor independent cell strain arisen from MSU-1.0 cells (29)
4C5/ST2, L210-6A/SB1	Derived from fibrosarcomas formed in athymic mice after injection of MSU-1.1 cells malignantly transformed by benzo[a]pyrene (30)
MA3-3/SB2, MB3-1/SF2	Derived from fibrosarcomas formed in athymic mice after injection of MSU-1.1 cells malignantly transformed by methylnitrosourea (31)
γ2-3A/SB1, γ4-2A/SFT2	Derived from fibrosarcomas formed in athymic mice after injection of MSU-1.1 cells malignantly transformed by γ-radiation (32)
NF15, NF80, NF85, NF89	Foreskin-derived finite life span normal human fibroblasts established in this laboratory
SHAC[a], NCI[a], HT1080[a], VIP:FT[a], 8387[b]	Human fibrosarcoma cell lines derived from patients' tumors and maintained in culture

[a] From American Type Culture Collection, Rockville, MD.

[b] From Dr. Stuart A Aronson, National Cancer Institute, Bethesda, MD.

Fig. 1. MET (A and B) and Sp1 (C and D) protein expression in human fibroblasts by Western blot analysis. The origin of each cell strain/line is listed in Table 1. Cell lines/strains in panels A and C are from the MSU-1 lineage (lane 1: LG1, lane 2: MSU-1.0, lane 3: MSU-1.1, lanes 4-9: malignant cell lines derived from fibrosarcomas formed in athymic mice by MSU-1.1 cells malignantly transformed in culture by various carcinogens). Cell lines in panels B and D are several normal fibroblast cell lines (lanes 1-5) and several human fibrosarcoma cell lines derived from patients' tumors (lanes 6-10). Cell lines that are marked with * are those showing high levels of both MET and Sp1.

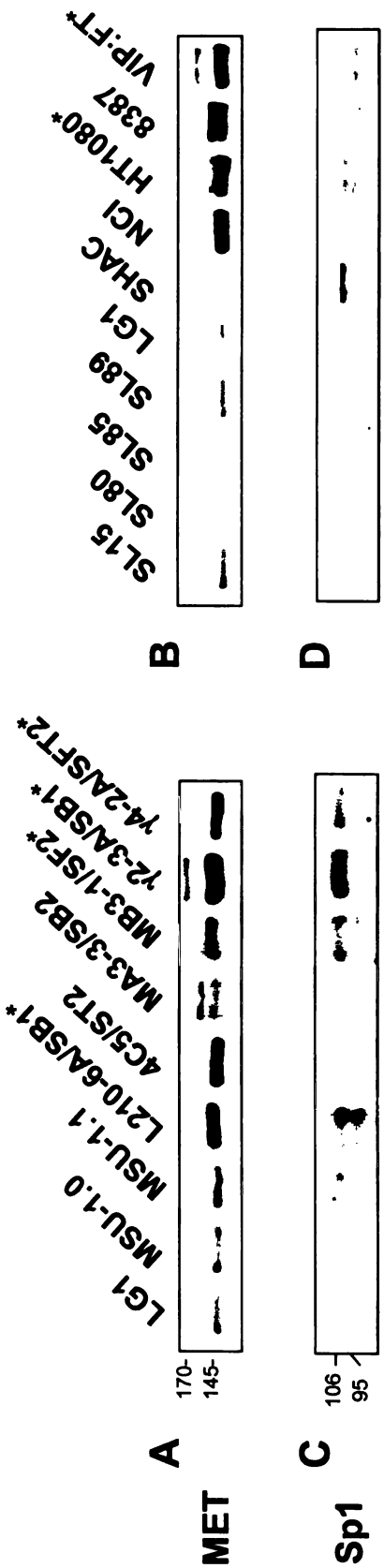

A
MET
170-
145-
LG1
MSU-1.0
MSU-1.1
L210-6A/SB1*
4C5/ST2
MA3-3/SB2
MB3-1/SF2*
γ2-3A/SB1*
γ4-2A/SFT2*
C
Sp1
106
95
B
SL15
SL80
SL85
SL89
LG1
SHAC
NCI
HT1080*
8387
VIP:FT*
D

Fig. 1

Table III- 2 Summary of the levels of expression of Sp1 and MET in human fibroblasts

The expression of Sp1 and MET were examined by Western blot analysis as shown in Figure 1. The levels of expression were quantified using the BioRad Quantity One program. The relative levels were compared to that of the normal fibroblast cell line LG1 (LG1 as 1.0).

