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Role of MET in SV40-induced mammary tumorigenesis

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Hunsur Narayan Sathish Babu

aus Krishnarajanagar, India.

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Professor Dr. Axel Temming
Leiter des Departments Biologie

HPI
Heinrich-Pette-Institut für Experimentelle Virologie
und Immunologie an der Universität Hamburg

Dr. Carol Stocking
AG Mol Path
HPI · Martinistraße 52 · 20251 Hamburg

Tel.: (+49)40-480-51-273
Fax.: (+49)40-480-51-187
e-mail: stocking@hpi.uni-hamburg.de

7. April 2010

Fakultät für Mathematik, Informatik und Naturwissenschaften

Universität Hamburg

Martin Luther King-Platz 2

D-20146 Hamburg

Sehr geehrte Damen und Herrn,

hiermit bestätige ich, dass die von Herr Sathish Babu mit dem Titel "Role of MET in SV40-induced mammary tumorigenesis" vorgelegte Doktorarbeit in korrektem Englisch geschrieben ist.

Mit freundlichen Grüssen,

Carol Stocking

Dr. Carol Stocking

Leiterin der FG Molekulare Pathologie
Heinrich-Pette-Institut
(Amerikanerin)

For those who are my two eyes;

my mother and sister

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ABSTRACT

Role of MET in SV-40 induced mammary tumorigenesis

Our laboratory generated and characterized a transgenic mouse model (WAP-T mice) suitable for molecular analysis of initiation and progression of mammary adenocarcinoma. Expression of SV40 early proteins in WAP-T mice is initiated during late pregnancy, and tumors develop from T-antigen expressing mammary epithelial progenitor cells that survived involution. Molecular analyses of the tumours by aCGH and gene expression profiling revealed that the *MET* gene is amplified and strongly expressed in nearly all undifferentiated tumors. Therefore, we focused on the role of MET during SV40-induced tumorigenesis.

Double staining for MET and SV40 T-antigen (T-Ag) in an established mammary epithelial tumour cell line called G2 from our mice showed that MET expression levels were directly proportional to T-Ag expression levels, indicating a role for T-Ag in regulating MET expression. Ectopic expression of T-Ag in mouse embryo fibroblasts with wild-type p53 (wtp53) or p53-null background revealed a significant T-Ag dependent increase in MET expression in cells with wtp53 compared to p53-null background. The data suggested that MET expression is repressed by wtp53, and that this repression is alleviated by p53 inactivation through complex formation with T-Ag. To confirm the role of wtp53 in MET expression, we investigated MET expression in mouse cG9 cells which express a temperature-sensitive p53 that is wild-type at 32°C and mutant at 39°C, respectively. We observed increased expression of MET when p53 was in a mutant conformation. The data further supported the postulated p53-dependent repression of the *MET* gene and the action of T-Ag that leads to transcriptional inactivation of p53 by complex formation. By ChIP assay we detected binding of p53 to the *MET* promoter, which was abolished when T-Ag was co-expressed. Silencing of MET using shRNA in T-Ag transformed cells abrogated these cells' ability to form colonies in soft agar, highlighting the role of MET in proliferation and substrate-independent growth. We conclude that p53 acts as a repressor of the *MET* gene. Alleviation of this repressor function by T-Ag is essential for T-Ag mediated cellular transformation.

ZUSAMMENFASSUNG

Die Funktion von MET in der SV40 induzierten Brustkrebsentstehung

In unserer Arbeitsgruppe wurde ein transgenes Mausmodell (WAP-T Maus) entwickelt und charakterisiert, mit dem die Entstehung eines Brust Adenokarzinoms auf molekularer Ebene analysiert werden kann. Die Expression des Transgens, der frühen SV40 Genregion, wird am Ende der Schwangerschaft durch laktotrophe Hormone induziert. Die Tumore entwickeln sich aus T-Antigen exprimierenden Epithelzellen der Brustdrüse, die die Involution überlebt haben. Molekularbiologische Untersuchungen dieser Tumore durch aCGH und Genexpressionsanalyse deckten eine Amplifikation und verstärkte Expression des MET Gens in nahezu allen undifferenzierten Tumoren auf. Aufgrund dessen wurde die Rolle von MET in der SV40 induzierten Tumorgenese genauer analysiert.

Die Immunfluoreszenz-Doppelfärbung von MET und SV40 T-Antigen (T-Ag) in einer aus unserem Mausmodell etablierten epithelialen Mammakarzinom Zelllinie (G2 Zelllinie) zeigte, dass sich die MET Expression direkt proportional zur Expression von T-Ag verhält. Dies weist auf eine Funktion von T-Ag in der Regulation der MET Expression hin. Die ektope Expression von T-Ag in Mausembryofibroblasten mit Wildtyp p53 (wtp53) oder p53-null Genotyp zeigte einen signifikanten, T-Ag abhängigen Anstieg der MET Expression in Zellen mit wtp53 im Vergleich zu p53-null Zellen. Daraus lässt sich schließen, dass die MET-Expression durch wtp53 supprimiert wird und diese Unterdrückung durch die Bildung von p53-T-Ag Komplexen revertiert wird. Um die negative Regulation der MET-Expression durch wtp53 zu bestätigen, wurde die MET-Expression in Maus cG9 Zellen untersucht. Diese Zelllinie exprimiert eine Temperatur sensitive Mutante von p53, welche bei 32°C Wildtyp-Konformation und bei 39°C Mutanten-Konformation besitzt. In Gegenwart von p53 in der Mutanten-Konformation wurde eine verstärkte Expression von MET beobachtet. Dieser Versuch bestätigte zudem die postulierte wtp53 abhängige Unterdrückung des MET Gens und die Rolle von T-Ag in der Inaktivierung von p53 durch Komplexbildung bestätigt werden. Mittels ChIP Analysen wurde eine Bindung von p53 an den MET Promotor nachgewiesen, die durch die Ko-Expression von T-Ag aufgehoben werden konnte. Die Reduktion der MET Expression durch spezifische shRNA in T-Ag transformierten Zellen hob deren Fähigkeit zur Koloniebildung in Softagar auf, was die Bedeutung von MET für Proliferation und Substrat unabhängiges Wachstum hervorhebt. Aus den vorliegenden Daten ergibt sich, dass p53 als Repressor des MET Gens fungiert. Die Aufhebung dieser Repressorfunktion durch Interaktion mit T-Ag ist entscheidend für die T-Ag vermittelte zelluläre Transformation.

I. INTRODUCTION

I.1 Cancer

Cancer is a complex disease, resulting from a progressive accumulation of genetic aberrations and epigenetic changes that enable escape from normal cellular and environmental controls (Weinberg, 1995).

1.1 Cancer Statistics

Lung, breast, colorectal and stomach cancers are the most prevalent types of human cancers in the world. While men are mostly affected by lung and stomach cancer, breast and cervical cancers are most common among women worldwide, with recent epidemiological studies pointing out to the increasing cases of lung cancer in females (WHO, 2006 http://www.who.int/cancer/en/). More than 11 million people are diagnosed with cancer every year. Cancer causes 7 million deaths every year (12.5%) worldwide. In 2005, 7.6 million people died of cancer out of 58 million deaths worldwide. It is estimated that there will be 16 million new cases every year by 2020. Based on projections, cancer deaths will continue to rise with an estimated 9 million people dying from cancer in 2015, and 11.4 million dying in 2030 (WHO, 2006).

The cancer statistics necessitates the inevitability to focus scientific research towards cancer, aimed at better understanding the causes at the molecular level and eventual preparation and use of novel therapeutics. Although the causes of cancer are at the genetic level, understanding the cancer cell physiology is also essential to unknot the complexity of cancer. Over the past 25 years, cancer research has developed a rich body of information about cancer cell physiology. Eventually, it has been transferred into knowledge, and several classifications have been made for cancer staging. The classification of different kinds of cancer based on the cell types is explained in the following section.

1.2 Types of Cancer

Depending on their degree of aggressive growth tumors are divided into two broad categories. Tumors that grow locally without invading adjacent tissues are classified benign, those that invade nearby tissues and form metastases are termed malignant. The

majority of primary tumors arising in humans are benign and harmless to their hosts, except in the rare cases where the expansion of these localized masses causes them to press on vital organs or tissues, or by releasing dangerously high levels of hormones that create physiologic imbalances in the body. For example, thyroid adenomas may cause hyperthyroidism when they release excessive thyroid hormone into the circulation; pituitary adenomas may release growth hormone into the circulation, which is a causative agent of an excessive growth of certain tissues, a condition known as acromegaly. Nevertheless, death by benign tumors is relatively uncommon. The vast majority of cancer-related mortality derives from malignant tumors. More specifically, it are the recurrences and metastases caused by these tumors that are responsible for some 90% of deaths from cancer (Sporn, 1996).

Cancers arising from epithelial cells are termed carcinomas. These carcinomas can be subdivided into two, namely squamous cell carcinomas and adenocarcinomas. Tumors that arise from epithelial cells lining the skin and the esophagus are squamous cell carcinomas. Tumors arising from epithelia that secrete substances into the ducts or cavities that they line are called adenocarcinomas.

Cancer derived from nonepithelial cells can be broadly classified into three different groups. In the first group, tumors arising from nonepithelial cells, for example from fibroblasts, adipocytes, osteoblasts, and myocytes, are called sarcomas (A relatively unusual tumor, an angiosarcoma, arises from precursors of the endothelial cells).

The second group of nonepithelial cancers arises from the various cell types that constitute the hematopoietic system. Among these are cells destined to form erythrocytes, plasma cells, as well as T and B lymphocytes. The term leukaemia (literally “white blood”) refers to malignant derivatives of several of these hematopoietic cell lineages that move freely through the circulation. Lymphomas include tumors of the lymphoid lineages

(B and T lymphocytes) that aggregate to form solid tumor masses, most frequently found in lymph nodes, rather than the dispersed, single-cell populations of tumor cells seen in leukemias.

The third and last major grouping of nonepithelial tumors arises from cells that form various components of the central and peripheral nervous system; they include gliomas, glioblastomas, neuroblastomas, schwannomas, and medulloblastomas.

Not all tumors fall neatly into one of these four large groups. For example, melanomas derive from melanocytes, the pigmented cells of the skin and the retina. Small-cell lung

carcinomas (SCLCs) contain cells with characteristics of neurosecretory cells, such as adrenal glands.

1.3 Cancer Cell Physiology

The past three decades of cancer research have led to phenomenal advancement in the field of cancer which has provided us a rich and complex body of knowledge, which reveals that cancer is a disease that involves dynamic changes in the genome, leading to alterations in cell physiology. There are six essential alterations in cell physiology that collectively dictate malignant growth. Almost all types of human tumors share these six capabilities (Figure 1). During tumor development, each of these physiological changes is acquired (Hanahan and Weinberg, 2000). The six acquired capabilities of cancer cells are

(i) Self-Sufficiency in Growth Signals
(ii) Insensitivity to Antigrowth Signals
(iii) Evading Apoptosis
(iv) Limitless Replicative Potential
(v) Sustained Angiogenesis
(vi) Tissue Invasion and Metastasis

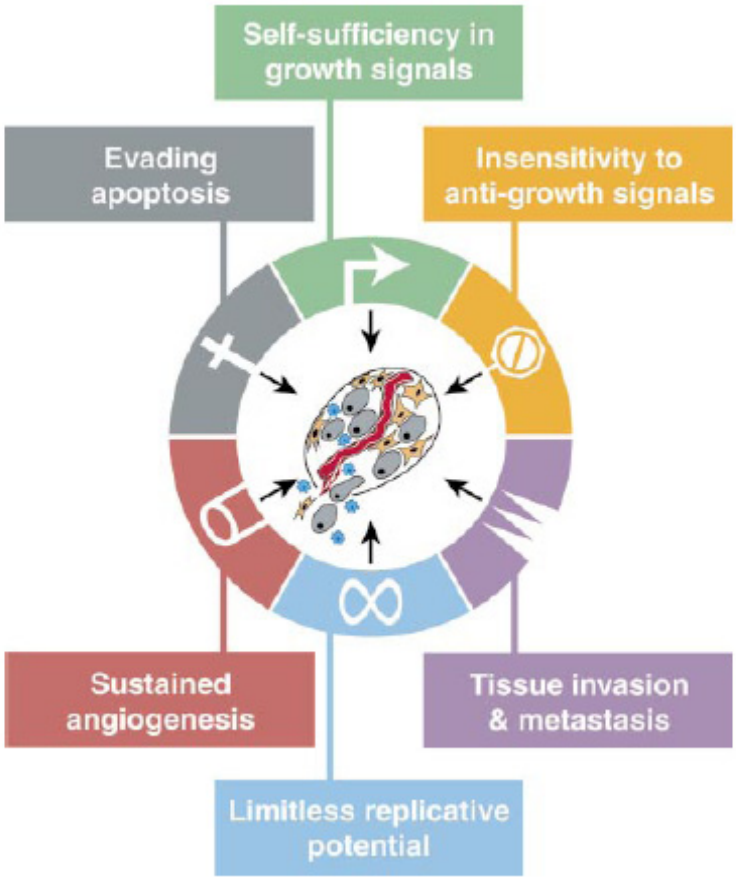

Figure 1: Acquired capabilities of cancer (Hanahan and Weinberg, 2000).

1.4 Oncogenes

Oncogenes are genes whose products (protein or RNA) stimulate or enhance the division and viability of cells, leading to eventual immortalization or transformation of cells. In the oncogenic state, oncogenes are activated and found in retroviruses and transformed cells. Oncogenes in non-transformed cells in their normal state are termed proto-oncogenes.

In other words, proto-oncogene is a gene whose protein product has the capacity to induce cellular transformation, given it sustains some genetic insult leading to constitutive activation.

In nature there are several processes which can transform proto-oncogenes to oncogenes. These are mainly retroviral transduction or retroviral integration, point mutations, insertion mutations, gene amplification, chromosomal translocation, and/or protein-protein interactions. More than 100 oncogenes have been identified at all levels of cell growth and death pathways, and are involved in the multistep process of tumorigenesis (Gronbaek and Guldberg, 2006).

1.5 Tumor Suppressor Genes

Tumor suppressor genes prevent cells from transformation. They either have a repressive effect on the regulation of the cell cycle, or promote apoptosis, and sometimes do both. In cancer, some of the tumor suppressor genes are inactivated either by mutation, deletion, or some other mechanisms. Tumor suppressor genes regulate diverse cellular activities, including cell cycle checkpoint responses, detection and repair of DNA damage, protein ubiquitination and degradation, mitogenic signaling, cell specification, differentiation and migration, and tumor angiogenesis. The functions of tumor suppressor genes can be divided into four major categories (Sherr, 2004) such as 1) Repression of genes that is essential for continuing the cell cycle thereby effectively inhibiting the cell division. 2) Coupling the cell cycle to DNA damage. If the damage can be repaired, the cell cycle can continue. 3) Inducing programmed cell death. For example, if the DNA damage can not be repaired, the cell usually initiates apoptosis, to remove the threat that it poses for the entire organism. 4) Some proteins involved in cell adhesion prevent tumor cells from dispersing, and thereby inhibit metastasis (Hirohashi and Kanai, 2003). More then 30 tumor suppressor genes have been understood sufficiently, including p53, BRCA1, BRCA2, APC, and Rb1.

1.6 Mechanisms of Inactivation of Tumor Suppressor Genes

Tumor suppressor genes are widely mutated in many cancers. Abnormalities of the p53 gene, in particular acquired mutations, have been found in more than 50% of the human cancers, including lung, colorectal and breast cancer, as well as many other cancers. Acquired changes in many other tumor suppressor genes also contribute to the development of non-inherited cancers. Although there are many mechanisms operating in the cancer-prone cells to eliminate tumor suppressor genes, the cells with inherited defects in one copy of a tumor suppressor gene lose their remaining good copy by six possible ways (Alberts et al., 2002). The normal copy of the gene can be eliminated by

point mutation, deletion, gene conversion, mitotic recombination, non-disjunction and duplication, and chromosome loss.

2. Breast Cancer

Breast cancer develops as a multi-step process and can be conceptualized to develop from normal epithelial cell or its precursor, progressing through non-proliferative disease (including apocrine change and duct ectasia), to epithelial hyperplasia and atypical hyperplasia, progressing further to ductal carcinoma in situ and ultimately invasive cancer (Kopans 1988). About 85% of breast carcinomas originate within the cells of the ducts, the remaining 15% begin in the cells that line the lobules.
Breast cancer is mainly categorised into three major types. They are

1. Noninvasive breast cancer
2. Invasive breast cancer
3. Metaplastic carcinoma

Noninvasive breast cancer as the term suggests is non-invasive where uncontrolled growth of cells arises and restricted only to breast duct. Ductal carcinoma in situ (DCIS) and Lobular carcinoma in situ (LCIS) are the two kinds of non-invasive breast cancer types where the former starts in ducts and the latter in lobules of the breast. DICS is the most common type of non-invasive breast cancer.

Invasive Breast Cancer is the most common type of all the breast cancers. Invasive (infiltrating) ductal carcinoma (IDC) is the most common type of invasive breast cancer. About 80% of invasive breast cancers are classified as invasive ductal carcinoma where cancer cells have penetrated the ductal wall and invaded surrounding breast tissue. The cells may then metastasize to other parts of the body through the bloodstream or lymphatic system.

Metaplastic carcinoma is uncommon, representing less than 5% of all breast cancers. Lesions contain several different types of cells that are not typically seen in other forms of breast cancer; frequently single palpable lesions often are associated with rapid growth and appear benign.

3. Mouse models

Animal models are commonly used as human surrogates in cancer research. Even though the use of animals in laboratory has been highly debated, the benefits of animal models, in particular mouse models, have revolutionised the scientific research to a great extent. The first and foremost important reason for the choice of mice is ethics; there are many situations where it is simply unethical to do direct human studies on cancer. Mice are small with a short life cycle compared to humans, which allows for rapid, economical results. Genetically modified mice are generated which are more prone to acquire certain types cancers, which are known as models. These models facilitate the study of specific cancers.

Tumor biology is a very complicated and very dynamic physiological system too. Mouse models of cancer give us an opportunity to understand this complex system. Efforts to model cancer in the mouse have produced fundamental insights into various aspects of cancer, including the action of oncogenes and tumor suppressor genes, the biology of tumor-host cell interactions, the factors that influence cellular responsiveness to chemotherapeutic agents, and the role of stem cells during cancer development and progression. Genetically engineered mice have become even more important in cancer gene identification and validation due to the immense information obtained from the of cancer genome atlas project. To summarise, these models have become increasingly important for identifying and validating new drug targets, and as advanced preclinical test systems for new drugs or drug combinations to combat cancer.

Mouse models for human cancers were generated generally by injecting human cancer cells under the skin and in the body cavity of the mouse. This resulted in tumor growth in the animal, and studies were undertaken to study the cancer process. Mice that developed a cancer condition were also used to screen compounds for cancer treatments. Utilizing standard breeding techniques, specialized inbred strains of mice have been developed that are particularly sensitive to certain cancer conditions.

The use of mouse models in cancer research has been greatly enhanced with the advent of genetic engineering. Genetic engineering is the technique of removing, modifying, or adding genes to the DNA of a species in order to change the characteristics of the species. Two primary techniques are involved: the random insertion of a gene called “transgenic mouse”, and specific gene targeting to remove or replace a gene called “knock-out” or “knock-in” mouse respectively. Of great current interest is also the

replacement of certain mouse genes with human genes. The resultant "humanized" animals are said to better models for cancer research. Our lab has generated and characterized transgenic mouse models suitable for molecular analysis of initiation and progression of mammary tumorigenesis. These are

1. SV40 - WAP-T mouse line (T1)
2. mutp53 -WAP mouse line (H22)

These two mouse lines were interbred to obtain the bitransgenic mouse line named as

3. WAP-T1-H22

3.1 SV40 - WAP-T mouse line (T1)

The transgenic BALB/c mouse based WAP-T mouse model was generated in our lab in 2000 by Schulze-Garg et al. WAP-T mice carry the SV40 early gene region under the control of the murine *whey acidic protein* (WAP)-promoter. The WAP promoter is hormonally and developmentally regulated by lactotrophic hormones like estrogen, prolactin, hydrocortisone, insulin (Burdon et al., 1991; Pittius et al., 1988). The expression of the transgenic SV40 early proteins T-Ag, small t and 17kT can be induced by mating and is directed to epithelial cells of Terminal Ductal Lobular Units (TDLUs) like mammary epithelial cells (MECs) of the differentiating and lactating gland. Hence, the WAP promoter has been successfully used for the generation of mouse models for breast cancer (Husler et al., 1998; Tzeng et al., 1993).

The WAP promoter is activated by lactotrophic hormones in differentiating mammary epithelial cells during late pregnancy and lactation (Andres et al., 1987). The transgene expression in the mammary epithelial cells in this line was up to 90%. As a consequence of transgene expression, WAP-T mice developed multifocal intraepithelial neoplasia like Ductal Carcinoma in situ (DCIS) of the mammary gland, which can further progress to become invasive carcinomas. In this model the occurrence of metastasis was rarely observed. The key feature of this model was that the tumors formed in this mouse model have a close similarity in histology to human tumors (Schulze-Garg et al., 2000). In this model loss of p53 function is due to SV40 T-Ag complexing the endogenous wtp53. This mouse line serves as model for DCIS-like carcinoma and invasive mammary adenocarcinoma (Schulze-Garg et al., 2000).

3.2 The mutp53 -WAP mouse line (H22)

The transgenic mutp53 -WAP mouse line (H22) was generated in our lab by Krepulat et al., in 2005. These transgenic mice carry a murine mutant p53 transgene harbouring a point mutation equivalent to the human tumor-derived hot spot mutation R273H (murine R270H) under the control of WAP promoter. Upon induction, this mouse line did not express mutp53. Experiments proved that epigenetic silencing was responsible for the suppression of mutp53 expression in this mouse line. While generating the mutp53 -WAP mouse line various mouse lines were also obtained, in which most of the induced MECs expressed the mutp53 transgene and most of these transgenic lines did not express mutp53 or expressed the transgene in less than 2% of the induced MECs. The expressing lines showed mouse line-specific mosaic expression patterns for the mutp53 transgenes. Even though transgene integration could be attributed to this phenomena, but genetic analysis of mice by Southern blotting did not reveal any correlation with numbers of integration sites and copy numbers of the transgene. This suggested that specific epigenetic factors might be responsible for these events. *In vitro* experiments showed a significant increase in mutp53 expression by testing the effect of T-antigen on the expression of stably integrated silent mutp53 transgenes in BALB/c 3T3 cells. To confirm this when T-antigen highly expressing line WAP-T1 (90% T-antigen-positive MECs upon induction) was crossed with line H22 (not expressing mutp53) led to re-expression of the silenced mutp53 transgene in about 1–2% of the induced cells in bitransgenic animals indicating that chromatin remodelling events occur via chromatin remodelling activity of the SV40 T-antigen.

3.3 WAP-T1-H22

This mouse line was generated by interbreeding mouse lines WAP-T(T1) x WAP-mutp53(H22). This bi-transgenic mouse model had an increased tumor incidence compared to the WAP-T1 mouse line. This was attributed to the "gain of function" activity of mutp53 (Heinlein et al., 2008). The bi-transgenic tumors showed worse clinical staging, histological grading, and enhanced metastasis. The tumors developed from this mouse model were subjected to various analyses like clinical staging, histological grading system, gene expression profiling, and comparative genomic hybridisation by Heinlein et al., from our lab. Molecular analyses of the tumours by Comparative genomic hybridisation (CGH) and gene expression profiling revealed that the *MET* gene was amplified and strongly expressed in nearly all undifferentiated tumours. From the above analyses WAP-T1-H22 was found to be a good mouse model for analysing mammary carcinogenesis.

4. The Simian Virus 40 (SV40) and cancer

SV40 is the most well characterized member of the *Polyomaviridae* family of small DNA tumor viruses. The SV40 genome consists of 5,243 base pairs of double-stranded DNA and encodes seven viral proteins in overlapping reading frames (Fanning and Knippers, 1992; Reddy et al., 1978). The viral genome encodes 3 structural proteins of the virus named as VP1, VP2, and VP3, and two proteins important for the viral life cycle i e., large T antigen (LT) and small t antigen (st) and also two small proteins of unknown function as agnoprotein and 17kT respectively (Sullivan and Pipas, 2002; Thimmappaya et al., 1978). In addition, the SV40 genome contains two strong transcriptional promoters, an origin of replication, several splicing sites, and a polyadenylation signal (Figure 2).

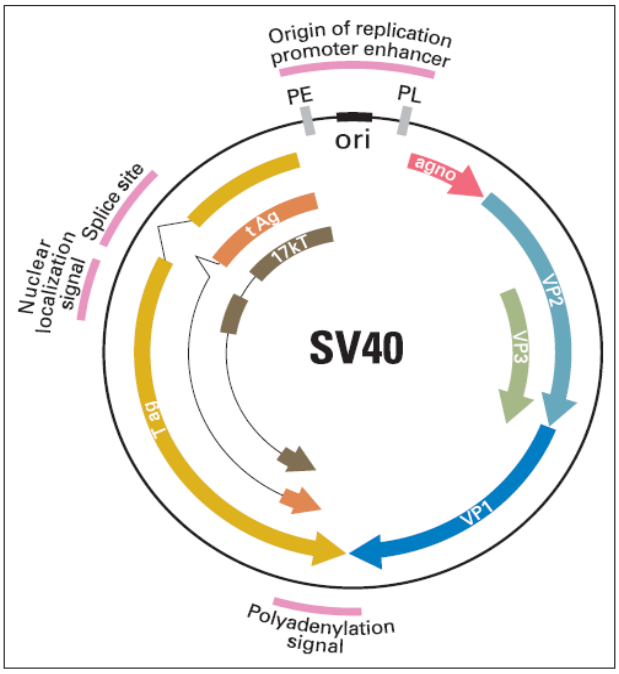

Figure 2: Simian Virus 40 (SV40) genome organization ; Regulatory elements of the SV40 genome used in the construction of laboratory plasmids are highlighted in red, including the small t antigen (t-Ag) intron splice site, the large T antigen (T-Ag) polyadenylation signal, promoter, enhancer, and origin of replication. The early (PE) and late (PL) promoters, origin of replication (ori), major SV40 viral proteins are also represented. agno, agnoprotein. (Poulin and DeCaprio, 2006).

SV40 virus naturally infects certain species of Asian macaques, in particular the rhesus monkey. Shortly after SV40 was discovered, several groups reported that the virus could form tumors in rodents as well as induce transformation of primary cultures of human cells (Eddy et al., 1962; Rabson and Kirschstein, 1962; Rabson et al., 1962; Shein and Enders, 1962). Many of these viral DNA elements have proven to be invaluable tools for molecular biology research and have been inserted into nearly every mammalian expression plasmid vector used to this day (Mulligan and Berg, 1980; Pipas, 1992). The very first opportunity to explore the molecular basis for cancer was led by the discovery that SV40 can induce tumors in certain laboratory animals and transform many types of cultured cells.

To study the molecular biology of breast cancer formation in greater details, various transgenic lines are established, which overexpress either key regulators of the cell cycle or tumor genes that disturb the balance between cell differentiation and/or the cell death program such as the SV40 LT and st-antigens (Schulze-Garg et al., 2000; Tzeng

et al., 1993). SV40 induces transformation by binding key cellular proteins like p53 and pRB and altering their activities. The mechanism known for functional inactivation of p53 and pRB by SV40 early proteins is by binding to the T-Ag by complex formation. (DeCaprio et al., 1988; Dyson et al., 1989b; Helt and Galloway, 2003; Levine, 1990). Addition to this, st interacts with the catalytic subunit of phosphatase PP2A which changes the specificity of PP2A by blocking its association with the PP2A regulatory subunit B56 (Moreno et al., 2004) which is required for the regulation of several cancer-associated pathways (Arroyo and Hahn, 2005).

Thereby, expression of SV40 early proteins mimics early genetic alterations common in human carcinogenesis. Expression of SV40 early proteins is absolutely required to initiate tumor development, but it is not sufficient and requires additional genetic alterations, which arise from the induction of genomic instability by T -antigen (Li et al., 2000; Ramel et al., 1995). Although there is no indication that the SV40 virus is involved in human breast cancer formation, the LT/st-antigens have been used as useful tools to study mammary gland differentiation and tumorigenesis. Both viral proteins mimic alterations etiologically linked with human breast cancer. The transgenic mouse models generated in our lab also exhibit distinct architectural and cytological features which closely resemble those commonly present in humans.

There are numerous mouse models generated using SV40 as an oncogene to study various cancer types. Listing all the models is beyond the scope of this thesis. The important mouse models for mammary tumorigenesis are mentioned below.

Table 1: SV40 Transgenic Mouse Models in Mammary Tumorigenesis

Transgene/Gene knockout	Promoters	Phenotype in mouse	Reference
SV40TAg	WAP; C3(1)	Hyperplasia, adenocarcinoma, soft tissue sarcoma, osteosarcoma, lung metastasis	(Husler et al., 1998; Maroulakou et al., 1994; Tzeng et al., 1993)
SV40Tag x BCL-2	WAP	Inhibition of apoptosis, shortened tumor latency	(Furth et al., 1999)
SV40TAg x bax	C3(1)	Accelerated tumorigenesis, decreased survival rates	(Shibata et al., 1999)
SV40Tag x Ki-Ras	C3(1)	Decreased tumor incidence in SV40Tag; Ki-ras+/- mice	(Liu et al., 2001)
SV40Tag x maspin	WAP	Reduced tumor growth, angiogenesis and metastasis	(Zhang et al., 2000)
SV40Tag	WAP	multifocal intraepithelial neoplasia like ductal carcinoma in situ (DCIS)	(Schulze-Garg et al., 2000)
SV40Tag x mutp53	WAP	Increased tumorigenesis	(Heinlein et al., 2008)

5. Tumor suppressor p53 and cancer

Until today more than 50,000 publications have examined the role and function of p53 since its discovery in 1979. This immense work on a single gene reflects the central role that p53 has in governing normal cell growth and consequently, in determining the outcome of genetic insults that may lead to malignant cell transformation and tumor cell growth. p53 was first discovered as a protein that could bind with the virally encoded LT, responsible for the transformation of cells by simian virus 40 (Lane and Crawford, 1979; Levine et al., 2006; Linzer and Levine, 1979). Antibodies raised against the LT immunoprecipitated a protein of 53kDa (hence the term ‘p53’) and for several years this protein was thought to be the product of an oncogene. It took almost 10 years of time since its discovery to know that p53 also acts as a tumor suppressor. The p53 level in normal cells is very low, but substantially increases in SV40 transformed cell lines. This led to the conclusion that p53 levels correlated with transformation, assigning p53 protein as an oncogene. These observations were concluded by the isolation of

molecular clones of p53, and was found that the overexpression of these cloned p53 genes was capable of inducing cell transformation in gene transfer assays. This further proved that p53 as an oncogene. This conclusion was found to be incorrect by the further studies carried out later which showed that p53 clones initially shown to induce transformation coded for mutant rather than wild type p53. When the normal p53 gene was isolated, it was found to lack transforming activity. The groups of both Arnold J. Levine and Moshe Oren demonstrated that overexpression of normal p53 in fact acted to inhibit rather than induce transformation, the behaviour expected for a tumor suppressor gene, and not an oncogene.

p53 is essential for the prevention of cancer development. In response to oncogenic or other cellular stress, p53 is activated and induces up-or down regulation of a variety of genes involved in cell cycle arrest, DNA repair, senescence, or apoptosis (Levine et al., 2006; Vogelstein et al., 2000; Vousden and Lu, 2002).

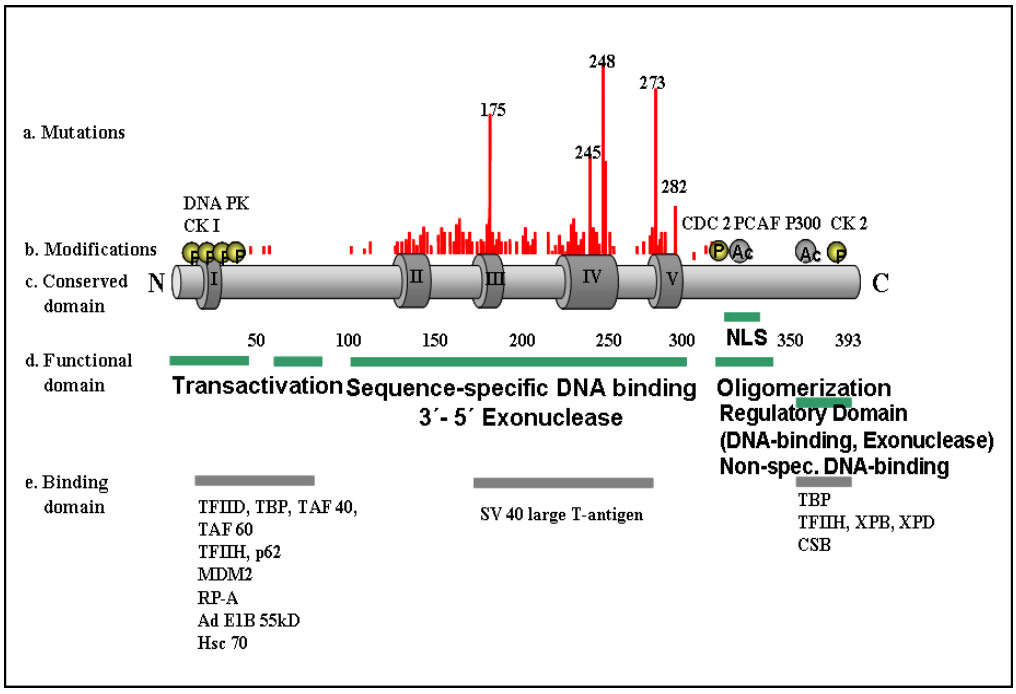

Figure 3: Schematic representation of p53: a) Mutations b) Post translational modifications of N & C terminus, P: Phosphorylation, A: Acetylation, c) Functionally conserved domains for DNA binding, d) Functional domains, e) Binding proteins of p53 for DNA interactions, and p53-protein complex formation (Albrechtsen et al., 1999).

p53 is active as a tetramer, with four identical chains of 393 residues (390 mouse). The N-terminal region consists of an intrinsically disordered transactivation domain (TAD) and a proline-rich region. It is followed by the central, folded DNA-binding core domain that is responsible for sequence-specific DNA binding; this domain is connected via a flexible linker to a short tetramerization domain that regulates the oligomerization state of p53. At its C terminus, p53 contains the regulatory domain (Figure 3). Mutation of the TP53 gene is the most frequent genetic alteration found in human cancer, and one in two tumors carries a p53 mutation. The current release of the TP53 mutation database of the International Agency for Research on Cancer (version R11; http://www-p53.iarc.fr) reports more than 17,000 cases of somatic p53 missense mutations, comprising more than 1200 distinct mutant proteins (Petitjean et al., 2007). The phenotypes associated with common p53 cancer mutations are diverse, and distinct transactivation spectra are observed, ranging from complete loss of function to promoter dependent activity at subphysiological temperature (Dearth et al., 2007; Kato et al., 2003; Menendez et al., 2006; Resnick and Inga, 2003). Above all, there is increasing evidence that the type of mutation correlates with cancer prognosis, as, for example, shown in clinical study on breast cancer (Olivier et al., 2006).

Table 2: p53 Transgenic and Gene Knockout Mouse Models in Mammary Tumorigenesis

Transgene/Gene knockout	Promoters	Phenotype in mouse	Reference
p53(null allele)		Mammary tumor rare in p53-/- mice. Frequent mammary tumors with BALB/C p53-/- mammary gland transplant	(Donehower et al., 1992; Jerry et al., 2000)
p53 (null allele) x WNT-1	MMTV-LTR	Accelerated tumorigenesis in p53-/-;wnt-1 mice	(Donehower et al., 1995)
p53 x Brca1		Increased tumor incidence in Brca1+/-; p53-/- mice	(Cressman et al., 1999)
p53-172H x MMTV	WAP	Accelerated tumorigenesis	(Chatterjee et al., 2002)
BRCA1 x p53	MMTV-Cre; WAP-Cre	Invasive carcinoma in Brcalko/co ; MMTV-CreTrp53+/- mice	(Xu et al., 1999)
BRCA2(conditional D of exon 11) x p53(conditional inactivation)	K14-Cre	Dysplasia, adenocarcinoma, adenosquamous carcinoma	(Jonkers et al., 2001)
SV40Tag x mutp53	WAP	Increased tumorigenesis	(Heinlein et al., 2008)

6. Mesenchymal epithelial transition factor (MET)

MET (mesenchymal-epithelial transition factor) is a proto-oncogene that encodes the protein MET, also known as hepatocyte growth factor receptor (HGFR). MET was originally identified as an activated oncogene product in a fusion protein resulting from chromosomal translocation in a human osteogenic sarcoma (HOS) cell line treated with the chemical carcinogen N-methyl- N'-nitro-N nitrosoguanidine (Cooper et al., 1984).

The MET signaling pathway has been shown to be involved in wide array of cellular responses including proliferation, survival, angiogenesis, wound healing, tissue regeneration, scattering, motility, invasion and branching morphogenesis.

6.1 Structure and functions of MET

The proto-oncogene product MET, expressed in both normal and malignant cells, is a disulfide linked α-β heterodimeric receptor tyrosine kinase (RTK). A partially glycosylated 170-kDa-precursor protein is produced after the primary MET transcript produces a 150-kDa polypeptide. This 170-kDa precursor is further glycosylated and then cleaved into a 50-kDa α-chain and a 150-kDa β-chain, which are linked via disulfide bonds. The mature MET heterodimer consists of a highly glycosylated and entirely extracellular α-subunit as well as a β-subunit with a large extracellular region, a membrane spanning segment, and an intracellular tyrosine kinase domain. The representative functional structures and domains of MET are shown in Figure 4, the Sema domain at the N terminus; PSI domain, found in plexins, semaphorins and integrins; IPT repeats, which are found within immunoglobulin, plexins and transcription factors; the transmembrane and the juxtamembrane domains; and the C-terminus intracellular tyrosine kinase domain. The C-terminal intracellular tyrosine kinase domain contains the phosphorylation sites Tyr1234 and Tyr1235 within the catalytic site, which positively modulates enzyme activity, whereas phosphorylation of Ser975 in the juxtamembrane segment down-regulates kinase activity. The carboxy-terminal tail includes another two critical tyrosines (Tyr1349 and Tyr1356) that once phosphorylated, act as a promiscuous docking site for the recruitment of several transducers and adaptors (Figure 4).

The natural ligand for MET is hepatocyte growth factor/scatter factor (HGF/SF), which is a disulfide-linked heterodimeric molecule produced principally by mesenchymal cells and acting primarily on MET expressing epithelial cells in an endocrine and/or paracrine fashion (Jeffers et al., 1996). Phosphorylation of multiple residues on MET activates its signalling pathway. MET undergoes autophosphorylation of specific tyrosine residues within the intracellular region upon ligand binding. The inherent kinase activity of MET is activated by phosphorylation of Y1230, Y1234 and Y1235 located within the activation loop of the tyrosine kinase domain. MET possess multisubstrate signal transducer docking site (Y1349VHVX3Y1356VNV) which gets activated upon the phosphorylation of Y1349 and Y1356 which are in the C-terminus.The multisubstrate docking site in mouse MET consists of the docking sites that bind Src homology-2 (SH2) domains, phosphotyrosine binding (PTB) domains, and

MET binding domains (MBD) of signal transducers and adapter proteins. Chimeric receptors containing this amino acid sequence can mediate cellular responses that are similar to those of MET, suggesting that this site is responsible for much of MET-mediated signal transduction.