Cell line	Sp1 level (fold)	MET level (fold)
Normal human fibroblast cell lines		
LG1	1.0	1.0
SL15	1.5	2.2
SL89	2.0	1.7
SL80	3.3	0.1
SL85	6.1	0.3
Non-transformed cell strains		
MSU-1.0	0.5	1.1
MSU-1.1	11.9	1.5
Human fibrosarcoma cell lines		
γ4-2A/SFT2	17.5	2.6
MB3-1/SF2	23.0	3.0
γ2-3A/SB1	29.8	6.3
L210-6A/SB1	32.5	3.0
HT1080	29.6	7.9
VIP:FT	30.0	9.0

Fig. 2. Sp1/Sp3 DNA binding activity in human fibroblasts by electrophoretic mobility shift assays. The origin of each cell line is listed in Table 1. 15 μg of nuclear extracts from each cell line were incubated together with a ^{32}P end-labeled oligonucleotide spanning -156/-119 region of the *met* promoter (5' ACCTTGTCGTGGGCGGGGCAG-AGGCGGGAGGAAACG 3'), in the presence (+) or absence (–) of anti-Sp1 or anti-Sp3 antibody. The binding reactions were separated on a 4% non-denaturing polyacrylamide gel followed by autoradiography (lanes 1-6: normal human fibroblast cell lines, lanes 7-12: human fibrobsarcoma cell lines). NE, nuclear extracts.

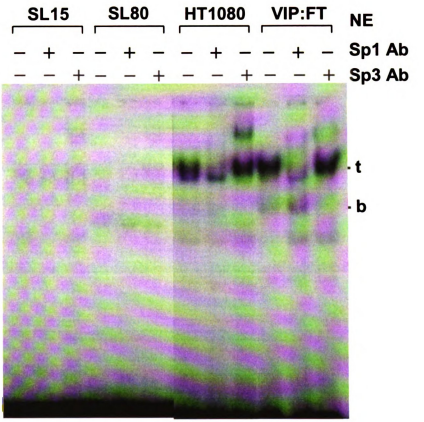
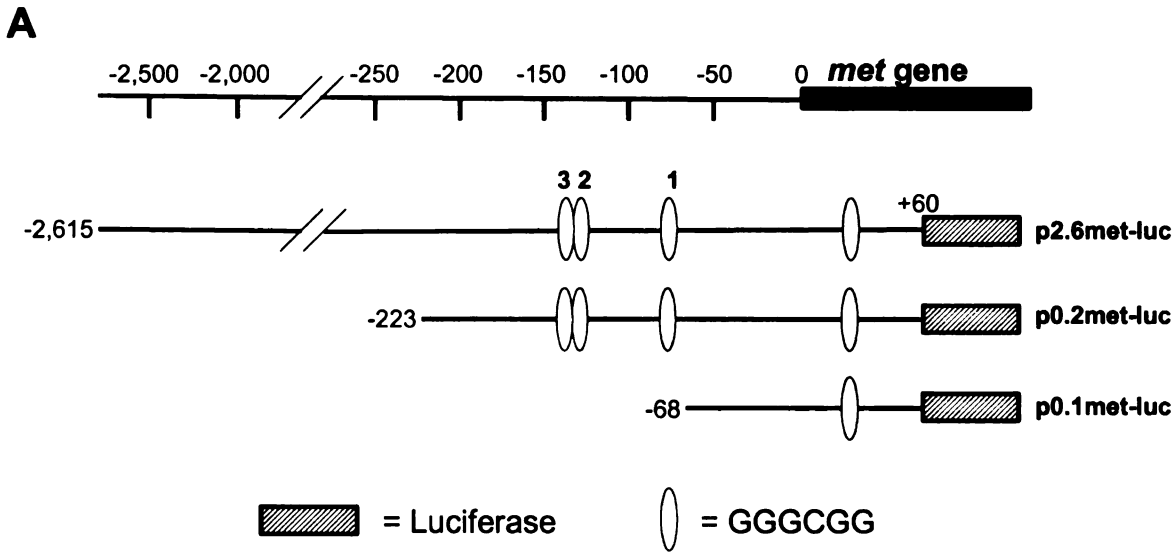