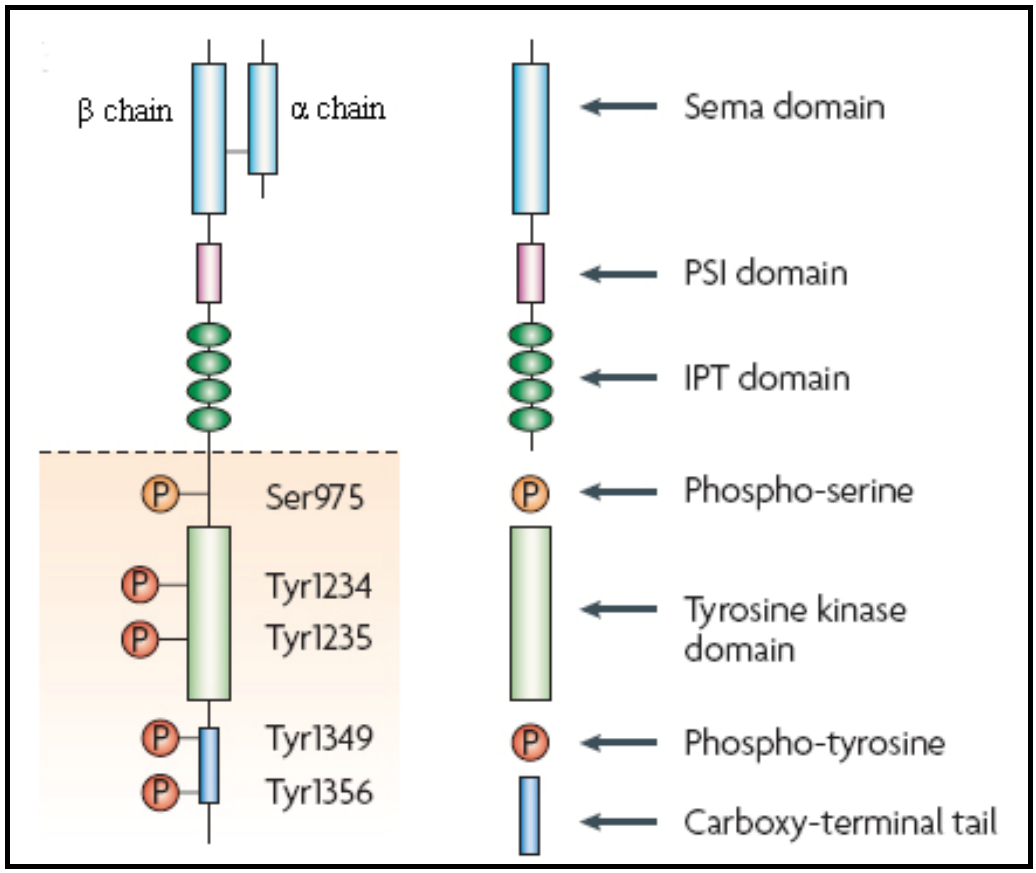

Figure 4: Structure of MET; MET is a disulphide-linked α/β heterodimer. The extracellular portion of MET is composed of three domain types Sema domain, PSI domain, IPT 1-4 repeats. The N-terminal 500 residues fold into a large Sema domain, which encompasses the whole α-subunit and part of the β-subunit. The Sema domain is followed by a PSI domain that spans about 50 residues and contains four disulphide bonds. The PSI domain is connected to the transmembrane helix via four IPT domains. The intracellular portion includes tyrosine kinase catalytic sites flanked by distinctive juxtamembrane and carboxy-terminal sequences containing phosphorylation sites (Comoglio et al., 2008).

Mutational analysis of the multisubstrate docking site suggests that Y1349 and Y1356 mediate the interactions with SHC, Src, and Gab1 while recruitment of Grb2, PI3-K, PLC-γ, and SHP2 is mediated by Y1356 alone (Furge et al., 2000). Regulation of cell morphogenesis is mediated via Y1365. Within the JM domain, the Y1003 residue has an important role in binding to proteins such as c-Cbl. Cbl binds and ubiquinates activated MET. Additionally, by recruiting the endophilin-CIN85 complex, Cbl

regulates MET internalization, thereby having an important functional role in HGF/MET signaling regulation (Petrelli et al., 2002).

MET signaling has been shown to trigger a variety of cellular responses, particularly a unique 'invasive-growth' program, that vary based upon the cellular context. In vivo, the invasive-growth programming from HGF/MET signaling is thought to be an integrated function of a variety of biological responses such as cell proliferation and survival, cell-dissociation/scattering, motility, induction of cell polarity, angiogenesis, wound healing, tissue regeneration, invasion, branching morphogenesis and tumor metastasis (Brinkmann et al., 1995; Chmielowiec et al., 2007; Comoglio and Trusolino, 2002; Kolatsi-Joannou et al., 1997; Maulik et al., 2002; Stella and Comoglio, 1999).

6.2 Role of MET in cancer

There is significant evidence supporting a role for MET in human tumorigenesis (Birchmeier et al., 2003). Amplification of the *MET* gene, with subsequent protein overexpression and constitutive kinase activation, has been reported in a number of human primary tumours, like gastric and oesophageal carcinomas (Hara et al., 1998; Houldsworth et al., 1990; Kuniyasu et al., 1992; Miller et al., 2006), non-small-cell lung (NSCL) carcinomas with acquired resistance to epidermal growth factor receptor (EGFR) inhibitors (Bean et al., 2007) and medulloblastomas (Tong et al., 2004). *MET* gene has been associated with metastatic progression in various tumors (Comoglio, 2001; Jeffers et al., 1996). High levels of MET have been correlated with metastatic spread of tumors and poor survival in patients with breast carcinoma (Camp et al., 1999; Ghoussoub et al., 1998), endometrial carcinoma (Wagatsuma et al., 1998), hepatocellular carcinoma (Ueki et al., 1997), colorectal cancer (Di Renzo et al., 1995) and renal carcinoma (Natali et al., 1996). MET activation has three main consequences in cancer like endothelial cell proliferation, activation of key oncogenic pathways; the Ras pathway, the PIK/STAT3 pathway; the ß-catenin pathway, and increased protease production and hence cell dissociation leading to metastasis.

II Rationale and aims of the study

The transgenic WAP-T mouse model generated in our lab is a good model to study mammary tumorigenesis. The tumors developed by these mice were subjected to various analyses like clinical staging, histological grading system, gene expression arrays, and comparative genomic hybridisation. One of the major findings of these studies revealed that the *MET* gene is overexpressed and amplified in undifferentiated tumors. This led us to study the molecular mechanisms of MET during tumorigenesis in cell culture systems.

Preliminary investigation for the expression and localisation of MET in mammary epithelial cells (MECs) by immunofluorescence technique showed differential staining pattern. Detection of MET using the same antibody through passage numbers zero to five demonstrated a gradually change in its localization from the cytoplasmic membrane to the nucleus. Double staining for MET and T-antigen in an established mammary epithelial tumour cell line from our mice showed that MET expression levels were directly proportional to T-Ag expression levels, indicating a role for T-Ag in regulating MET expression. Therefore, the specific aims and questions of my thesis were:

- To investigate the localization of MET in various cell lines
- Does MET get translocated into the nucleus?
- Does nuclear MET exists?
- Does T-Ag regulate MET expression?
- Is p53 a repressor of MET?
- To analyze the effects of MET silencing in SV40 transformed cells.

III.1 Materials

IV.1.1 List of chemicals, kits used

The chemicals and reagents used in this study were purchased from Sigma, Merck, Biomol, AppliChem, Roche and New England Biolabs. Cell culture materials were obtained from Falcon, Gibco BRL and Pan, other plastic materials and equipment were purchased from Falcon, Sarstedt, Whatman, Nunc, Biorad, Eppendorf GmbH, Brand, Protean, Schleicher & Schuell, Engelbrecht, Biozym, Hellma, VWR, and Greiner.

2-Propanol	Merck, Darmstadt
30 % Acrylamide/Bisacrylamide (37.5:1)	Serva, Heidelberg
40 % Acrylamide/Bisacrylamide (19:1)	Serva, Heidelberg
Agarose VII, low melting	Sigma-Aldrich, Steinheim
Ampicillin	USB, Cleveland, USA
Aprotinin (Trasylol®)	Bayer, Leverkusen
APS	Sigma-Aldrich, Steinheim
Bacto-Agar	Difco, Detroit, USA
Bacto-Yeast extract	Difco, Detroit, USA
Bacto-Trypton	Difco, Detroit, USA
Bradford reagent	BioRad, München
Bromophenol blue	Merck, Darmstadt
BSA, Fraction V	Roche, Basel, Switerzerland
Chloroquine	Sigma-Aldrich, Taufkirchen
Coomassie Brilliant Blue R-250	Serva, Heidelberg
Coverslips-glass	Marienfeld, Lauda-Königshofen
DAPI	Invitrogen, Karlsruhe
DEPC	Sigma-Aldrich, Steinheim
DMEM-Powder	Gibco BRL, Karlsruhe
Dimethylsulfoxide	Merck, Darmstadt
Donkey Serum	Dianova, Hamburg
DTT	Serva, Heidelberg
EDTA	Sigma-Aldrich, Steinheim

EGTA	Sigma-Aldrich, Steinheim
Ethidiumbromide	Roche, Basel, Switzerland
FCS	PAA, Pasching, Austria
Filterpaper Whatman 3 MM	Schleicher und Schüll, Dassel
Glycerol	Serva, Heidelberg
Glycogen	Roche, Basel, Switzerland
Goat serum	Dianova, Hamburg
HEPES	Sigma-Aldrich, Steinheim
Kanamycin	Serva, Heidelberg
Leupeptin	Biomol, Hamburg
L-Glutamine	PAA, Pasching, Austria
Milk Powder	Lüneburg, Hamburg
2-Mercaptoethanol	Sigma-Aldrich, Steinheim
Mowiol® 4-88 Reagent	Calbiochem, Schwalbach
Nonidet_ P40 (NP-40)	Fluka, Neu-Ulm
dNTPs	MBI Fermentas, Heidelberg
OPTI-MEM® I Reduced Serum Medium	Gibco/BRL, Invitrogen, Paisley
Parafilm "M"	American National Can, Greenwich
Paraformaldehyde	Merck, Darmstadt
Pefablock SC	Biomol, Hamburg
Penicillin/Streptomycin	Biochrom, Berlin
Pepstatin A	Sigma-Aldrich, Steinheim
Phenol-Chloroform-Isoamylalcohol (25:24:1)	Biomol, Hamburg
Polyethylenimine	Gibco/BRL, Invitrogen, Paisley
Proteinase K, rec.	Roche, Basel, Switzerland
Phenol/C/I (25:24:1)	Roth, Karlsruhe
X-ray film CEA	PMA, Bode, Hamburg
Protein-G-Sepharose	Amersham Pharmacia Biotech, Freiburg
SeaKem© LE Agarose	Lonza, Wuppertal
Sodium deoxycholate	Sigma-Aldrich, Steinheim
SuperSignal West Dura Extended D Substrate	Pierce, Rockford, USA
TEMED	Sigma-Aldrich, Steinheim
Triton X-100	Sigma-Aldrich, Steinheim

Tris Base	Sigma-Aldrich, Steinheim
Trypsin	Biochrom, Berlin
Tween 20	Fluka, New Ulm
Vectashield H-100	Vector Laboratories, USA
Zeocin	Invitrogen, Karlsruhe

KITS

cDNA synthesis	MBI, Fermentas, Heidelberg
M-PER Mammalian protein extraction kit	Pierce, Perbio Science, Bonn
NE-PER Nuclear and cytoplasmic extraction reagents	Pierce, Perbio Science, Bonn
NucleoBond® PC 500	Macherey-Nagel, Düren
Power SYBR® Green PCR Master Mix	Applied Biosystems, Darmstadt
QIAshredder	QIAGEN, Hilden
RNeasy® MiniElute	QIAGEN, Hilden
RNase-Free DNase set	QIAGEN, Hilden
RevertAid™ H-Minus First Strand	
SuperSignal® West Dura Extended Duration Substrate	Pierce, Perbio Science, Bonn
ThermoScriptTMRT-PCR System	Invitrogen, Paisley, UK
Turbo DNA-free™	Ambion, Huntington

1. 2 List of buffers and solutions

All buffers were prepared using MilliQ water and autoclaved whenever it was needed.

Cell culture:

PBS	137mM NaCl; 2.7mM KCl; 4.3mM Na_2HPO_4; 1.5mM KH_2PO_4 pH 7.4 autoclaved.
2X Trypsin	2% (v/v) Trypsin 5 mM EDTA in PBS, pH 8.0 sterile filtration
DMEM	133.8 g DMEM-Powder 37 g $NaHCO_3$ in 10 l sterile ddH_2O, pH 7.1 with HCl,
FACS cell sorting buffer	0.1mEDTA in 1x PBS
PEI	Polyethylenimine in sterile ddH_2O, 1µg/µl concentration sterile filtration

Protein Biochemistry

Leammli Buffer (SDS-PAGE) 10x	0.25M Tris-HCl pH 8.9; 0.2M Glycine, 0.1% (w/v) SDS
Lysis buffer	1% SDS; 50mM Tris-HCl pH 7.5; 10mM EDTA
PBS/Glycine	137mM NaCl; 2.7mM KCl; 4.3mM Na2HPO4; 1.5mM KH_2PO_4 pH 7.4; 125mM Glycine

SDS loading buffer 2x	100mM Tris HCl pH 6.8; 5% SDS; 0.05% (w/v) Bromophenol blue; 5% (v/v) 2-Mercaptoethanol; 60% (v/v) Glycerol
SDS loading buffer 6x	130mM Tris HCl pH 6.8; 4.6% SDS, 0.05% (w/v) Bromophenol blue; 5% (v/v) 2-Mercaptoethanol; 60% (v/v) Glycerol
SDS-stacking gel 10% (for 5 mini gels)	15 ml Acrylamide/Bisacrylamide 11.25 ml 4x Tris/SDS-buffer pH 8.8 18.75 ml H_2O 150 µl 10% APS 30 µl TEMED
SDS-resolving gel (for 5 mini gels)	3.9 ml Acrylamide/Bisacrylamide 7.5 ml 4x Tris/SDS-buffer, pH 6.8 18.3 ml H_2O 150 µl 10% APS 30 µl TEMED
TBST	1xTBS; 0.5% (v/v) Tween 20
Transfer buffer 10x	0.5M Tris-HCl pH 8.3; 1.92M Glycine

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DEPC water	0.1 % (v/v) DEPC in dd H_2O; dissolved overnight at RT, autoclaved
TAE 50x	2M Tris-HCl pH 8.0; 1M Acetic acid, 0.05M EDTA
TE Puffer	10mM Tris-HCl pH 7.5 to 8.0; 1mM EDTA

1.3 Primary antibodies

Name	Description
Anti-EpCAM	rat monoclonal antibody against EpCAM (BD Pharmingen).
Anti - p53	goat polyclonal antibody against p53 (Santa Cruz).
Anti - Laming	goat polyclonal antibody against laming (Santa Cruz).
Anti - Tubulin	rat monoclonal antibody against tubulin (Santa Cruz).
Anti- MET (B-2)	mouse monoclonal antibody raised against a peptide mapping within a C-terminal cytoplasmic domain of MET p140 of mouse origin used for the detection of the 145 kDa β chain of MET (Santa Cruz).
Anti- MET (C-28)	rabbit polyclonal antibody raised against a peptide mapping within a C-terminal cytoplasmic domain of MET of human origin used for the detection of the 145 kDa β chain of MET (Santa Cruz).
Anti- MET (N-16)	goat polyclonal antibody raised against a peptide mapping within an N-terminal extracellular domain of MET of human origin used for the detection of the 145 kDa β chain of MET (Santa Cruz).
Anti-MET (CVD13)	rabbit polyclonal antibody raised against a peptide mapping within a C-terminal cytoplasmic domain of MET of human origin used for the detection of the 145 kDa β chain of MET (Zymed).

Name	Description
Anti- pMET (Tyr 1349)	rabbit polyclonal antibody raised against a short amino acid sequence containing phosphorylated Tyr 1349 of MET of human origin used for the Tyr 1349 phosphorylated MET (Santa Cruz).
Anti- pMET (Tyr 1234/1235)	rabbit polyclonal antibody raised against a short amino acid sequence containing phosphorylated Tyr 1234/1235 of MET of human origin used for the Tyr 1349 phosphorylated MET (Cell Signaling).
Anti-MET (N1)	rabbit polyclonal antibody raised against a peptide mapping within a N-terminal cytoplasmic domain of MET of mouse origin used for the detection of the 145 kDa β chain of MET (Eurogentec).
Anti-MET (N2)	rabbit polyclonal antibody raised against a peptide mapping within a N-terminal cytoplasmic domain of MET of mouse origin used for the detection of the 145 kDa β chain of MET (Eurogentec).
Anti-MET (C1)	rabbit polyclonal antibody raised against a peptide mapping within a C-terminal cytoplasmic domain of MET of mouse origin used for the detection of the 145 kDa β chain of MET (Eurogentec).
Anti-MET (C2)	rabbit polyclonal antibody raised against a peptide mapping within a C-terminal cytoplasmic domain of MET of mouse origin used for the detection of the 145 kDa β chain of MET (Eurogentec).

1.4 Secondary antibodies

Secondary antibodies used for Western Blotting

Name	Description
Sheep anti-Mouse IgG (H+L)	HRP coupled antibody raised against mouse IgGs in sheep (Biomol).
Goat anti-Rat IgG (H+L)	HRP coupled antibody raised against rat IgGs in goat (Biomol).
Donkey anti-rabbit IgG (H+L)	HRP coupled antibody raised against rabbit IgGs in donkey (Biomol).

Secondary antibodies used for indirect immunofluorescence

Name	Conjugate	Company
Donkey anti-Rabbit IgG (H+L)	Alexa555	Molecular Probes, Invitrogen
Donkey anti-Mouse IgG (H+L)	Alexa555	Molecular Probes, Invitrogen
Donkey anti-Mouse IgG (H+L)	Alexa488	Molecular Probes, Invitrogen
Donkey anti-Goat IgG (H+L)	Alexa488	Molecular Probes, Invitrogen
Donkey anti-Goat IgG (H+L)	Alexa568	Molecular Probes, Invitrogen
Goat anti-Mouse IgG (H+L)	Alexa568	Molecular Probes, Invitrogen
Goat anti-Rabbit IgG (H+L)	Alexa488	Molecular Probes, Invitrogen
Goat anti-Rat IgG (H+L)	Alexa488	Molecular Probes, Invitrogen
Goat anti-Rat IgG (H+L)	Alexa568	Molecular Probes, Invitrogen

1.5 Bacterial and mammalian cell culture medium

Bacterial media were autoclaved and antibiotics were added to warm media later.

1	LB-medium	10 g/l Bacto-tryptone 10 g/l NaCl 5 g/l Yeast extract
2.	LB- ampicillin medium	100 mg/l ampicillin in LB-medium
3.	LB ampicillin plates	20 g/l agar in LB-Medium 100 mg/l ampicillin

Mammalian cell culture medium were made or purchased from commercial companies.

1. DMEM
2. DME F12
3. RPMI
4. Quantum 286 Complete Medium for Epithelial Cells (QEM)

1.6 Eukaryotic cell lines

Name	Description
3T3	fibroblasts cell line from BALB/c mouse embryo tissue which are spontaneously immortalised (Aaronson and Todaro, 1968).
3T3 LT	fibroblasts cell line from BALB/c mouse embryo tissue which are stably transfected with LT (from our laboratory).
10-1	fibroblasts cell line from BALB/c mouse embryo tissue which are null for p53 gene on both alleles (Harvey and Levine, 1991).
10-1 LT	fibroblasts cell line from BALB/c mouse embryo tissue which are stably transfected with LT (from our laboratory).
EcoPack™ 2-293	packaging cell line (Cat. No. 631507) is a human embryonic kidney, HEK 293-derived cell line designed for rapid, transient production of high-titer, ecotropic retrovirus (Clontech).
A431	cell line derived from an 85-year-old female with epidermal carcinoma. (Giard et al., 1973)
AML12	murine non-transformed hepatocyte cell lines derived from mice transgenic for transforming growth factor alpha (Wu et al., 1994).
cG9	rodent fibroblasts carrying the temperature sensitive p53 mutant codon 135 Ala to Val a temperature downshift to 32.5°C results in the induction of wt-like p53 activities (Michalovitz et al., 1990)
mKSA	SV40-transformed BALB/c mouse fibroblasts (Kit et al., 1969).

Name	Description
SV3T3	SV40 transformed BALB/c 3T3 cells (Deppert et al., 1987).
G2	in-house established mammary epithelial tumour cell line from WAP T1-H22 founder mouse which express high amount MET protein

1.7 Plasmid constructs

Name	Description
pGIPZ lentiviral shRNAmir library	The pGIPZ lentiviral shRNAmir vector is the pGIPZ lentiviral vector containing short hairpin sequences to knockdown MET gene.

1.8 Mouse strains

BALB/c strain was used for all the primary cell culture preparation. WAP-T1 transgenic mouse was generated in our by Krepulat (Krepulat et al., 2005).

III.2 METHODS

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2.1.1. Plasmid isolation

All plasmid isolations were done using the kit NucleoBond® PC 500 from Macherey-Nagel following the manufacturer's instructions.

2.1.2. Polymerase chain reaction

(Saiki et al., 1988)

Amplification of DNA fragments was performed in a 10-50µl reaction mix with thin-walled PCR tubes in PCR cyclers.

The following reaction mixture was used for a standard PCR:

Template DNA 25ng

Forward primers 10 µM

Reverse primer 10µM

Nucleotides (dNTPs) 10 mM

Taq Polymerase 1-2 units

2.1.3. DNA gel electrophoresis

DNA fragments were separated by horizontal electrophoresis in DNA electrophoresis chambers (BioRad) using agarose gels. Agarose gels were prepared by heating 1-2 % agarose (w/v) (depending on the size of DNA fragments) in 1xTAE buffer till all the agarose was dissolved and then poured onto DNA gel trays. The gel was covered with 1xTAE buffer; the DNA samples were mixed with DNA sample buffer and pipetted into the sample pockets. The gel was run at constant voltage (10 V/cm gel length) until the orange G dye had reached the end of the gel. Afterwards, the gel was stained with ethidium bromide staining solution for 20 minutes and documented.

2.1.4. Determination of DNA/RNA concentration

DNA/RNA concentrations were determined spectroscopically using ND110 (Nano Drop). The absolute volume necessary for measurement was 1 µl. DNA/RNA was diluted appropriately with 10mM Tris EDTA before taking the reading. Concentration was determined by measuring the absorbance at 260 nm and 280 nm. The concentration of DNA/RNA was calculated from the absorbance at 260nm. A ratio of A_{260}/A_{280} between 1.8 and 2 indicated sufficient purity of the DNA/RNA preparation.

2.1.5. RNA isolation and Reverse Transcription

For RNA isolation RNeasy® MiniElute from QIAGEN Company was used. Protocol followed was according to the manufacturer's instructions.
RevertAid™ H-Minus First Strand cDNA Synthesis kit from MBI, Fermentas was used for *in-vitro* reverse transcription with 1µg of total RNA.
Reaction mixture followed is a follows:

1. Reaction mixtures

Template RNA	1µg
Oligo $(dT)_{18}$ primer (0.5 µg/ µl)	1µl
DEPC H_2O	to 12µl

Mixed gently and centrifuged

2. Incubated the mixture at 70°C for 5 min. Chill on ice, spin down and place the vial back on ice.
3. Added the following components in the indicated order:

5X reaction buffer	5µl
RiboLock™ RNase Inhibitor (20 u/µl)	1µl
10 mM dNTPs mix	2µl

Mixed gently and centrifuged.

4. Incubated at 37°C for 5min
5. RevertAid™ H Minus M-MuLV

Reverse Transcriptase (200 u/µl)	1µl
Final volume	20µl

6. Incubated the mixture at 42°C for 60min
7. Stop reaction by heating at 70°C for 10min. Chilled on ice.
8. The total volume of 20µl was diluted 5 times with sterile water and 0.5-1µl was used for further PCR.

2.1.6. Quantitative Real Time PCR

RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantification. Even though there are different kinds of methods available for RT-PCR our lab opted for SYBR green method since its most the simplest and most economical format for detecting and quantitating PCR products in real-time reactions. SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases which is detected by laser.
The total reaction mix was 10µl which consisted of following ingredients:

2x Power SYBR Green PCR Master Mix	5 µl
Primer A	0.5 µl from 10 µM concentration
Primer B	0.5 µl from 10 µM concentration
cDNA	1.0µl from 0.5ng/µl cDNA concentration
DEPC water	2.0 µl

The PCR reaction conditions were as follows

Step 1
95°C 10 min Initial denaturation and activation of enzyme
Step 2 – 40 X
96°C 15 s Denaturation
60°C 1 min Amplification/Elongation
Step 3 –Dissociation curve
95°C 15 s
60°C 1 min
95°C 15 s
60°C 15 s

Analysis of results was done using Comparative Ct Method. This involves comparing the Ct values of the samples of interest with a control or calibrator such as a non-treated sample or RNA from normal tissue. The Ct values of both the calibrator and the samples of interest are normalized to an appropriate endogenous housekeeping gene like actin, GAPDH or HPRT. HPRT was the best housekeeping gene for our experiments

The comparative C_t method is given as the $2^{-[delta][delta]Ct}$ method, where

$[delta][delta]C_t = [delta]C_{t,sample} - [delta]C_{t,reference}$

Here, $[delta]C_{T,sample}$ is the C_t value for any sample normalized to the endogenous housekeeping gene and $[delta]C_{t,\ reference}$ is the C_t value for the calibrator also normalized to the endogenous housekeeping gene.

2.1.7 Chromatin Immunoprecipitation (ChIP) Assay

ChIP is an important technique used to study the protein-gene interactions in vivo, identifying a particular factors (transcription factors or histone complexes) interaction with its respective cis-elements in the genome. We followed the protocol Mammalian ChIP-on-chip provided by Agilent Technologies. The assay was performed according to the guide book.

III.2.2 Cell Biology Methods

2.2.1. Cultivation of eukaryotic cells

Adhesive mammalian cells were cultured as a monolayer in polystyrene cell culture dishes. Dulbecco's Modified Eagle medium (DMEM), Roswell Park Memorial Institute (RPMI), Quantum 286 Complete Medium for Epithelial Cells was used. This medium was then enriched with 5-10% fetal calf serum (FCS) and with or without 1X penicillin/streptomycin and fungizone solution. For primary cultures like mammary epithelial cells at times 1-5% of mouse serum was used and also the medium was treated with or without hormones like insulin, prolactin, estradiol, and hydrocortisone. Primary mammary epithelial cultures were incubated at normoxic atmosphere like 21%

oxygen (O_2), 5% carbon dioxide ($CO_{2)}$ balanced with nitrogen (N_2) at 37°C and other cell lines were cultured were incubated at 37°C in a CO_2-incubator with 5% CO_2.

2.2.2 Splitting and freezing of cells

Depending on the cell type, the cells were split in a ratio varying from 1: 2 to 1:20. The cells were washed with sterile 1X PBS and 1-2ml of 2X trypsin was added. The Petri dish was incubated for 2-5 minutes at 37°C for faster detachment of cells. 2-5ml of respective medium based on the type of cells containing 5-10% FCS was added. This ensured that the protease activity of Trypsin was competitively inhibited by the protein content of the FCS and cellular proteins were protected from further destruction. The cell suspension was transferred to Falcon tubes and spun at 300g for 5 minutes. The medium was siphoned off and the cell pellet was dissociated by adding fresh medium and plated according to the need of future experiments. If counting was necessary the cells were counted using *Neubauer* cell counting slide.
The cells were frozen using the freezing medium. The freezing medium contained 95% FCS and 5% DMSO. The cell pellet was resuspended using freezing medium and 1 ml aliquot of the cell suspension was pipetted into pre-cooled cryo tubes. The cryo tubes were placed in precooled cryobox and stored in -80°C for a week and later transferred to liquid nitrogen tank for long term usage. To reculture the frozen cells, the cryo tube was rapidly thawed at 37°C and the cells were immediately transferred into Falcon tube containing 10ml of respective pre-warmed medium. This suspension of cells was spun at 300g for 5 minutes and plated in appropriate cell culture dishes.

2.2.3 Preparation of primary mammary epithelial culture

Isolation and maintenance of primary mammary epithelial cells;

To isolate epithelial cells we adopted the protocol from Smalley et al., with minor modifications. The animal model used was BALB/c and BALB/c -WAP-T1 mouse. The protocol outline is as follows

- Breast tissue/tumors was aseptically dissected
- Washed with media (DMEM F12/Quantum Epithelial media)
- Minced mechanically using scalpels until no visible tissue clump is seen
- Collected the minced tissue and washed with media
- Incubated with 5ml of media containing 0.4% collagenase for > 1 hour at 37ºC in revolving incubator
- Centrifuged for 5 min at 300g and discarded the supernatent
- Incubated with 3ml of Cell Dissociation buffer for 15 mins at 37ºC on revolving incubator
- Centrifuged for 5 min at 300g and discarded the supernatent
- Trypsinized with 3ml for 5 mins at 37ºC and media with 10% FCS was added to quench trypsin
- Centrifuged for 5 min at 300g and discarded the supernatent
- Washed the cells twice with the media
- Plated $>10^6$ cells with media supplemented with or without hormones like insulin, prolactin, estradiol, hydrocortisone and antibiotics.

2.2.4. Plating of cells on matrigel coated plates

The Matrigel Basement Membrane Matrix is a solubilised basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in ECM proteins. Its major component is laminin, followed by collagen IV, heparan sulphate proteoglycans, entactin and nidogen. It also contains Transforming Growth Factor (TGF) ß, fibroblast growth factor, tissue plasminogen activator and other growth factors which occur naturally in the EHS tumor. At room temperature, the Matrigel matrix polymerizes to produce biologically active matrix material resembling the mammalian cellular basement membrane.

Matrigel was thawed at 4°C overnight. The Petri plates with or without coverslips were precooled at 4°C. The matrigel and media were mixed at 1:1 ratio and smeared on the surface of the Petri plate. The Petri plate was placed in incubator for 30 minutes at 37°C which allowed matrigel solidify and polymerise. The plates were then washed with media to remove the excess of matrigel and the cells were plated.

2.2.5. Immunofluorescence staining (IFS) and Immunohistochemistry (IHC)

2.2.5a Indirect - Immunoflourescence staining:

Immunoflourescence was used to investigate the expression and localisation of specific proteins. Indirect-immunofluorescence is a method of immunofluorescence staining in which the first antibody, that is directed against the antigen to be localised, is used unlabelled and the location of the first antibody is then detected by use of a fluorescently labelled anti-IgG i.e., against IgGs of the species in which the first antibody was raised.
The experimental procedure followed is as follows:

1. Cover slips containing 60-80% confluent cells were transferred to 6 well petriplates and were washed with 2 ml 1X PBS twice for 3 minutes on a rotating shaker.

2. The cell were fixed with 4% paraformaldehyde in PBS buffer (pH 7.4) by adding 1ml/well and incubated for 10 minutes at room temperature.

3. The cells were permeabilized with 1 ml of 1% Triton X-100 in PBS (pH 7.4) per well for 10 minutes.

4. The cover slips were washed 3 times with 2ml of 1X PBS

5. The cells were covered with blocking buffer (0.5% BSA, protease free) or with serum of species same as secondary antibody in PBS pH 7.4 to minimise non-specific adsorption of the antibodies to the cover slip. This was done by placing 100µl of BSA solution on a clean parafilm and placing the cover slip upside down. This setup was incubated in a humidified chamber at room temperature for 30 minutes

6. Primary antibody was diluted in blocking buffer at desired concentration based on antibody concentration. For majority of the antibodies dilution ratio of 1:100 was used. The cover slips were removed from blocking buffer and placed on

Whatmann paper to remove excess of blocking buffer. 100µl of diluted primary antibody mixture was aliquoted on clean parafilm. The cover slip was placed upside down and this set up was placed again in a humidified chamber for 1 hour.

7. 200 µl of PBS/0.1% Tween 20 was underlayed for the smooth removal of cover slip from the parafilm. The cover slips were facing cells-side-up were placed in 6 well culture plate and washed 3 times of 3 minutes time at room temperature with PBS/0.1% Tween 20

8. Secondary antibody was diluted in blocking buffer at desired concentration based on antibody concentration. For majority of the antibodies dilution ratio of 1:300 or 1:500 was used. The coverslips were placed on Whatmann paper to remove excess of wash buffer. 100µl of diluted secondary antibody mixture was aliquoted on clean parafilm. The cover slip was placed upside down and this set up was placed again in a humidified chamber for 30 minutes.

9. The washing step was carried out like the same way like after the incubation with primary antibody.

10. The bound antibodies were fixed with 4% paraformaldehyde in PBS (pH 7.4) by adding 800 µl per well and incubating at room temperature for 10 minutes. For DAPI staining 2 µl DAPI (2 µl/ml stock) was added at this step.

11. Next, the cover slips were washed with PBS/0.1% Tween 20 (pH 7.4) 3 times of 3 minutes time at room temperature. The cover slips were mounted using VECTASHEILD mounting medium and allowed to dry at room temperature. The cells are now ready for microscopic viewing.

2.2.5b Immunofluorescence staining on cryosections

Immunofluorescence staining on cryosections is once again a method to investigate the expression and localisation of specific proteins in tissue sections using fluorescent molecules tagged for secondary antibodies for the detection of unlabeled primary antibodies. The protocol followed is just same as immunofluorescence staining for cells with the exception that here tissue material is used instead of cells grown on coverslips.

2.2.5c Immunohistochemistry (IHC)

Immunohistochemical staining is a valuable tool for detecting specific antigens in tissues by means of antigen-antibody interactions, the site of antibody binding being identified either by direct labelling of the antibody, or by use of a secondary labelling method. The experimental procedure followed is as follows:

1. Fixed the dissected tissues in formalin for overnight.
2. Embedded the tissue in paraffin.
3. Sectioned the tissues 5-10 micrometers thick using a microtome and floated on a 40°C water bath containing distilled water.
4. Transferred the sections onto glass slides. The slides were allowed to dry overnight
5. Deparaffined samples by incubating sections 2-3 times in xylene for 10 minutes each.
6. Hydrated samples by placing twice in 100% ethanol for 3 minutes each, then in 95%, 70%, 50%, 30% ethanol for 2 minutes each. Washed in ddH2O for 2 minutes
7. Antigen retrieval was performed dipping the slides in citrate buffer and placed in microwave. Once the buffer started boiling the knob was turned to from 800W to 200W. The setting for maintained for next 25 minutes.
8. The slides were washed for 5 minutes each, 3 times in 1xTBST buffer (pH7.6).
9. The surface around the tissue material was cleaned and encircled with DAKO pen which create a hydrophobic boundary to hold the liquid around the tissue material.

10. The endogenous peroxidase was inactivated or blocked by incubating for 5-15 minutes with 0.1% hydrogen peroxide in PBS.
11. The slides were washed for 5 minutes each, 3 times in 1xTBST buffer (pH7.6).
12. Primary antibody was diluted at 1:10,000 ratio using DAKO antibody diluent. The antibody mixture was dropped on tissue material and placed in humidified chamber and placed in cold room for overnight.
13. The slides were washed for 5 minutes each, 3 times in 1xTBST buffer (pH7.6)
14. The secondary antibody provided by the DAKO kit was added and incubated for 15 minutes and placed in humidified chamber.
15. The slides were washed for 5 minutes each, 5 times in 1xTBST buffer (pH7.6).
16. The slides were treated with 3, 3'-Diaminobenzidine (DAB) to produce a brown staining wherever primary and secondary antibodies are attached. The reaction was stopped by washing in running water when a uniform brown color first becomes visible on the section.
17. The slides were counterstained by dipping twice into Hematoxylin solution.
18. The slides were than washed thoroughly in water.
19. Then slides were then subjected for dehydration through 5 changes of alcohol (95%, 95%, 100% and 100%). Cleared in 3 changes with xylene. The slides were dried and the coverslips was placed on the tissue material using mounting solution. Allowed to dry overnight and the slides were ready for microscopy observation.

2.2.6 Soft agar assay

This assay is designed to assay a cell's ability to grow unattached to a surface and therefore suspended in agar. Anchorage independent growth is one of the hallmarks of transformation, which is considered the most accurate and stringent *in vitro* assay for detecting malignant transformation of cells.

Agarose VII low melting, for cell culture was used. 1.2% and 0.7% agarose was prepared using ddH_2O and autoclaved.

Preparation of ground agar

For a total volume 20 ml:
2ml of FCS
9ml of 1.2% agarose
9ml of 2xDMEM medium
All the above ingredients were placed in 40 ºC water bath. After mixing all the ingredients an aliquot of 1-1.5 ml was poured into 5cm Petri plate very slowly taking care not to induce bubble formation. The plates were placed in the laminar hood for 30 minutes.

Preparation of top agar

Total volume 15 ml (for quadruplet)
1.5ml of FCS
6.75ml of 0.7% agarose
6.75ml of 2xDMEM medium.
All the above ingredients were placed in 50 ºC water bath. The cells subjected for assay were counted to have approximately 1.125 x 10^5 and pelleted in 50ml Falcon tubes. The cell pellet was resuspended by adding 6.75 ml of 2xDMEM medium and mixed with rest of the ingredients i.e., 6.75ml of 0.7% agarose and 1.5ml of FCS. 4 ml of this mixture was aliquoted into Petri dishes containing solidified ground agar. Care was taken not induce any bubble formation and performed quickly so that the agarose do not get solidified. These plates were than allowed to stay in the hood for another 30 minutes for complete solidification of top agar and then transferred to 37 ºC humidified incubator. Photograph was taken every 3 days till 21 days. After 21 days the plates were stained with 0.5ml of 0.005% crystal violet for more than an hour. Colonies were counted and also the plates were scanned.

2.2.7 Fluorescence-activated cell sorting (FACS)

FACS is a method for sorting a suspension of biologic cells into two or more containers, one cell at a time. It is based upon specific light scattering and fluorescence characteristics of each cell. In FACS the cell suspension is entrained in the centre of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell being in a droplet. Just before the stream breaks into droplets the flow passes through a fluorescence measuring station where the fluorescence character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately prior fluorescence intensity measurement and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. FACS was performed in our experiments to select cells. For FACS the cells were resuspended in sterile PBS containing 0.1M EDTA. The cells were sorted into medium containing antibiotics. The cells were either plated or used for RNA isolation or for total cell lysate preparation.

III.2.3. Protein biochemistry

2.3.1 Preparation of cell lysates

Total cell lysates was prepared by using M-PER kit and cytoplasmic, nuclear fractions were prepared using N-PER kit from Pierce.

2.3.2 Protein quantification

Protein quantification by Bicinchoninic Acid assay method or Bradford method
Protein concentrations were determined with the BC Assay protein Quantification Kit or Bradford as per manufacturer’s instructions.

2.3.3. SDS-PAGE

Protein samples were subjected to 8-10% (depending on the molecular weight of the protein of interest) SDS-PAGE under reducing conditions using the Mini-Protean II system (Bio-Rad) using standard protocols (Laemmli, 1970). Gel composition is given in the materials section. After polymerization of the gels, the chamber was assembled as described by the manufacturer's protocol. Samples were diluted with appropriate amount of 6X SDS sample buffer followed by boiling for 10 minutes at 95ºC and 10-50 µg of total protein of each sample was loaded per well. The assembly was filled with 1X SDS running buffer and the gel was run at a constant voltage of 60V for approximately 30 minutes and then at 100V till the end of the run. The run was stopped when the bromophenol blue running front had reached the end of the gel. Gels were either stained or subjected to Western blotting.

2.3.4. Electrophoretic transfer of proteins

Proteins were transferred after SDS-PAGE (see above) onto a Hybond nitrocellulose or Biotrace PVDF membrane using a MINI TRANSBLOT-apparatus (Bio-Rad). After equilibration of the polyacrylamide gel in transfer buffer for approximately 5 minutes, a blotting sandwich was assembled as described in the manufacturer's protocol. Proteins were transferred at 4°C in blot buffer at constant voltage (60V for 2h). Prestained protein markers from Bio-Rad Laboratories were used as a molecular weight marker and to monitor successful protein transfer after tank blotting.

2.3.5. Immunochemical detection of electrophoretically transferred proteins

After the electrophoretic transfer, membranes were removed from the sandwiches and placed protein-bound side-up in polyacrylic vessels. Membranes were washed once in PBST for 5 minutes and were subsequently blocked for 1 h in PBST with 5% skimmed milk powder under gentle shaking at room temperature. Incubation with an appropriate antibody diluted in PBST with 5% skimmed milk powder, was performed either for 60 minutes at room temperature or overnight at 4°C. The primary antibody solution was removed and membranes were washed 4x15 minutes with PBST under constant shaking. The appropriate horseradish peroxidase (HRP)-conjugated secondary antibody

was applied at a concentration varying from 10 ng/ml to 1 ng/ml in PBST with 5% skimmed milk powder for 60 minutes at room temperature. The membrane was washed three times by incubating with PBST with constant shaking and changing the PBST at ten minute intervals. Immunoreactive bands were visualized using the chemiluminescence detection reagents. The membrane was soaked for 5 minutes in detection solution (1:1 mixture of kit-supplied reagent I and II of enhanced chemiluminiscence detection system). The detection solution was drained off and the blot was placed between transparent plastic sheets. The membrane was exposed to BIOMAX ML (Kodak) for several time intervals, starting with a 5 seconds exposure to 2 hours.

2.3.6 Stripping and re-probing of Western blots

For detection of an additional protein on the immunoblot, the nitrocellulose membranes were stripped from bound antibodies by shaking blots in stripping buffer "Restore" from 10 – 30 minutes at 55oC. Blots were washed again two times with PBST and again subjected to immunochemical detection as described above.