Fig. 2

Fig. 3. Functional analyses of human *met* 5'-flanking sequences in human fibrosarcoma cell line HT1080. A. Schematic representation of human *met* promoter-luciferase chimeric constructs. Various lengths of the 5' flanking region of human *met* gene were inserted before the luciferase reporter gene. The numbers are relative to the transcription initiation site. B. *met* promoter-luciferase activities in HT1080 cells at 48 hr after transient co-transfection with 1.5 pmole of the constructs shown above and 0.01 μg of the CMV promoter-*Renilla* luciferase control plasmid. The relative luciferase activities (with the activity of the promoter-less pGL3-basic vector as 1.0) are presented after normalization for the control *Renilla* luciferase activities. C. Effects of mutations in the Sp1 sites on human *met* promoter activity. The wild-type Sp1 site (GGGCGG) was mutated to GTTCAT using Stratagene QuikChange Mutagenesis Kit. HT1080 cells were co-transfected with 1.5 pmole of the wild type template (0.2met-luc or 2.6met-luc construct) or mutated constructs and 0.01 μg CMV promoter-*Renilla* luciferase control plasmid. The relative luciferase activities (with the activity of the appropriate wild type construct as 100%) are presented after normalization for the control *Renilla* luciferase activities.


A
-2,500
-2,000
-250
-200
-150
-100
-50
0
met gene
3 2
1
+60
-2,615
p2.6met-luc
-223
p0.2met-luc
-68
p0.1met-luc
= Luciferase
= GGGCGG