IV.2.4 Virology Methods

2.4.1 Lentivirus Production:

Production of Lentiviral particles through transfection of 293T cells with Polyethylenimine (PEI). The protocol followed to generate Lentiviral particles is as follows:

1. 293T cells were grown to a confluence of 60% on a 10cm dish with DMEM +10% FCS medium.
2. 1ml of Optimum medium was taken in a small eppendorf tube into which following components were added
    a. 3µg of plasmid DNA (shRNA construct)
    b. 1.5 µg R8.91 (packaging plasmid)
    c. 0.75 µg MD2.G (pc VSV-G = VSV glycoprotein)
    d. 50 µl of Polyethylenimine
    Vortexed and spun briefly.
3. Incubated for 10 min at room temperature.

4. Medium from the cells was removed and 3ml of Optimum medium was added, then the content of the eppendorf tube prepared before was added and mixed gently.
5. After 6 hours the medium was changed and 5ml of DMEM +10% FCS containing Penicillin/Streptomycin was added.
6. Incubated the cells in the incubator at 37°C with 5% CO_2
7. Virus was harvested after 24/36/48/72 hours by removing the media containing viral particles in 10ml syringe and passed the liquid through a sterile filter (pore size 0.44 micron). Made 1ml aliquots in cryoplastic tubes and stored them at -80°C.

2.4.2 Concentration of Viral particles

Chilled the rotor by spinning at 1000 rpm at 4° for 1-2 hours. Fresh viral particles harvested were filtered using 0.44 micron cellulose acetate filter. The filtrate was transferred into an ultraclear centricon tube and placed into SW28 rotor. Spun at 21,000 rpm for 3 hours at 4°C. The supernatant was poured off and aspirated the last drop from the lip. Added DMEM medium, shaken on ice and allowed the pellet dissolve overnight. This concentrated virus was either used immediately or stored at -80°C for later usage.

2.4.3 Transduction of cells with Lentiviral particles

The target cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in 5cm Petri dishes. When the cells are in log phase *i.e.,* overnight/one day post plating the cells were transduced by adding one to two ml of virus particles. As positive control 3T3 cells were used. Usually 1 ml of virus was enough to infect 3T3 cells in most of the virus generated during our experiments. The cells were infected twice within 48 hours. After 48 hours the cells were washed with sterile 1x PBS and selection medium was added or subjected to FACS based on the experiment.

IV Results

IV.1 Cellular, molecular and biochemical analysis of MET

IV.1.1 Isolation, characterization and maintenance of primary epithelial cells from mammary glands and mammary tumors

To isolate mammary epithelial cells (MECs) we adopted the protocol from Smalley et al., with minor modifications. The animal models used were WAP-T1 and BALB/c wild type mice. Mammary glands and tumor tissue were used to prepare primary cultures from virgin mice, postweaning mice and mice carrying tumors. The protocol is described in detail in the Methods section. Approximately 10^6 cells were plated on polystyrene (Nunc delta) Petri dishes. These freshly prepared cells hereafter are named as passage zero which increases in ascending order as passage 1, 2, 3, and so on (P0, P1, P2..,). For some experiments, the cells were plated on feeder layer, matrigel coated plates, and for a few more experiments cells/tissue were embedded in matrigel and placed in Petri dishes.

Different media like Roswell Park Memorial Institute medium (RPMI), Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture (DME F12), Quantum Epithelial Medium (QEM) were used to culture these cells. The media were prepared with (5-10%) or without fetal calf serum, and also with (2-5%) or without mouse serum. The media also were prepared with or without hormones insulin, prolactin, β estradiol, hydrocortisone, and dexamethasone. QEM with 5 % FCS was observed to be the best medium to grow primary mammary epithelial cells and was then used as standard medium. Glass cover slips were placed in the Petri plates for immunostaining. The cells usually reached confluence after 5 days in majority of preparations.

Table 1: WAP-T1 mouse list for cell culture preparation

Mouse No	Mouse Code	Description	T-Ag expression in primary cell culture	Duration of T-Ag expression in cell culture
1	WAPT1#100	10 days pp*	Negative	
2	WAPT1#88	8 days pp	Negative	
3	WAPT1#35	30 days pw**	Negative	
4	WAPT1#28	35 days pw	Negative	
5	WAPT1#27	60 days pw	Negative	
6	WAPT1#55	91 days pw	Positive	2 passages
7	WAPT1#88	90 days pw	Positive	2 passages
8	WAPT1#55	12 days pw	Negative	
9	WAPT1#16	virgin	Negative	
10	WAPT1#17	virgin	Negative	
11	WAPT1#26	virgin	Negative	
12	WAPT1#79	virgin	Negative	
13	WAPT1#50	tumor	Positive	1 passage
14	WAPT1#08	tumor	Positive	2 passages
15	WAPT1#100	tumor	Positive	2 passages
16	WAPT1#03	tumor	Positive	1 passages

* postpartum, ** postweaning

The above table contains the list of mouse used to culture primary mammary epithelial cells. The initial goal of our study was to establish a cell culture model to study the role of SV40 T-Ag mediated tumorigenesis where the expression of the *WAP* promoter could be turned on and off to activate T-Ag expression using lactotrophic hormones in primary mammary epithelial cells derived from mammary glands and tumors of WAP-T1 mice. The table lists the status of mice and the duration of T-Ag expression in cell culture in our study.

Mammary glands and tumor tissue were used to prepare primary cultures from virgin mice, post-weaning mice and mice carrying tumors. We expected the expression of T-Ag in all the post-weaning mice and mice carrying tumors' cell in cell culture. But, the expression of T-Ag was detected only in few primary culture preparations, for example WAP-T1#55, WAP-T1#88, WAP-T1#50, WAP-T1#08, WAP-T1#100, and WAP-T1 #03. Out of 12 animals in which T-Ag expression should be present we observed T-Ag expression only in 6 mice (Table 1). The expression of T-Ag was detected in few cells at passage number 0, 1 and 2, and was lost in the subsequent passages (Table 1 and Figure 6).

IV.1.2 EpCAM as marker of epithelial cells

In order to evaluate the phenotype of our primary culture, we used Epithelial Cell Adhesion Molecule (EpCAM). EpCAM is a Ca^{2+} independent homophilic adhesion molecule which is proposed to play an important role in the development and normal function of epithelial tissue. In the mouse, EpCAM is a 40-42kDa cell-surface glycoprotein expressed on epithelial cells of various organs. For example, thymic epithelial cells, thymic dendritic cells, immature thymocytes, a small subset of peripheral T lymphocytes, intestinal epithelium, kidney-collecting tubule epithelium, keratinocytes, Langerhans cells, lymph node and splenic dendritic cells. EpCAM is a well established marker to detect cells of epithelial origin (Bergsagel et al., 1992; Borkowski et al., 1996; Farr and Anderson, 1985; Nelson et al., 1996; Taguchi et al., 1999). We used this protein marker to examine the phenotype our cultures. The detailed protocol for immunofluorescence staining of EpCAM is described in the Methods section. This result clearly demonstrates all our primary culture preparations were indeed of mammary epithelial cells.

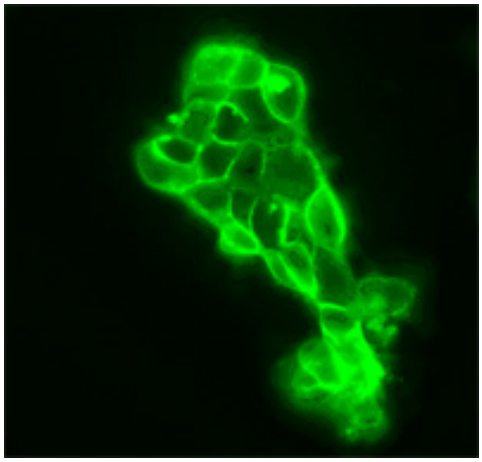


Figure 5: Immunofluorescence staining for EpCAM in primary mammary epithelial cells (MECs); The green fluorescence represents the membranous expression of EpCAM from WAPT1# 55 mice after 5 days post plating, were fixed with 4% PFA and stained for EpCAM. The cells were isolated from mammary glands.

IV.1.3 Expression and loss of SV40 Tumor antigen (T-Ag) in primary cell cultures

To evaluate the expression of SV40 Tumor antigen (T-Ag) by immunofluorescence in primary mammary epithelial cell cultures from WAP-T1 transgenic mice two different antibodies were used namely, rabbit polyclonal R15 anti-SDS-T and mouse monoclonal PAb 419. Expression of T-Ag was detected in few primary culture preparations for example WAP-T1#55, WAP-T1#88, WAP-T1#50, WAP-T1#08, WAP-T1#100, and WAP-T1 #03. Out of 12 animals in which T-Ag expression should be present we could observe T-Ag expression only in 6 mice (Table 1). The expression of T-Ag was detected in few cells at passage number 0, 1 and 2, and was lost in the subsequent passages (Table 1 and Figure 6).

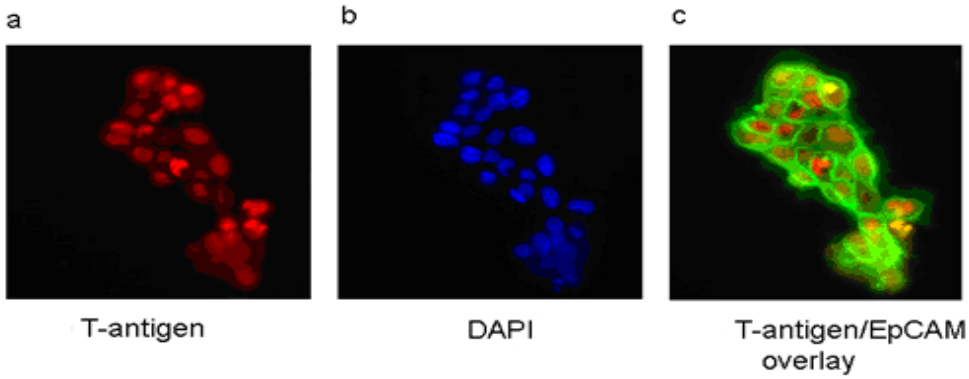


Figure 6: Expression of T-Ag in primary mammary epithelial cell culture. Red and green color represents the expression of T-Ag and EpCAM, respectively. Nuclei counter stained with DAPI (blue color). The cells from WAPT1# 55 mice after 5 days post plating were fixed with 4% PFA and double stained for LT and EpCAM. a) T-Ag b) DAPI c) T-Ag and EpCAM overlay**.**

To confirm whether T-Ag expression was indeed present in the tumors prior to primary culture preparation, the specimens were examined by immunohistochemistry and cytospin analysis of primary tumor cells. The strong expression of T-Ag was detected in the tumor tissues and cells (Figure 7a & b).

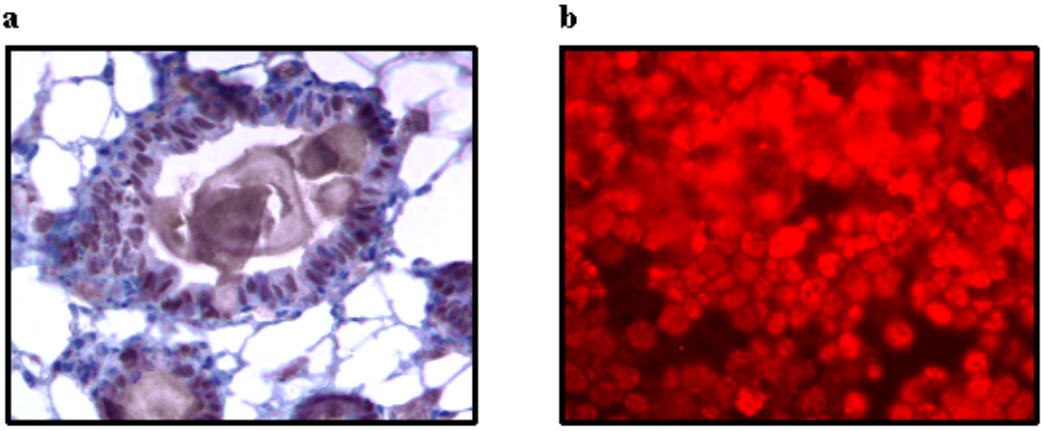


Figure 7: Immunostaining of T-Ag ; a) Immunostaining on paraffin section showing the presence of T-Ag; Paraffin embedded sections were made from tumors of WAPT1#100 mice and fixed with formalin and counterstained with Hematoxylin.
b) Immunofluorescence staining of cells (cytospin) showing the presence of T-Ag; Primary mammary epithelial culture was prepared from WAPT1#100 mice tumor. 10°5 cells were suspended in 200µl PBS and made to attach on special glass slide by centrifuging. The cells were fixed with 4% PFA and stained for T-Ag.

IV.1.4 Silencing of *whey acidic protein* (*WAP*) promoter in cell cultures

As described in the Introduction, T-Ag expression in the WAP-T1 transgenic mouse is under the control of *whey acidic protein* (*WAP*) promoter. The *WAP* promoter is induced by lactotrophic hormones like prolactin, progesterone, insulin, and glucocorticoids. Since sustained expression of T-Ag was not observed in the primary cultures in all the mice preparations, we decided to investigate the expression of *WAP* gene at the transcript level in the cells/tissues plated on Petri plates (primary cultures). Towards this, a primary culture was prepared from tumors of WAP-T1#100 mouse. After plating the cells total RNA was isolated at every 12 hours over a period of 0-72 hours. As can be observed from the figure 8 the *WAP* mRNA level could be detected only at 0 hour time point and was completely shut down within 12 hours after the cells were brought into culture. Not surprisingly, transcript of T-Ag was also lost within 12 hours period since T-Ag is under the control of *WAP* promoter (Figure 8).
Tzeng et al., also tried to culture T-Ag positive primary mammary epithelial cells from their WAP-SV-T/t mouse model for breast cancer but were not successful since the since primary mammary cells exhibited a spontaneous apoptosis in cell culture (Tzeng et al., 1998).

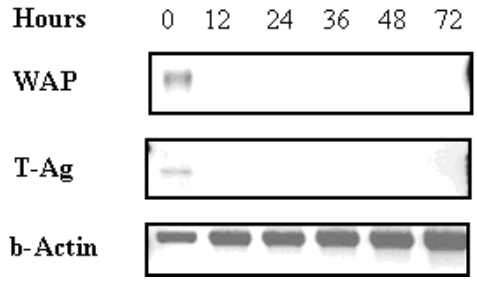


Figure 8: Silencing of *WAP* gene in cell culture: Primary mammary tumor culture was prepared from tumor raised in WAPT1#100 mice. RNA was isolated at regular intervals as shown in the figure. RT-PCR was performed using WAP, T-Ag and b-actin specific primers. Expression of T-Ag and WAP was found only at zero hour and absent in rest of the time periods indicating inactivation endogenous and transgenic *WAP* promoters within 12 hours after plating the cells.

From the literature its known that the *WAP* promoter is tightly regulated by complex interactions between lactogenic hormones, extracellular matrix and intercellular interactions, (Hobbs et al., 1982). Hence we decide to culture the cells in 2 dimensional

(2D) and 3 dimensional (3D) culture systems. For 2D culture, the cells were plated on matrigel coated plates and for 3D culture small pieces of tissue (explants) were embedded in matrigel and placed in petriplates. After 12 hours post plating total RNA was isolated from the explants. cDNA synthesis was performed using 1 μg of total RNA and PCR was performed to amplify WAP and T-Ag transcripts. Again there was no expression of both WAP and T-Ag (Figure 9) indicating that WAP promoter activity shuts off when the explants are brought to cell culture.

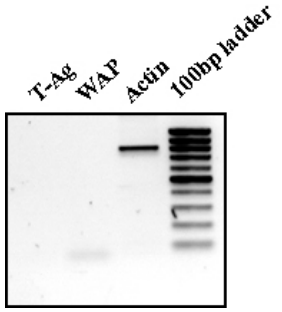


Figure 9: Silencing of *WAP* promoter in explants: Tissue explants culture was prepared from different tumors from WAPT1#100 mice. RNA was isolated after 12 hours post plating. RT-PCR was performed using WAP, T-Ag and actin specific primers. No expression of T-Ag or WAP was found indicating inactivation endogenous and transgenic *WAP* promoters within 12 hours of post plating the tissue explants.

IV.1.6 Expression of MET in mammary tumors

MET gene amplification is a feature of undifferentiated mammary tumors. (Heinlein *et al*., unpublished data). This led us to undertake more extensive cellular and biochemical approaches to study MET in tumors. Towards this it was necessary to prepare primary cultures from the tumors. Primary mammary epithelial cell culture was prepared from the tumors obtained from the mouse numbered as WAP-T1#75. The cultured cells were propagated for more than forty passages. Immunofluorescence staining was performed for primary and the propagated cells from this culture using two antibodies against MET namely, anti-mouse (MET-B2) and anti- rabbit (C28).

MET staining by the B2 antibody was membranous as expected (Figure 10). However, the staining pattern by the C28 antibody was surprising. The localisation of MET

gradually moved to nucleus along with passage numbers 0 to 5. Initially at passage zero (freshly prepared primary culture) MET was localised on the membrane, at passage one partially on the membrane, at passage two almost to the nucleus, and finally at passage five MET localised only to the nucleus (Figure 11). Subsequent passages up to 40 showed MET being localised only in nucleus with C28 antibody.

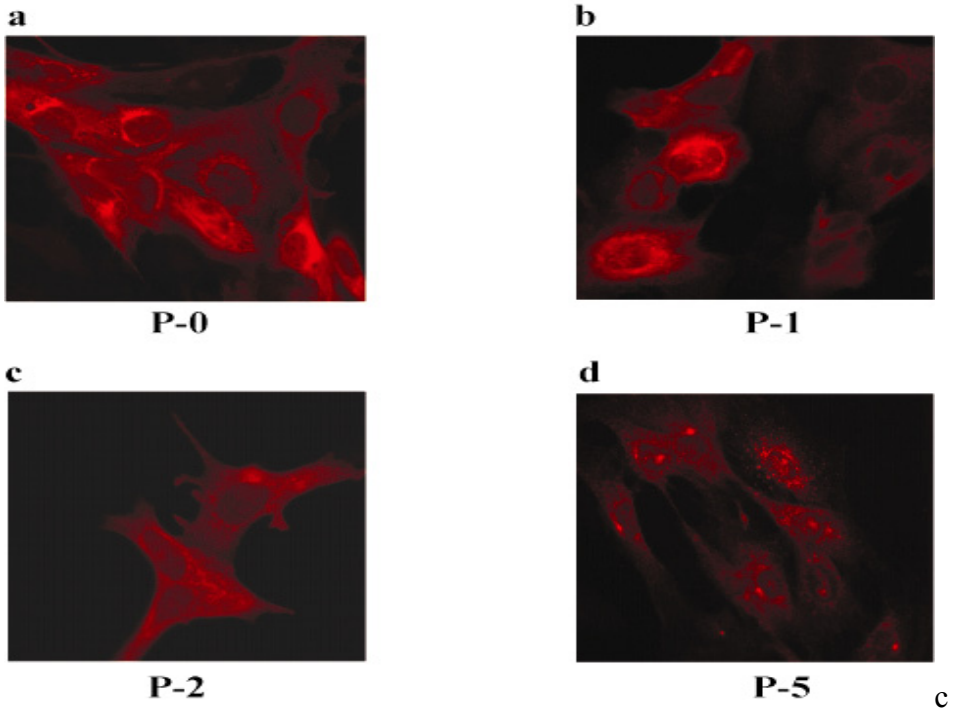


Figure 10: Immunofluorescence staining of WAP-T1#75 cells with the B2 antibody; MET staining using the B-2 antibody shows membranous and cytoplasmic staining in passage zero (Fig: a), passage 1 (Fig: b), passage 2 (Fig: c), and passage 5 (Fig:d).

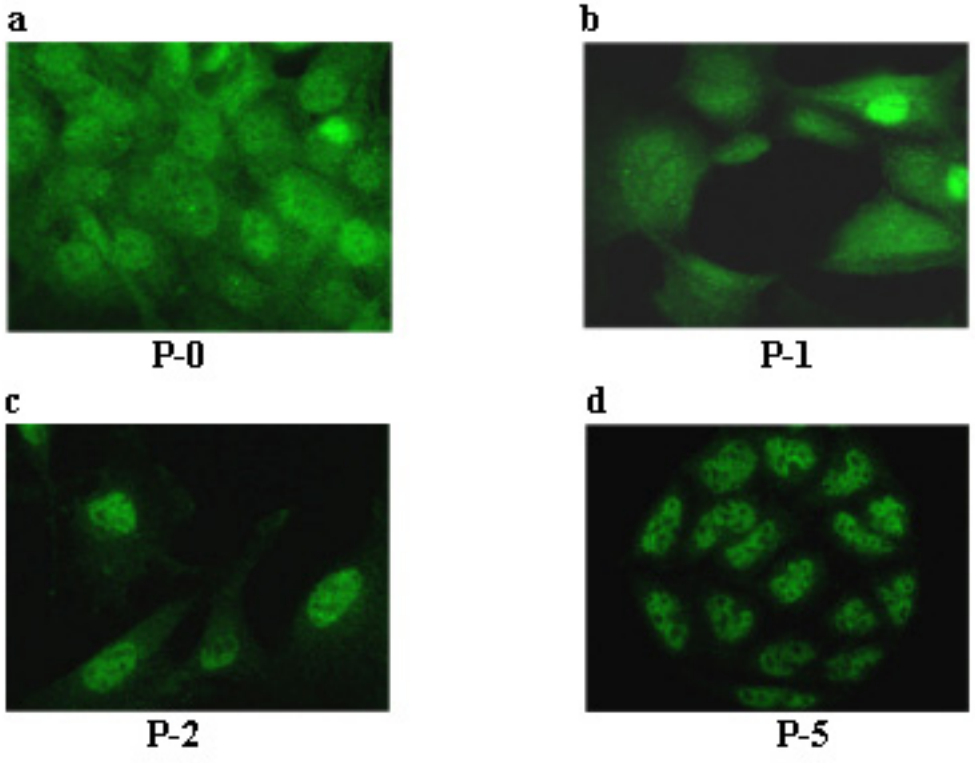


Figure 11: Immunofluorescence staining of WAP-T1#75 cells with the C28 antibody; MET staining using the C28 antibody shows membranous and nuclear staining in passage zero (Fig: a), passage 1 (Fig: b), passage 2 (Fig: c), and passage 5 (Fig:d).

IV.1.7 Localisation of MET

A growing list of membrane proteins has been found to translocate to the nucleus. For example, members of the epidermal growth factor (EGF) and fibroblast growth factor family of receptor tyrosine kinases have been shown to translocate to the nucleus as either intact receptors, or for ErbB4, as a truncated fragment, activating transcription of target genes (Komuro et al., 2003).

Nuclear staining of MET with the C28 antibody led us to investigate the localisation of MET using different antibodies recognising N terminal and C terminal epitopes of MET. In order to prove the localisation of MET in the nucleus, various anti-MET antibodies (Table 3) were obtained and tested for immunofluorescence staining in various cell lines of both human and mouse origin. The subcellular fractions were analysed for Western blotting. The main aim was to experimentally validate the existence of nuclear MET.

Table 3: List of antibodies tested for MET

No	Antibody	Host species	Immunogen	Reactivity	Company
1	MET-B2	Mouse monoclonal	Peptide from C-terminal of mouse origin	mouse	SCBT
2	MET-C28	Rabbit polyclonal	Peptide from C-terminal of human origin	human, <mouse, rat	SCBT
3	CVD13	Rabbit polyclonal	Peptide from C-terminal of human origin	human	ZYMED
4	MET-N16	Goat polyclonal	Peptide from N-terminal of human origin	mouse, rat, human	SCBT
5*	MET-p1234	Rabbit polyclonal	Peptide from C-terminal of human origin	mouse, rat, human	Cell Sign
6*	MET-p1349	Rabbit polyclonal	Peptide from C-terminal of human origin	mouse, rat, human	SCBT

* didn't work properly so omitted for future analysis

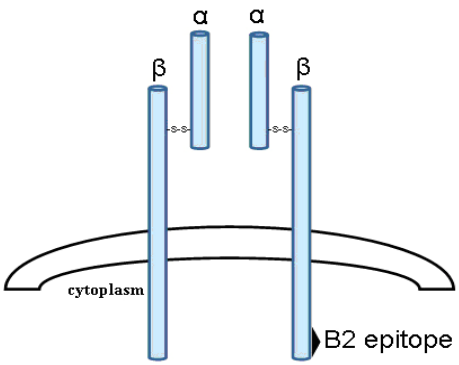
1.7 Detection of MET in different cell lines

1.7.1 A431 cell line

MET (C-28), CVD13, and MET-N16 antibodies according to manufactures guidelines can detect human MET and also to a lesser extent mouse MET. So, as positive controls we wanted to test both human and mouse cell lines which are known to express MET protein. A431 is a human epithelial carcinoma cell line derived from derived from an 85-year-old female with epidermal carcinoma (Fabricant et al., 1977). This human tumor epithelial cell line expresses high level of MET.

1.7.1a. Localization of MET with the antibody B2

Antibody	Host species	Immunogen	Reactivity	Company
MET-B2	Mouse monoclonal	Peptide from C-terminal of mouse origin	mouse	SCBT



MET

MET (B2) is recommended by the supplier for detection of the 145 kDą chain of MET of mouse and rat origin by Western blotting, immunoprecipitation and immunofluorescence staining. The cartoon above depicts the epitope on the MET molecule recognized by the B2 antibody.

Immunofluorescence showed a cytoplasmic staining with the B2 antibody (Figure 12), whereas it did not detect the MET protein by Western blotting using protein fractions from the cytoplasm and the nucleus. Anti-Lamin and anti-Tubulin antibodies were used as a positive control for protein detection in the nuclear and cytoplasmic compartment, respectively (Figure13).

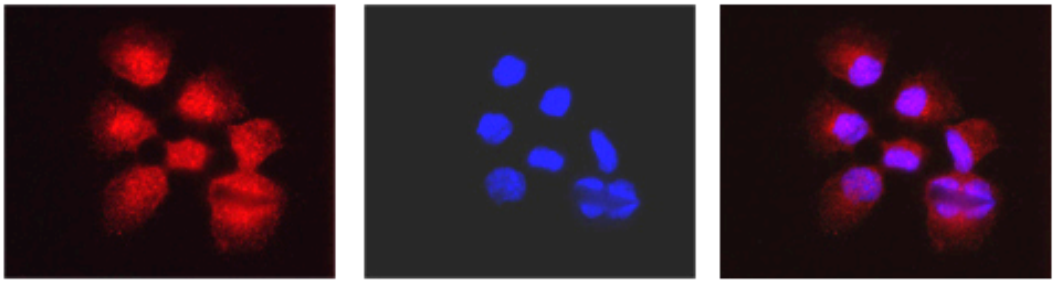


Figure 12: Immunofluorescence staining of A431 cells with the B2 antibody; Cells were fixed using 4% PFA and stained for MET with the B2 antibody. Red fluorescence represents the membranous localisation of MET. Nuclei were counterstained with DAPI.

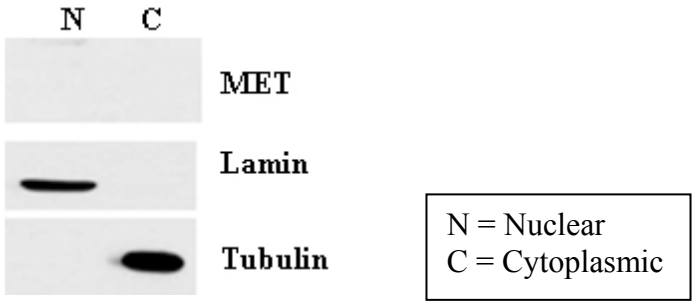
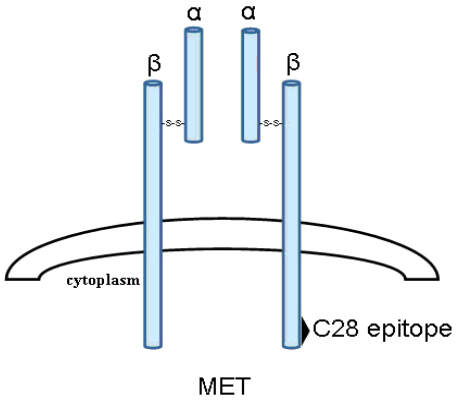


Figure 13: Western blot analysis of MET expression in A431 cells did not detect MET in nuclear or cytoplasmic fractions; Nuclear and cytoplasmic fractions were prepared using NE-PER kit and equal volume (50 µg) was loaded and probed with B2 antibody. Lamin and Tubulin served as controls for nuclear and cytoplasmic fractions respectively.

1.7.1b Localization of MET with the C-28 antibody:

Antibody	Host species	Immunogen	Reactivity	Company
MET-C28	Rabbit polyclonal	Peptide from C-terminal of human origin	human, < mouse, rat	SCBT

MET (C-28) is recommended for detection of the 145 kDa β chain of MET of human and, to a lesser extent, mouse and rat origin by immunofluorescence staining and Western blotting. The following cartoon depicts the epitope on the MET molecule recognized by the C-28 antibody.



Immunofluorescence staining showed nuclear staining with the C28 antibody (Figure 14) in A431 cells. This result was contradicting since the antibody datasheet by the supplier clearly shows membranous staining for the same A431 cell line. Western

blotting results showed the presence of MET both in nuclear and cytoplasmic fractions. In the cytoplasmic fraction the MET precursor (170kDA) was also detected (Figure 15).

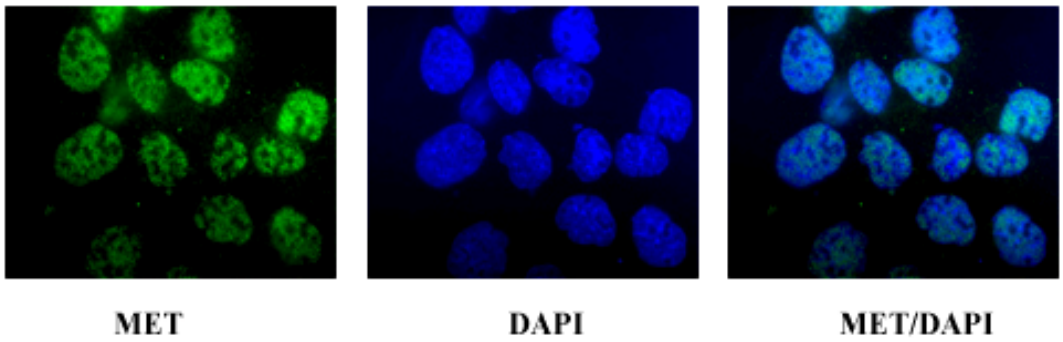


Figure 14: Immunofluorescence staining of A431 cells with C28 antibody; Cells were fixed using 4% PFA and stained for MET with the C28 antibody. Green fluorescence indicates the nuclear localisation of MET. Nuclei were counterstained with DAPI.

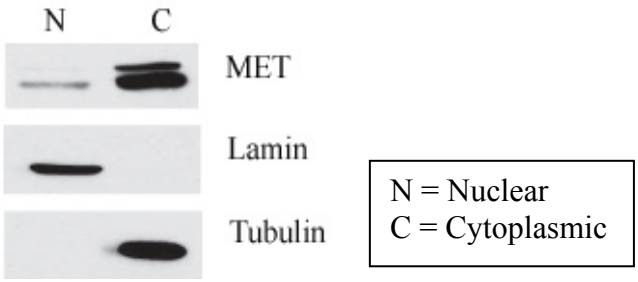


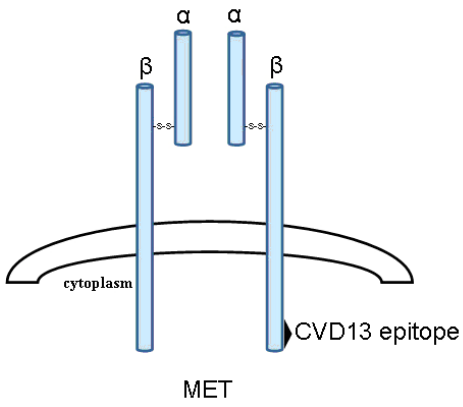
Figure 15: Western blot analysis of MET expression in A431 cells showing the presence of MET in nuclear and cytoplasmic fractions; Nuclear and cytoplasmic fractions were prepared using the NE-PER kit and equal volumes (50 µg) were loaded and probed with C28 antibody. In the cytoplasmic fraction the MET precursor was also detected. Lamin and Tubulin served as controls for nuclear and cytoplasmic fractions, respectively.

1.7.1c MET localization with CVD13 antibody:

Antibody	Host species	Immunogen	Reactivity	Company
CVD13	Rabbit polyclonal	Peptide from C-terminal of human origin	human	ZYMED

Immunogen for the CVD13 antibody was a synthetic peptide derived from the human MET sequence, conjugated to a carrier protein. ZYMED did not reveal in its catalogue details about the immunogen and the specificity of the antibody. Communication with the company's technical support revealed that the antibody detects

an epitope located in the C-terminal end of MET and that it is recommended for the detection of the 145 kDa β chain of MET of human origin by Western blotting and immunofluorescence staining. The following carton depicts the CVD13 epitope in MET molecule.



The immunofluorescence staining with CVD13 in A431 cells shows cytoplasmic staining and also nuclear staining (Figure 16). The immunoblot also depicts the existence of MET in both cytoplasmic and nuclear fractions (Figure 17).

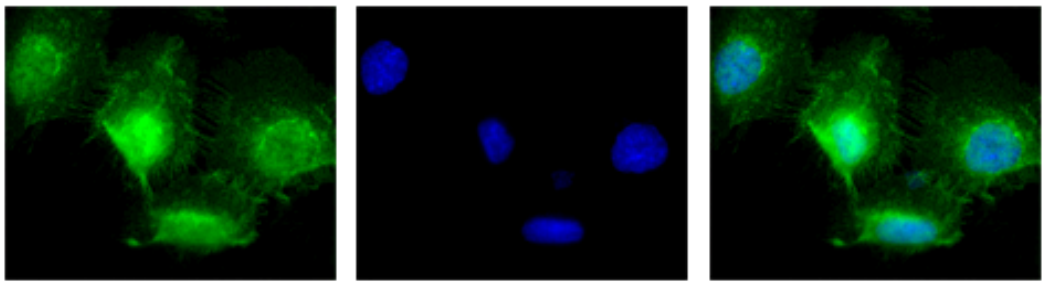


Figure 16: Immunofluorescence staining with the CVD13 antibody: Cells were fixed using 4% PFA and stained for CVD13 antibody. Green fluorescence represents the nuclear and cytoplasmic localisation of MET. Nuclei counter stained with DAPI.

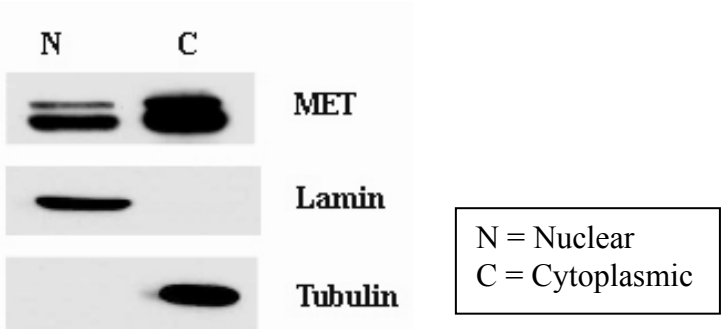
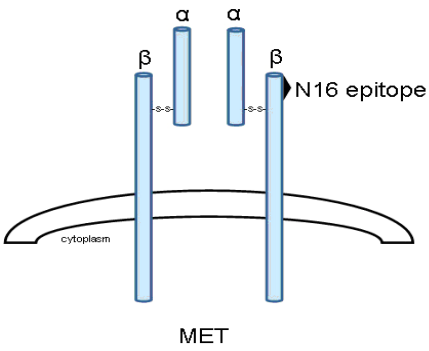


Figure 17: Western blotting showing presence of MET in nuclear and cytoplasmic fractions; Equal volume (50 µg) of nuclear and cytoplasmic fractions were loaded and probed with CVD13 antibody. In both the fractions the MET precursor is also detected. Lamin and Tubulin served as controls for nuclear and cytoplasmic fractions respectively.

1.7.1d MET localization with N-16 antibody:

Antibody	Host species	Immunogen	Reactivity	Company
MET-N16	Goat polyclonal	Peptide from N-terminal of human origin	mouse, rat, human	SCBT

MET (N-16) is recommended for detection of the 145 kDa β chain of MET of mouse, rat and human origin by Western blotting and immunofluorescence staining. The cartoon below depicts the epitope position recognized by N-16 antibody.



Immunofluorescence shows cytoplasmic staining and also to a lesser extent nuclear staining (Figure 18). In immunoblot MET was detected both in cytoplasmic and nuclear fractions (Figure 19).

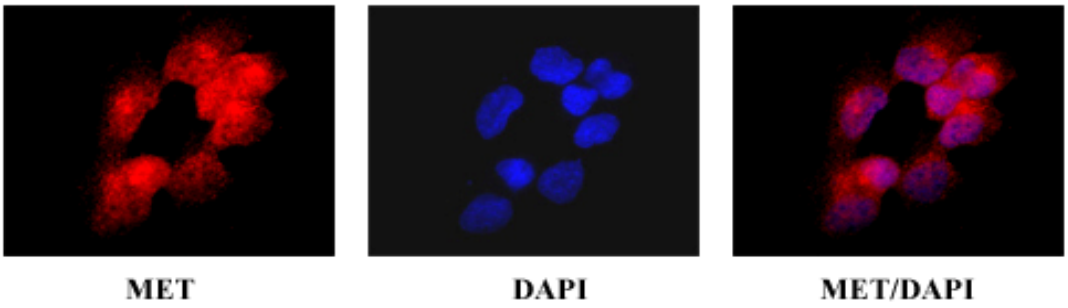


Figure 18: Immunofluorescence staining with the N-16 antibody; Cells were fixed using 4% PFA and stained for N-16 antibody. Red fluorescence represents the nuclear and cytoplasmic localisation of MET. Nuclei counter stained with DAPI.

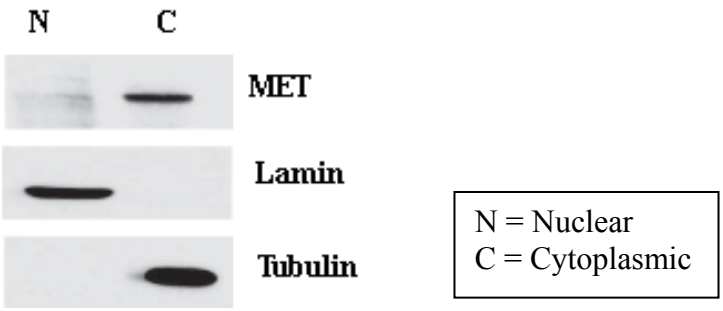


Figure 19: Western blotting shows presence of MET in the cytoplasmic fraction and to significantly lesser extent in the nuclear fraction; equal volume (50 µg) of nuclear and cytoplasmic fractions were loaded and probed with N-16 antibody. Lamin and Tubulin served as controls for nuclear and cytoplasmic fractions respectively.

1.7.2 AML12 Mouse hepatocyte cell line and mKSA cell line

After investigating human cell line A431 for MET expression and its localisation we wanted to test these antibodies in mouse cell lines. As mentioned before our laboratory has generated WAP-T mouse model in which MET gene amplification is observed so we wanted to test mouse cell lines specifically, later to understand the role of MET in SV40 derived carcinogenesis. Towards this AML 12 and mKSA cell lines were selected. AML12 is a murine non-transformed hepatocyte cell lines derived from transgenic mice (Wu et al., 1994). The mKSA cell line is a SV40 transformed Balb/C mouse kidney cells (Kit et al., 1969) and are highly tumorigenic. These two cell lines were selected for further cytological and biochemical analysis.

1.7.2a MET localization with B2, C28, CVD13 and N16 antibodies:

The immunofluorescence staining performed with B2 antibody showed the membranous localisation (Figure 20a & e), and Western blotting showed MET only in the cytoplasmic fraction (Figure 21). The immunofluorescence staining analysis with the C28 antibody shows only nuclear staining (Figure 20b & f) and Western blot did not show any the presence of full length MET in either the nuclear or the cytoplasmic fractions (Figure 21). The datasheet from the manufacturer mentions that this antibody can also detect mouse MET to lesser extent than human MET, but we could not see any signal for full length MET by Western blotting, whereas in immunostaining we see a clear nuclear localisation of MET. The immunofluorescence staining analysis with the CVD13 antibody shows both cytoplasmic and nuclear localisation of MET (Figure 20c & g). But, the same antibody does not detect any full length MET, both in nuclear and cytoplasmic cellular fractions by Western Blotting (Figure 21). The immunofluorescence staining with N-16 antibody shows membranous localisation for AML12 cells (Figure 20d & h) and for mKSA cells both membranous and nuclear staining, whereas in immunoblotting no full length MET is detected either in neither nuclear fraction nor cytoplasmic fraction (Figure 21).