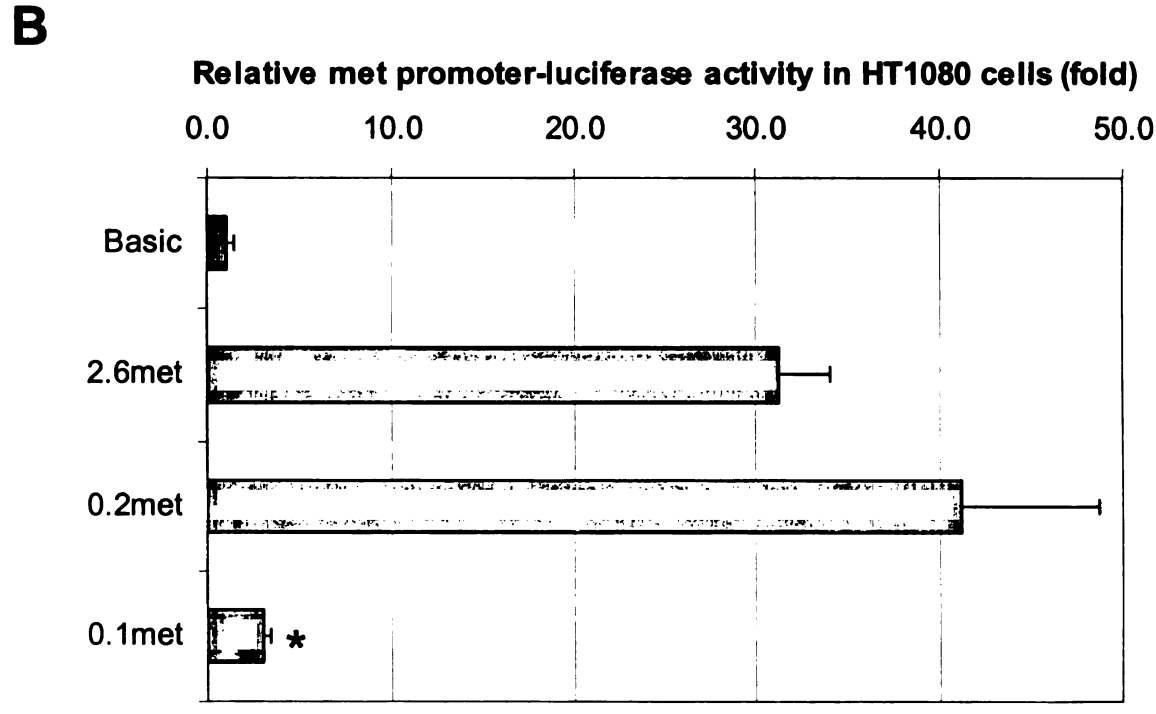
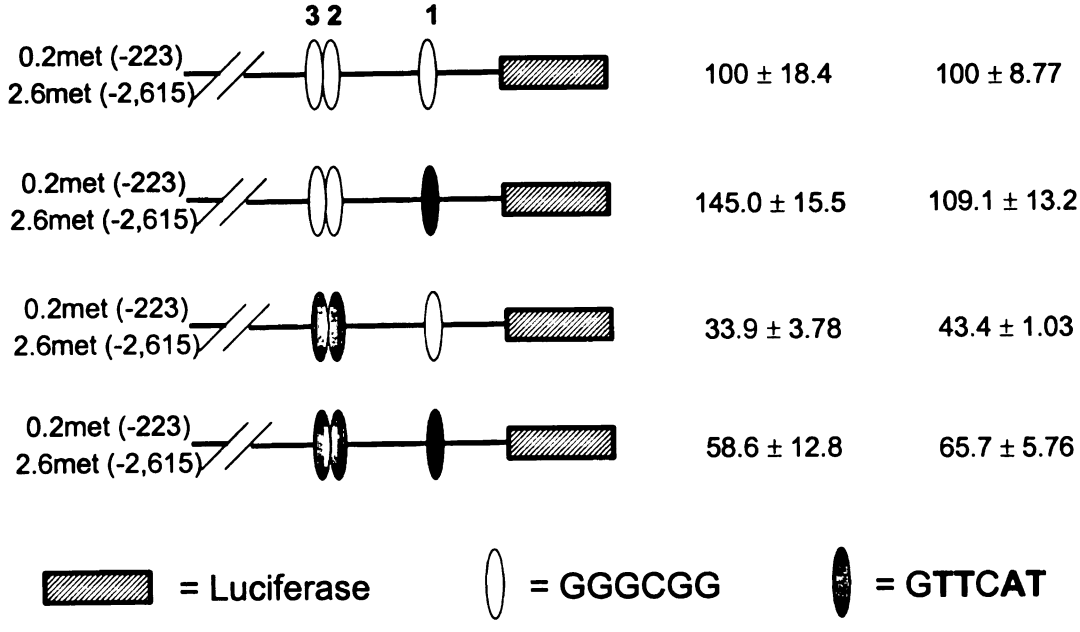

B
Relative met promoter-luciferase activity in HT1080 cells (fold)
0.0
10.0
20.0
30.0
40.0
50.0
Basic
2.6met
0.2met
0.1met
*

Fig. 3

C



	% of 0.2met	% of 2.6met
0.2met (-223) 2.6met (-2,615)	100 ± 18.4	100 ± 8.77
0.2met (-223) 2.6met (-2,615)	145.0 ± 15.5	109.1 ± 13.2
0.2met (-223) 2.6met (-2,615)	33.9 ± 3.78	43.4 ± 1.03
0.2met (-223) 2.6met (-2,615)	58.6 ± 12.8	65.7 ± 5.76

Fig. 3 (cont'd)

Fig. 4. Comparison of the *met* promoter activities in two human fibrosarcoma cell lines, γ2-3A/SB1 and HT1080, and two normal human fibroblast cell lines, LG1 and SL89. The cells were co-transfected with 1.5 pmole of the 2.6met-luc construct and 0.1 μg of the CMV promoter-*Renilla* luciferase control plasmid. Cell lysates were collected at various time points post transfection. The relative luciferase activities (with the activity of the LG1 cell line at 0 hr time point as 1.0) are presented after normalization for the control *Renilla* luciferase activities and the protein concentration of the lysates.