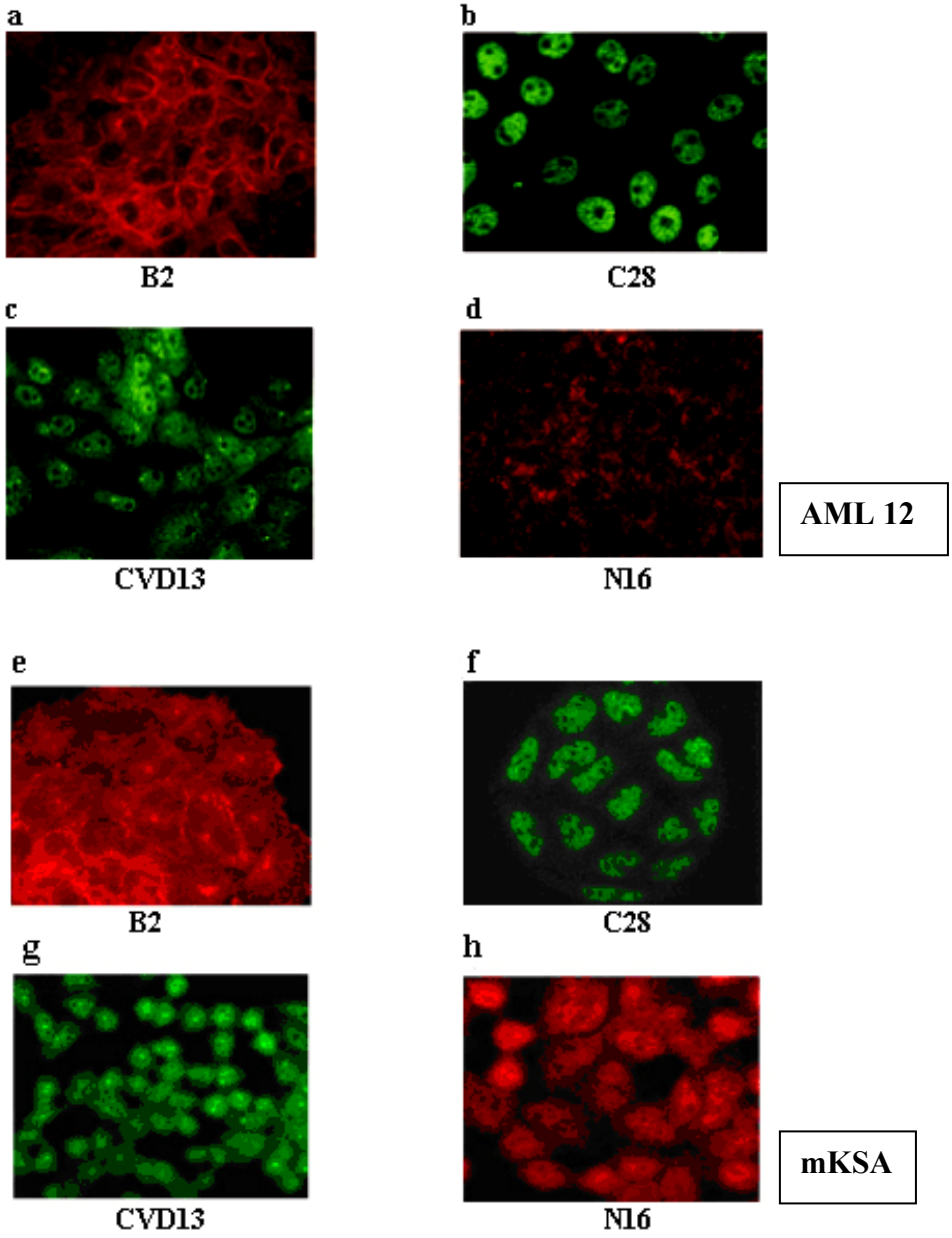


Figure 20: Immunofluorescence staining of AML12 and mKSA cells using different antibodies; Cells were fixed using 4% PFA and stained for with antibodies like B2 (Fig: 20a & e), C28 (Fig: 20b & f), CVD13 (Fig: 20c & g), and N16 (Fig: 20d & h) against MET.

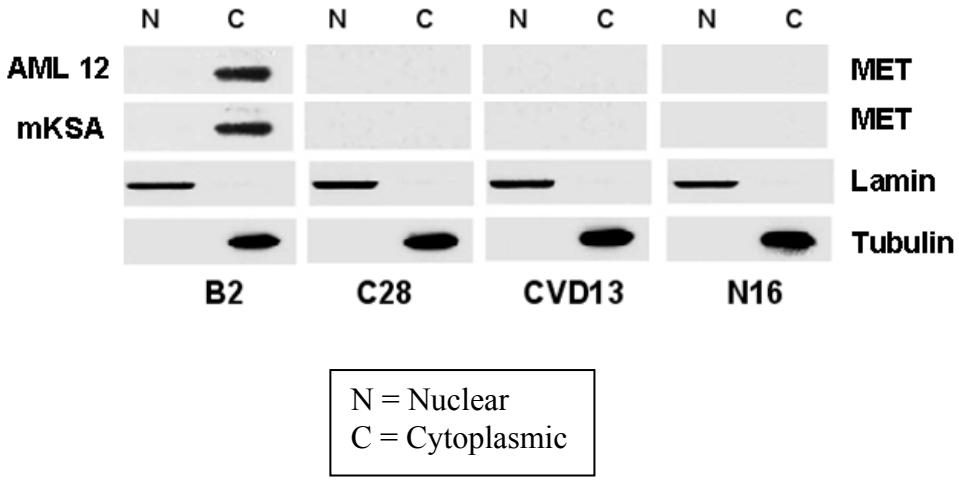


Figure 21: Western blot analysis of MET expression in AML12 and mKSA cells line; Nuclear and cytoplasmic fractions were prepared using NE-PER kit and equal volume (50 µg) was loaded and probed with B2, C28, CVD13, and N16. Lamin and Tubulin served as controls for nuclear and cytoplasmic fractions, respectively.

The results of immunofluorescence staining and Western blotting using MET antibodies B2, C28, CVD13, and N16 in A431, AML12 and mKSA cells can be commented as follows;

Immunofluorescence staining for A431 cells with B2 antibody showed cytoplasmic staining, C28 antibody showed nuclear staining and not cytoplasmic staining as expected, CVD13 and N16 antibody showed both cytoplasmic and nuclear staining. Western blotting results for A431 cells with B2 antibody showed no presence of MET, but with C28, CVD13 and N16 antibodies MET was present both in nuclear and cytoplasmic fractions. The presence of nuclear MET was convincing in A431 cells using CVD13, and N16 antibodies as they showed the presence of MET both in immunofluorescence staining and Western blotting. C28 antibody exhibited little ambiguity since it showed only nuclear staining in immunofluorescence but not cytoplasmic staining as seen in Western blots. B2 antibody even though it showed cytoplasmic staining of MET in immunofluorescence could not detect any MET in Western blotting since its raised against mouse MET and not able to detect human MET. This may be due to the fact that the accessibility of epitope might change during Western blot process whereas in the immunofluorescence the epitope is still accessible to antibody.

In mouse cell lines AML12 and mKSA even though CVD13 and N16 antibody showed both nuclear and cytoplasmic staining and also C28 showing exclusive nuclear staining

in immunofluorescence, we couldn't detect presence of any MET by Western blot analysis. This is due the fact that these antibodies were raised against human MET and were not able to detect mouse MET even though their data sheets suggested that they could partially detect mouse MET. The immunofluorescence results may be once again attributed to epitope accessibility phenomena as mentioned before. B2 antibody showed only cytoplasmic staining which was also confirmed by Western blotting which detected MET only in cytoplasmic fraction.

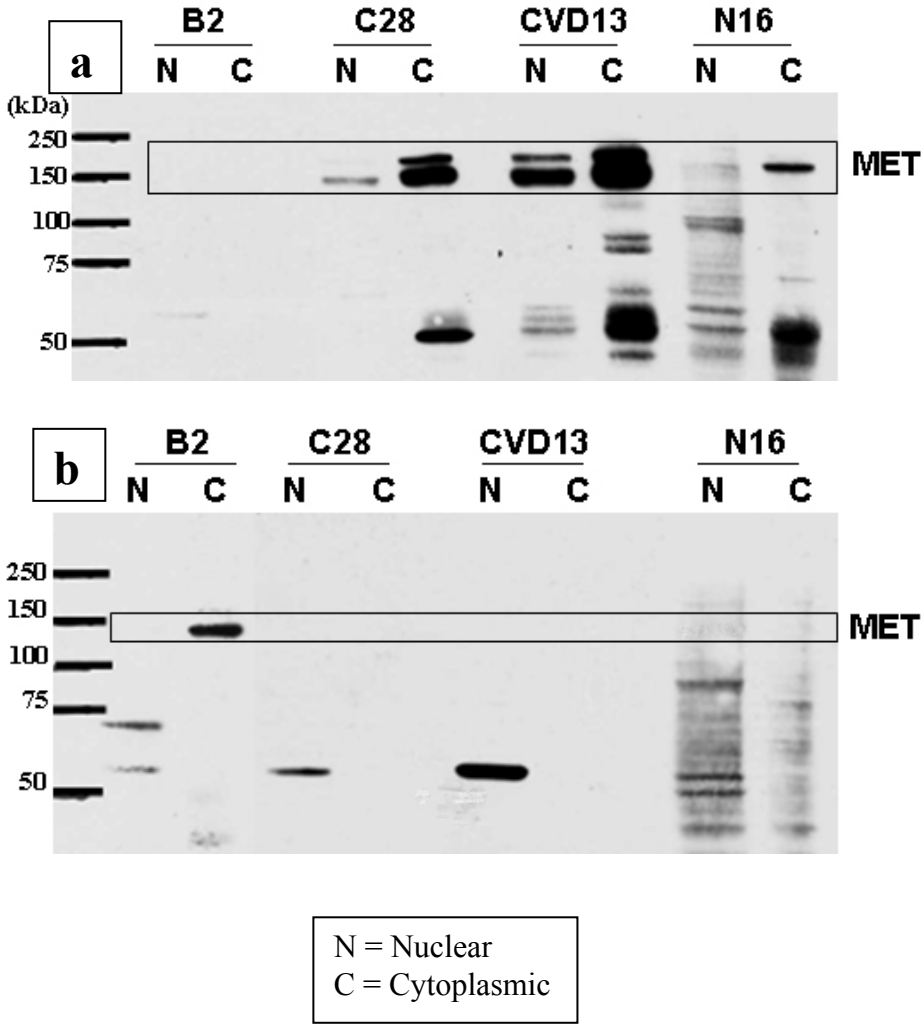


Figure 22: Western blot analysis of MET expression; a) A431 cells; b) AML12 cells; Nuclear and cytoplasmic fractions were prepared using NE-PER kit and equal volume (50 µg) was loaded and probed with B2, C28, CVD13, and N16 antibodies.

At that stage of our experiments Pozner et al., reported the existence cleaved form of MET existing in nucleus which is 60kDa length in A431 and HEK293 cell lines. Gomes et al., also showed that full length MET enters nucleus to initiate calcium signalling in SkHep1 cell line. Both Pozner and Gomes et al., showed this in human cell lines. To support Pozner findings we could also see cleaved products of MET in nuclear fractions of AML12 cells which is a mouse cell line on Western blot (Figure 22). We were more interested to show this phenomenon in mouse cells. This would lead us to study our WAPT mouse model in greater detail with context to the involvement of MET during tumorigenesis where MET amplification was already revealed in this mouse model. We wanted to further analyze using molecular biology techniques to find any evidences for the existence nuclear MET.

1.7.4 5`and 3´ RACE PCR for MET transcript

Four MET transcripts of different length have indeed been detected in different cell lines (Giordano et al., 1989; Park et al., 1986). Hence we wanted to test whether any shorter form of transcripts are present. To test this we selected the WAPT1#08 P40 tumor mammary epithelial cells (TMECs) which showed exclusive nuclear MET. RACE (rapid amplification of cDNA ends)-PCR was performed to identify the 5' and 3' end sequences of MET. RACE-PCR is a technique to obtain the 5' and 3' end sequences and also full length sequence of an RNA transcript found within a cell. The 5' and 3' RACE-PCR products were as expected indicating no shorter isoforms. (Figure 23)

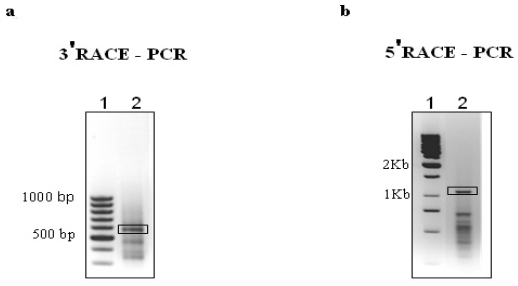


Figure 23: Agarose gel electrophoresis of 3' and 5' RACE PCR products; After reverse transcribing with oligo (dT) primer to obtain cDNA, PCR was performed with oligonucleotide PCR-CDS and MET specific primer for 5' RACE PCR; and with oligonucleotide Mbo20 and MET specific primer for 3' RACE PCR. After PCR, 5µl aliquots were mixed with sample buffer and applied to a 1% (w/v) agarose gel and subjected to electrophoresis.

Truncated and alternative spliced forms of growth factor receptors in several cells have been described. It has been identified that two distinct C-terminal truncated forms of the protein is encoded by the MET proto-oncogene. These truncated forms are detectable in the GTL-16 human gastric carcinoma cell line, in which the *MET* gene is amplified and over expressed (Giordano et al., 1988), and also in other carcinoma cell lines with normal levels of MET expression. In mouse, there is an alternatively spliced variant transcript that has a 141-base pair deletion with an in-frame deletion of 47 amino acids in the JM domain (Lee and Yamada, 1994). Hence we wanted to check whether there are any alternative spliced variants of MET present in TMECs of later passages which showed exclusive nuclear MET by immunofluorescence staining.

We wanted to check whether there are any alternative spliced variants of MET present in different passage numbers of TMECs. 19 pairs of primers were designed encompassing the full length transcript of MET (Figure 24). The primers were designed in such a way that they would overlap and the PCR product size was restricted to less than 500bp so that any splice variants would be seen visually on agarose gel. The 19 contig are represented in alphabetical order A to S.

cDNA was prepared taking equal volume RNA (1µg) from mKSA cell line (positive control) and TMECs from WAPT1 # 08 mice from passage number 0, 1, 3 , 5 and 40 represented as P0, P1, P3, P5 and P40 respectively. PCR didn't reveal the presence of any alternative spliced forms or truncated forms of MET. All the contig were present, but the amount of transcript decreases gradually from passage to passage showing high level in P0 and low in P40 (Figure 25).

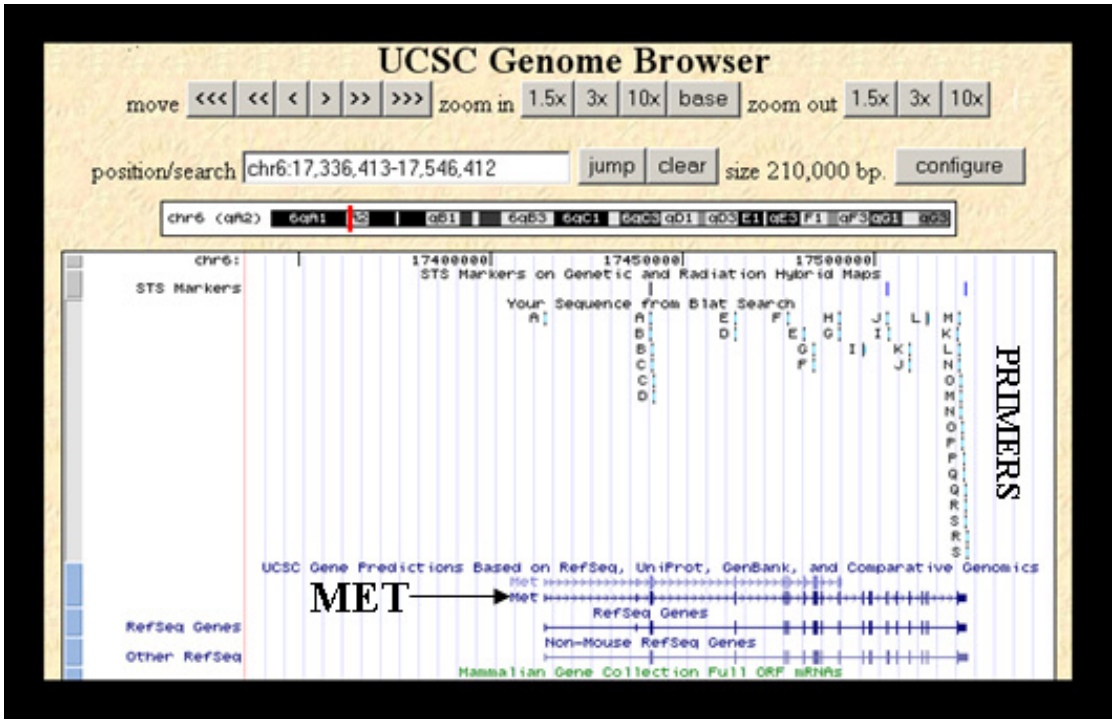


Figure 24: Graphical representation showing the localisation of 19 contig (A-S) encompassing whole MET mRNA transcript using UCSC Genome browser tool.

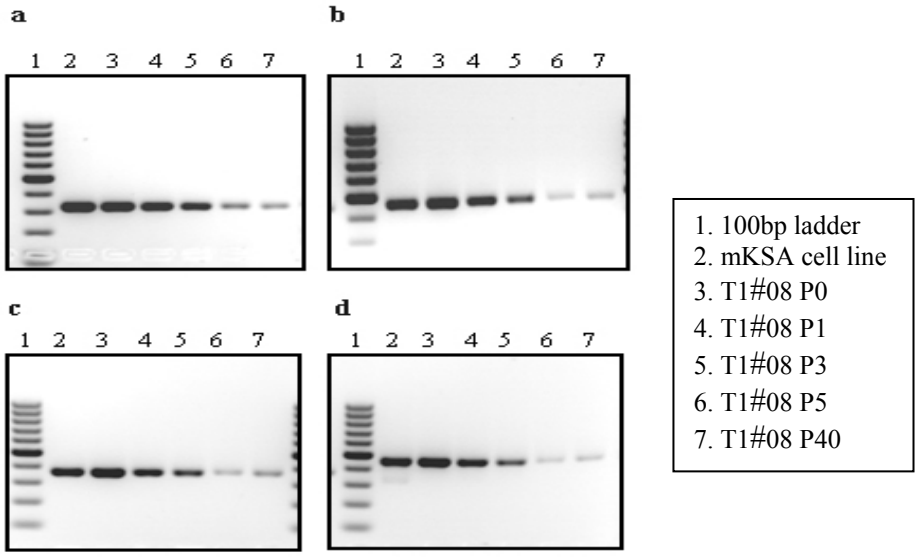


Figure 25: Representative gels of PCR products of the C, E, H, I contig indicating the gradual decrease in mRNA transcript level of MET gene in WAPT1# 08 TMECs; After reverse transcribing with oligo(dT) primer to obtain cDNA, PCR was performed with oligonucleotide pairs encompassing whole MET gene. After PCR, 5µl aliquots were mixed with sample buffer and applied to a 1% (w/v) agarose gel and subjected to electrophoresis. MET expression gradually decreases, showing high expression in earlier passages and low expression at later passages. mKSA cell line was used as positive control.

From the above two molecular biology experiments we could conclude that neither variation in transcript length nor the existence of spliced forms of MET existed in WAPT1# 08 TMECs.