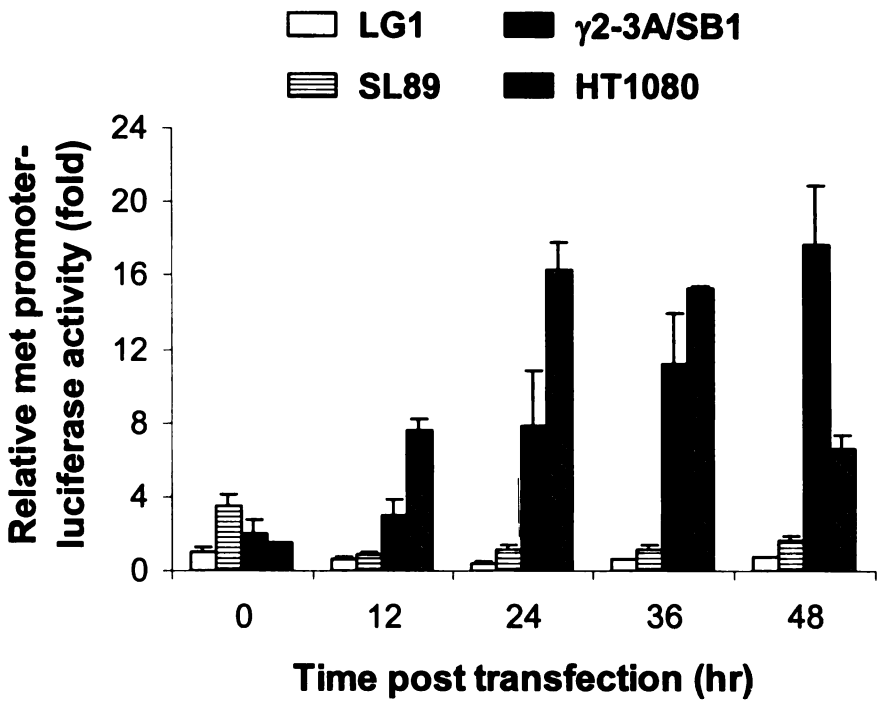

LG1
γ2-3A/SB1
SL89
HT1080
Relative met promoter-luciferase activity (fold)
24
20
16
12
8
4
0
0
12
24
36
48
Time post transfection (hr)

Fig. 4

Fig. 5. Evidence that the transcription factor Sp1 activates human *met* promoter activity. The normal human fibroblast cell line, LG1, was co-transfected with various amounts (0-0.8 μg) of the expression plasmid for Sp1 (pCMV-Sp1 cDNA), 1.5 pmole of the 2.6met-luc construct and 0.1 μg of CMV promoter-*Renilla* luciferase control plasmid. Cell lysates were collected at various time points post transfection. The relative luciferase activities (with the activity of the cells at 0 hr time point in the absence of Sp1 expression plasmid as 1.0) are presented after normalization for the control *Renilla* luciferase activities and the protein concentration of the lysates.

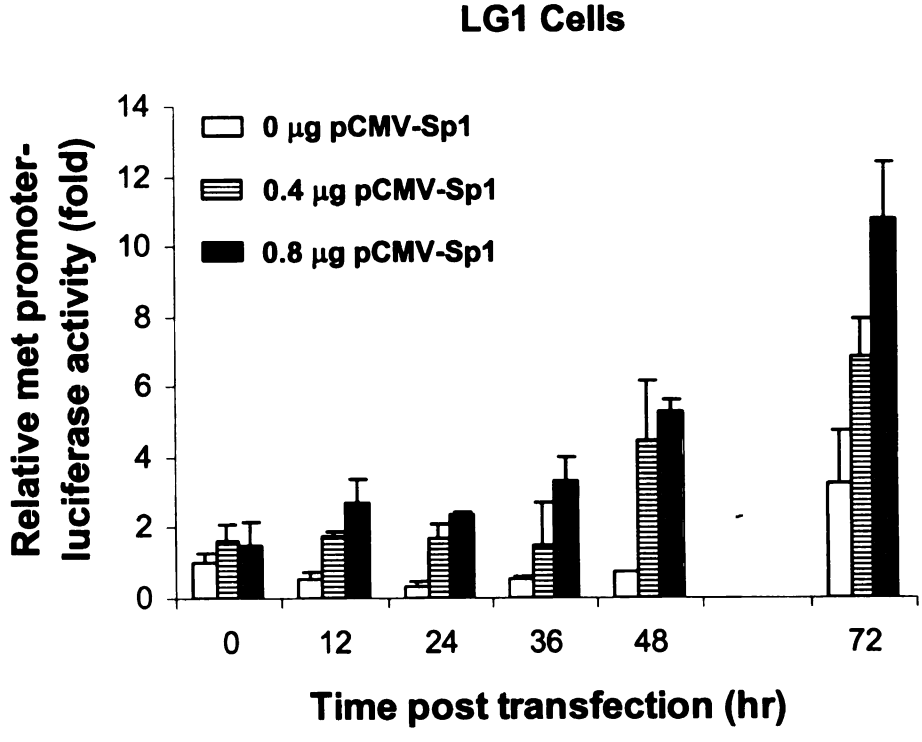

LG1 Cells
Relative met promoter-luciferase activity (fold)
0 µg pCMV-Sp1
0.4 µg pCMV-Sp1
0.8 µg pCMV-Sp1
14
12
10
8
6
4
2
0
0
12
24
36
48
72
Time post transfection (hr)

Fig. 5

Fig. 6. Reduction of MET protein expression in human fibrosarcoma cell line HT1080 by interfering with and inhibiting Sp1 DNA binding using a transcription factor decoy. Double stranded decoy oligonucleotides containing consensus Sp1 binding sequence (5' ATTCGATCGGGGCGGGGCGAGC 3') or mismatched control oligonucleotides (5' ATTCGATCGGTTAAGTGCGAGC 3') were delivered into HT1080 cells using LipofectAMINE. Cell lysates were collected at various times after the treatments. 50 μg protein of cell lysates was analyzed for MET protein expression by Western blot analysis. Actin was used as an unrelated control protein whose expression was not affected by either the decoy or the mismatched control treatment. TFD, transcription factor decoy; MC, mismatched control.

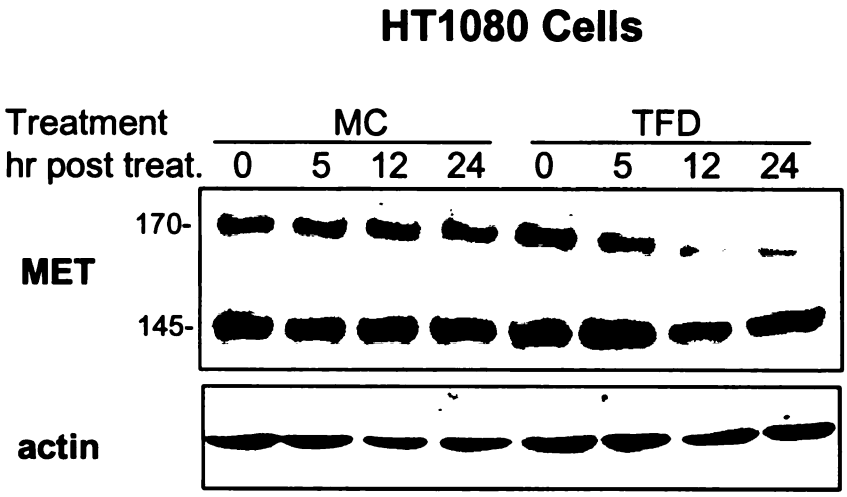

HT1080 Cells
Treatment
MC
TFD
hr post treat.
0
5
12
24
0
5
12
24
170-
MET
145-
actin

Fig. 6

DISCUSSION

In this study, we have demonstrated that MET over-expression is a common feature of human fibrosarcomas, and a majority of the human fiborsarcoma cell lines with high levels of MET shows coordinately high levels of Sp1. Functional analysis of the *met* promoter revealed that the tandem Sp1 sites in the proximal promoter region are important for the promoter activity in human fibroblasts. Two human fibrosarcoma cell lines (γ2-3A/SB1 and HT1080) with high Sp1 levels exhibited high *met* promoter activities as compared to that of two normal human fibroblast cell lines (LG1 and SL89) with low Sp1 levels. Furthermore, transfection of the LG1 cell line with an expression vector carrying Sp1 cDNA led to a dose-dependent increase of the *met* promoter activity, whereas inhibition of Sp1 binding to DNA by a decoy led to a dramatic reduction of MET level in the HT1080 cell line. Therefore, transcriptional up-regulation by Sp1 is a major mechanism for MET over-expression in human fibrosarcomas.

The *met* gene expression is inducible by various extracellular stimuli, such as EGF, HGF, IL-1, IL-6, TPA, or TNF-α (39-41). Previous work (21, 40) has characterized the human *met* promoter as a TATA-less promoter that lacks TATAA and CCAAT boxes, a feature many housekeeping and constitutively expressed genes have in common. The proximal *met* promoter region is highly GC-rich and contains multiple binding sites for transcription factor Sp1. In this study, we further characterized the important roles of those GC boxes for the *met* gene expression. The tandem GC boxes 2 and 3 were found to be important positive elements for the functional activity of the *met* basal promoter since disruption of these two sites by mutations significantly reduced the *met* promoter-luciferase activity. These two GC boxes are separated by only 6 base pairs. Clustering of GC boxes in close proximity to the transcription start point is common in

TATA-less promoters (42-44). Binding of Sp1 to tandem GC boxes may have a synergistic effect on transactivation of promoters (45).

The study presented here does not rule out that other transcription factors may participate and/or cooperate with Sp1 for the regulation of *met* gene expression. In addition to multiple Sp1 sites, there is also an AP2 site (21) and multiple Ets sites (30) in the -223 to -68 proximal *met* promoter region. A study by Gambarotta et al. (30) showed Ets could transactivate the *met* expression in a human gastric carcinoma cell line MKN1. However, when we tested the Ets protein expression in our human fibroblast cell lines, the Ets levels were the same for the tumor cells and the normal cells (unpublished studies). Thus, it seems unlikely that Ets is directly responsible for the over-expression of MET we observed for human fibrosarcomas. In addition, a study by Liu (21) demonstrated that there is also a positive regulatory element in the distal *met* promoter region (-2615 ~ -1621), which contains a cAMP response element (CRE) and an IL-6 response element (IL6RE). Those sites could also participate in the regulation of *met* promoter activity.

Our study demonstrates that the transcription factor Sp1 was over-expressed in a majority of the human fibrosarcoma cell lines tested, and that such over-expression of Sp1 was responsible for the over-expression of MET in those cell lines. As a ubiquitously expressed regulatory transcription factor, Sp1 is involved in the expression of many different genes, including housekeeping genes and genes involved in cell growth and differentiation as documented by more than 2600 citations (23). However, few studies have examined whether Sp1 plays a role in tumor growth and malignant progression. The first study addressing this issue was reported in 1992 by Kitadai et al (25). They found simultaneous over-expression of *Sp1, TGF-α, TGF-β,* c-*Erbb2* and *EGFR* in human gastric carcinomas and hypothesized that over-expression of Sp1 causes rapid tumor growth through simultaneous over-expression of these growth

factor/receptor genes. The next study related to this issue was published in 2000 by Zannetti et al (26), who showed that there is coordinate up-regulation of Sp1 DNA-binding activity and uPAR in human breast carcinomas. More recently, Shi et al (27) found correlated high levels of VEGF and Sp1 in human pancreatic adenocarcinoma cell lines and showed that constitutive Sp1 activity is essential for differential constitutive VEGF expression in these cell lines. Furthermore, an Sp1 decoy transfected into a lung adenocarcinoma cell line, A549, and a glioblastoma multiforma cell line, U251, was shown to suppress the expression of VEGF, TGF-β1 and TF, as well as the cell growth and invasion activities in culture (46). Our results, taken together with these studies, demonstrate that over-expression of transcription factor Sp1 is found in various types of human tumors. What is more, it appears that when over-expressed, Sp1 can function as an oncoprotein by simultaneously up-regulating a variety of genes with consensus Sp1 binding sites, such as *TGF-α*, *TGF-β*, c-*erbb2*, *EGFR*, *VEGF*, *PDGF-B*, *met*, *uPAR*, *MMP2* and hTERT (21, 47-55). In turn, the up-regulation of these target genes can lead to specific tumorigenic properties, such as uncontrolled cell growth, immortalization, invasion and angiogenesis, although the specific genes being up-regulated might depend on the cell type and the cell context. In the present study, we demonstrated the link between up-regulation of Sp1 and up-regulation of MET in human fibrosarcomas. We are now testing several of the other genes listed above to see whether they are also up-regulated by Sp1 in human fibrosarcomas. Studies of the effects of over-expression of Sp1 cDNA in the infinite life span human fibroblast cell strain MSU-1.1 and/or of down-regulation of the Sp1 level in human fibrosarcoma cell lines should provide further evidence as to whether Sp1 can act as a oncoprotein when over-expressed.

Because we found that high levels of transcription factor Sp1 is a major mechanism for MET over-expression in human fibrosarcomas, an important question is

the mechanism(s) responsible for Sp1 over-expression in human tumors. We are now testing whether Sp1 is up-regulated by gene amplification or by control of transcription. The 5' flanking region of human Sp1 gene was recently shown to lack TATAA and CCAAT boxes (56). The proximal promoter region is GC rich and has multiple binding sites for Sp1. Thus, over-expression of Sp1 protein, such as we found in human fibrosarcomas, would form a positive feedback loop for its own gene expression. Also, Sp1 protein can be phosphorylated and/or glycosylated, changes that could affect its DNA binding capacity (57-62). Furthermore, the functional Sp-mediated transcription might well depend on the ratio of Sp1 and Sp3, a closely related Sp family protein, which is also ubiquitously expressed and has the same DNA binding sequence as Sp1. Sp3 has mostly been reported as a repressor, though it can function as an activator for transcription as well (63-65).

In summary, our data demonstrates that high expression of MET is a common feature of human fibrosarcomas, and up-regulation by high levels of transcription factor Sp1 is a major mechanism for such over-expression of MET. These results suggest that when over-expressed, Sp1 can function as an oncoprotein, by simultaneously up-regulating a variety of genes with consensus Sp1 binding sites, and that the up-regulation of specific target genes can result in specific transformed properties. More understanding of the molecular basis of Sp1 regulation may provide insights into ways to suppress the tumorigenicity and invasion of malignant human cells, and ultimately lead to the design of new therapeutics.

ACKNOWLEDGEMENTS

We want to acknowledge that this study originates from an observation by our colleague Xi-De Wang that showed MET was over-expressed in some fibrosarcoma cell lines derived from patients' tumors as well as in some malignant cell lines in the MSU-1 lineage. We thank Dr. Robert Tjian at University of California, Berkeley, CA for providing us with the Sp1 expression plasmid (pCMV-Sp1 cDNA) and Drs. Timothy Zacharewski and Jason Matthews at Michigan State University, East Lansing, MI for helping us with the dual luciferase assays.

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