1.8 Knockdown of MET using shRNA to investigate the specificity of antibodies

To investigate the specificity of the antibodies we decided to knockdown the expression of MET in TMECs from WAP-T1#03-P82 cells which exhibited exclusively nuclear MET using lentivirus based MET specific shRNA transduction. A small or short hairpin RNA (shRNA) is a sequence of RNA expressed from DNA template and processed into small RNAs to guide RNAi-mediated target mRNA degradation. Four plasmid constructs containing a shRNA sequence cloned into the lentiviral vector pGIPZ were purchased from OpenBiosystems Company. The sequence of shRNA is as follows

shMET 11 – 1955bp 5' CCTATGTAGATCCTGTAATAA 3' -1995 bp
shMET 26 – 975bp - 5' GGCCAATCTTGCTAAGCAAAT 3' - 995 bp
shMET 70 – 4053bp 5' CGCTACTTATGTGAATGTAAA 3' - 4073 bp
shMET 87 – 2847bp 5' GCTCTTCCTGTGGATGAGAAA 3' - 2867 bp

Recombinant lentiviruses were produced by co-transfecting 293T cells with the lentivirus expression plasmid and packaging plasmids using the Polyethylenimine transfection method. After transfection the medium was changed after 6 hours. Then viral particles were harvested, filtered, and concentrated at 24 and 48 hours time intervals after transfection. The infectious titer was determined by infecting 293T cells. WAP-T1#03-P82 cells which showed exclusively nuclear MET were selected for infection with lentivirus. The cells were infected twice at 24 and 48 hours. After 48 to 72 hours post infection, the cells were subjected for antibiotic selection with QEM containing 2µg/ml puromycin to establish a stable cell line expressing shRNA. The cells were subjected for Fluorescence Activated Cell Sorting (FACS) for Enhanced Green Fluorescent Protein (eGFP) expression. About 95% cells were positive for eGFP. RNA was isolated from these cells to prepare cDNA for Quantitative Real-Time PCR (qRT-PCR) and the total cell lysate was also prepared for Western blot analysis.

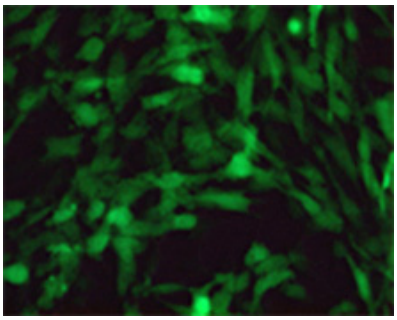


Figure 26: WAP-T1#03-P82 cells expressing eGFP infected with MET shRNA.

Western blotting with total lysates from FACS sorted WAP-T1#03-P82 using MET-B2 antibody showed a significant knockdown of MET with shMET 70 construct. Other 3 shRNA constructs showed moderate to weak knockdown of total MET protein expression (Figure 27). qRT-PCR showed up to 90% down regulation of MET transcript (Figure 28). When probed with C28 and N16 antibodies which showed the cleaved forms of MET in Western blotting (Figure 22) we couldn't see any down regulation with any of the constructs doubting the specificity of these antibodies.

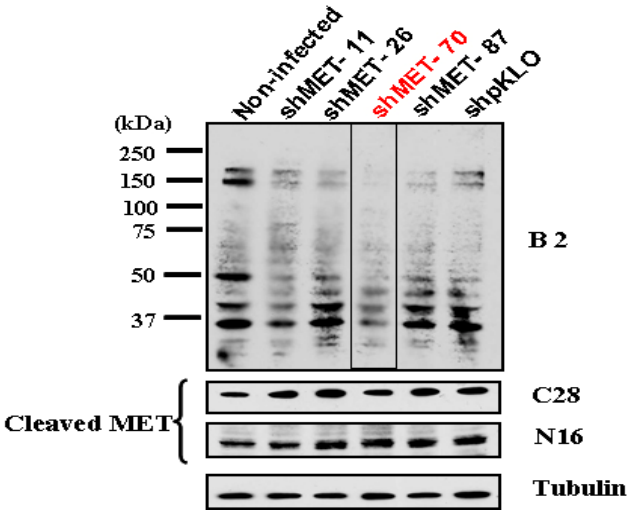


Figure 27: Western blot analysis of total cell lysates of WAPT1#03 P82 cells transduced with MET shRNA; 50µg of total lysate was loaded and probed using MET-B2, C28, N16 and Tubulin antibodies. Down regulation of MET expression is observed only for the full length 140kDa and partially for 50kDa product. No down regulation is seen for the cleaved forms of MET when probed with C28 and N16 antibodies doubting the specificity of these antibodies. shpKLO vector was used as mock and Tubulin was used as internal control.

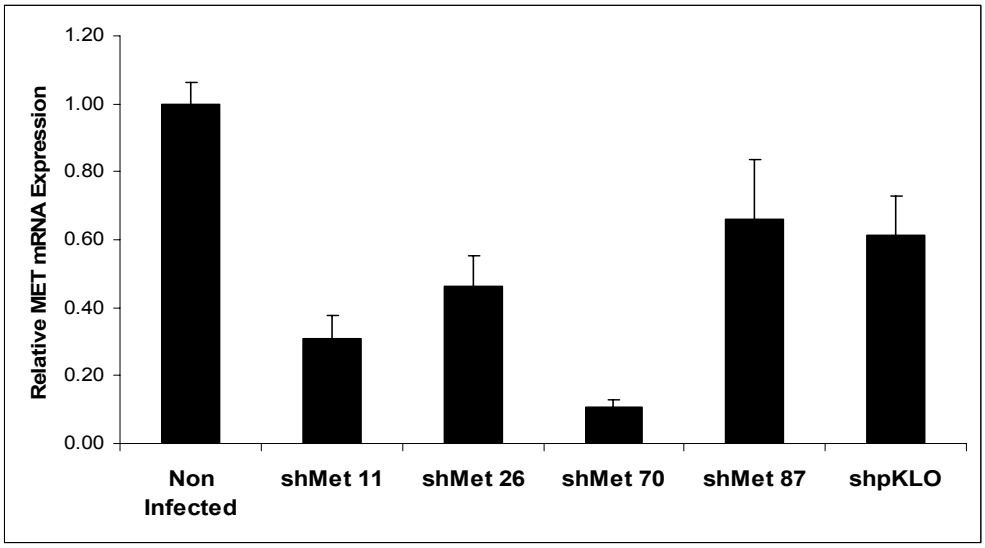


Figure 28: Validation of shRNA lentiviral system; Total RNA extracted from WAPT1#03 P82 cells transduced with different shRNA constructs was subjected to qRT-PCR using MET specific primers and normalized against actin RNA. The bar graph represents the down regulation of MET expression up to 90% using the shMET 70 construct.

Immunofluorescence staining for MET was performed for WAP-T1#03 P82 stable cell line expressing shMET 70, using MET B2 and MET C28 antibody (Figure 29) to confirm the down regulation of MET. The results were contradicting since there was no appreciable reduction of MET expression by immunofluorescence staining method especially with C28 antibody immunofluorescence staining (Fig 29c, 29d) where as Western blotting (Figure 27) and qRT-PCR (Figure 28) showed significant knockdown of full length MET expression with B2 antibody. This led us to question the specificity of antibodies. Therefore, we decided to raise our own custom antibody against MET.

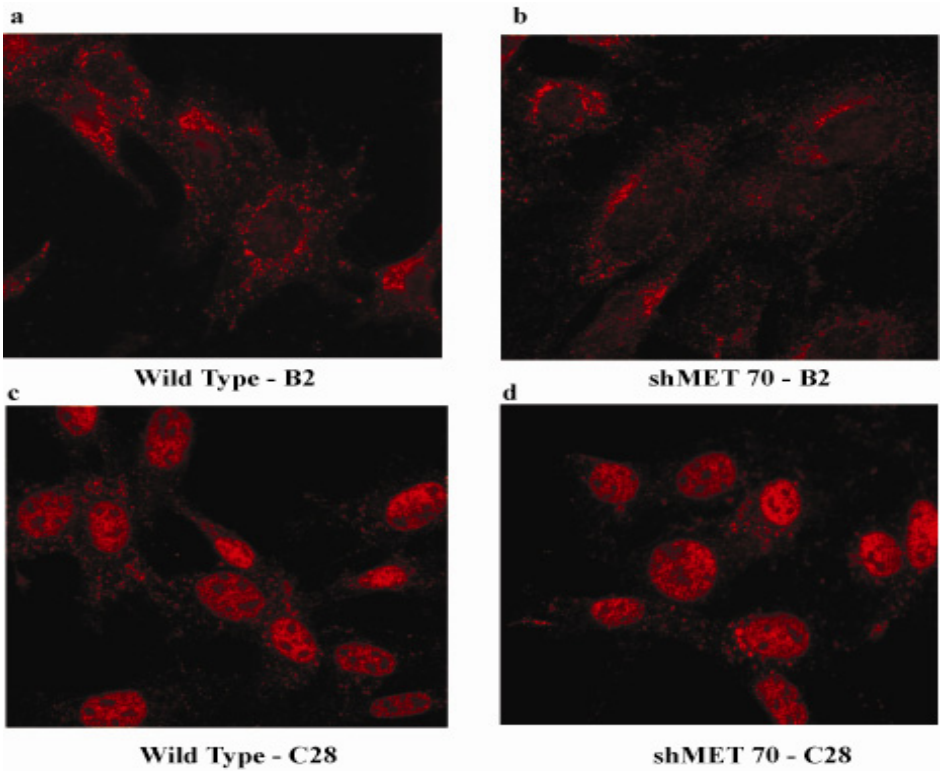


Figure 29: Immunofluorescence staining of MET knockdown cells of WAP-T1#03-P82 stable cell line with shMET-70; Staining was performed using MET B2 and MET C28 antibodies. No difference seen between wild type and knockdown cells, particularly using the MET C28 antibody.

1.9 Raising custom polyclonal antibodies against MET

Testing with above commercial antibodies lead us to more ambiguous results for the confirmation of nuclear MET. Hence we decided to raise custom polyclonal antibodies against MET using rabbit as host animal species. Designing of peptides, synthesis of peptides, immunising, raising of antibodies, bleeding, and affinity purification of antibodies was outsourced to Eurogentec Company.

Two peptides N1 & N2 for N termini and two peptides C1 & C2 for C termini of β chain of MET were designed using peptide designing software. The localisation of peptide sequences are depicted in the cartoon below (Figure30). The sequence of peptides designed is mentioned below.

a) QSKPDSAEPVNRSAVC – N 1
b) RNSSGCEARSDEYRTE – N 2
c) CWHPKAEMRPSFSE – C 1
d) SLLPSQDNIDGEGNT – C2

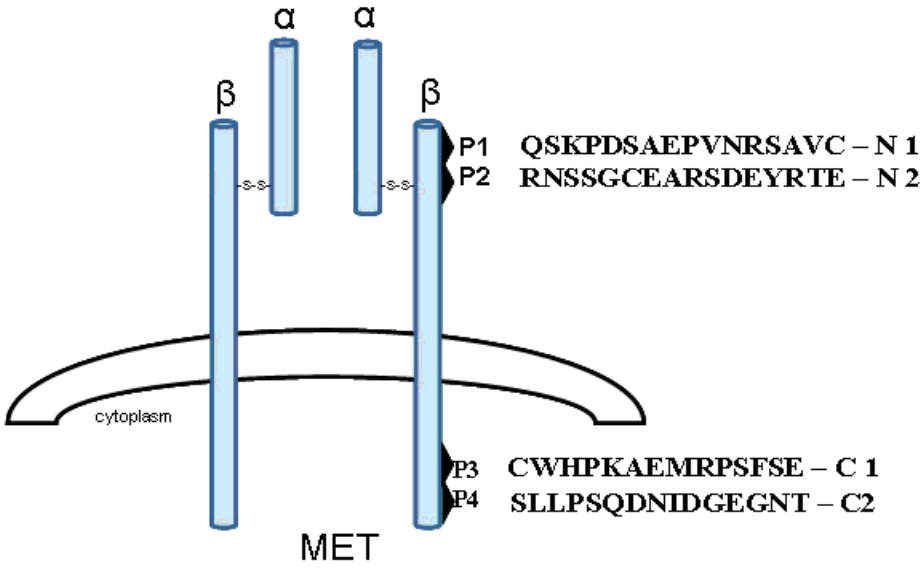


Figure 30: Cartoon depicting the epitopes in the β chain of MET; N1 and N2 were designed to recognise the N-terminus of MET and C1 and C2 to recognise the C-terminus of MET.

Two rabbits were immunised with a mixture of peptides N1 & N2 and two rabbits were immunised with a mixture of C1& C2 peptides, respectively. The antibodies were named as N1, N2, C1 and C2. Western blotting performed using the final bleed from the animals resulted in many non-specific bands. The affinity purified antibodies were obtained after passing the serum through affinity columns carrying each individual peptide namely N1, N2, C1 and C2. The antibodies were diluted to have a final concentration 200µg/ml and tested by Western blotting analysis.

1.10 Failure of customised antibodies

According to the standard procedures while raising customised antibodies the first bleed and final bleed after immunisation were collected after the respective time points and used for detection of MET by Western blotting. Jurkat cells were used as negative control for MET and mKSA cell line was used as positive control to test the antibodies. The first bleed and the final bleed showed a lot of non-specific signals in mKSA cell lysate and Jurkat cell lysate too. The affinity purified antibodies were tested in various dilution series like 1:50, 1:100, 1:300, and 1:500 for Western blotting. For N1, N2 and C2 no dilution series worked efficiently. A dilution range of 1:300 was optimal without too many non-specific signals for C1 antibody (Figure 31) but not satisfactorily since there were signals from Jurkat cell lysate too which is MET negative. The other antibodies like N1 and N2 showed only non-specific bands, and C2 antibody did not give any signal.

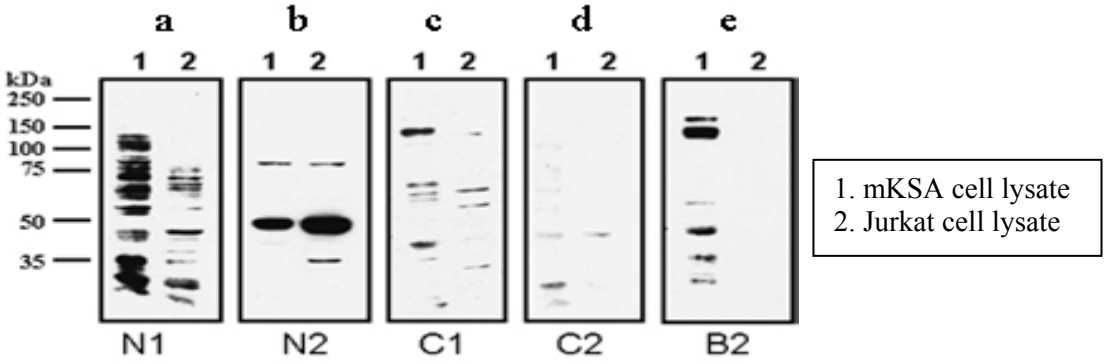


Figure 31: Western blotting using custom antibodies; a) N1 antibody, b) N2 antibody, c) C1 antibody, d) C2 antibody, e) B2 antibody used as positive control. 50µg of total cell lysate was loaded onto each lane. mKSA cell lysate was used as positive control and Jurkat cell lysate as negative control.

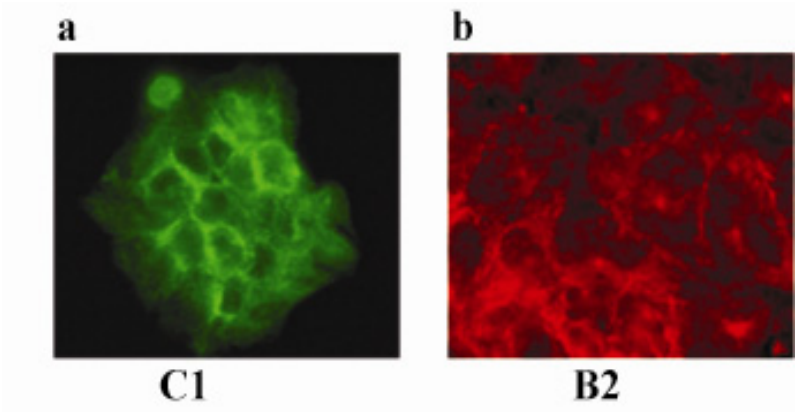


Figure 32: Immunofluorescence staining of MET in mKSA cells using the C1 and B2 antibodies: The cells were fixed with 4% PFA and stained for expression of MET using C1 antibody (a) and B2 antibody (b). The green fluorescence shows membranous, but no any nuclear expression of MET using the C1 antibody. The red fluorescence represents membranous expression of MET using the B2 antibody.

Our main goal for raising custom antibodies was to prove the existence of nuclear MET. Hence, we required an antibody which works well both in Western blotting as well as in immunofluorescence staining. Since only the C1 antibody worked to some extent in Western blotting, immunofluorescence staining was performed using this antibody on mKSA cells. The C1 antibody showed good membranous staining (Figure 32a), like the B2 antibody (Figure 32b) and no nuclear staining. Through this series of experiments it was not possible to prove the existence of nuclear MET in mKSA or in any mouse cell lines.

IV.2 Role of MET in SV40-induced mammary tumorigenesis

IV.2.1 Expression of T-Ag correlates with expression of MET

Various mouse models of mammary tumorigenesis have been generated till date using SV40 as an oncogene to initiate tumorigenesis (see Table 1). These models have led us to understand the molecular events of tumorigenesis in greater details. The very important features to use these models are like they are reproducible *in vivo* systems which mimic the human pathology. Hence they provide us with unique opportunities to study multi-step carcinogenesis and the biology of tumor progression, from the initiation of dysplasia through the development of invasive and metastatic carcinoma. The key feature of SV40 is to functionally inactivate the tumor suppressor gene p53 and Rb (DeCaprio et al., 1988; Dyson et al., 1989b; Levine, 1990) which are frequently

inactivated in human breast cancer (Cox et al., 1994; Osborne et al., 1991) Hence the inactivation of these tumor suppressor genes leads to increased proliferation by way of defective cell cycle regulation and impaired activation of apoptotic pathways. During this process many genes do get up regulated. One of main event that can occur at this stage is gene amplification. It's a selective increase in the number of copies of a gene coding for a specific protein without a proportional increase in other genes. Several cancer genes do undergo this change. As mentioned earlier, our lab has generated transgenic WAP-T mouse model in which *MET* gene was over expressed and amplified in undifferentiated tumors. Cell lines can be derived from transgenic models which can mimic tumorigenesis and thus can provide convenient and reproducible *in vitro* systems. Likewise our laboratory also generated a cell line from WAP-T mouse tumor and this cell line was named as G2 cell line. This is an immortal mammary epithelial cell line with cancer stem cell properties.

We performed co-staining for T-Ag and MET in G2 cell line. This cell line expresses T-Ag and also MET. We could observe that there was direct correlation between T-Ag and MET. In cell culture, like in G2-derived tumors, T-Ag showed heterogeneous expression levels. Interestingly, MET expression levels were directly proportional to T-Ag expressions levels in these cells (Figure 33). This provided us with a hint indicating a role for T-Ag in the regulation MET expression.

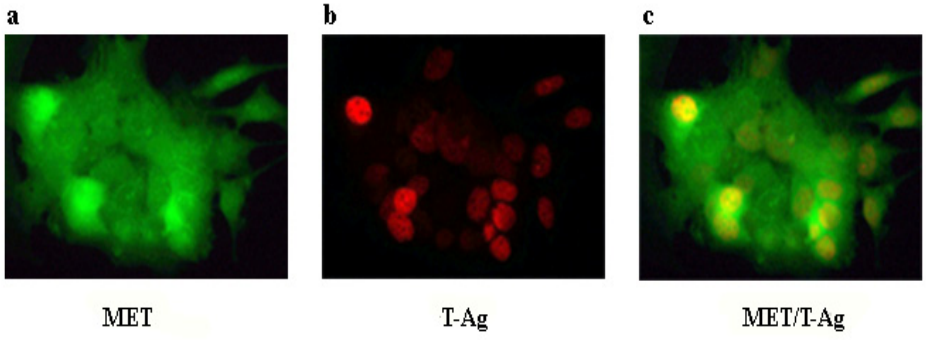


Figure 33: Immunofluorescence staining of MET and SV40 T-Ag in G2 cells; the cells were fixed with 4% PFA and stained for the expression of MET and T-Ag. The green fluorescence represents the expression of MET (a) and red fluorescence represents the expression of T-Ag (b). We can observe that cells strongly expressing T-Ag exhibit a higher expression of MET.

IV.2.2 Expression analysis of MET in different cell lines

The correlation between T-Ag and MET expression in G2 cells was an interesting phenomenon. Hence we were interested to investigate T-Ag and MET expression levels in different cell lines with or without T-Ag. Dr. Hermannstädter from our laboratory established cell lines stably expressing Large T-antigen (LT) in 3T3 and 10-1 cells through retroviral transduction. The cells expressing LT were named 3T3 LT and 10-1 LT cells along with control vector-transduced cells. cDNA was prepared taking equal volume RNA (1µg) from all the cell lines and qRT-PCR was performed.
The cell lines subjected for MET expression are as follows.

1. BALB/c 10-1 (10-1)
2. BALB/c 10-1 LT (10-1 LT)
3. BALB/c 3T3 (3T3)
4. BALB/c 3T3 LT (3T3 LT)
5. G2
6. mKSA
7. SV3T3

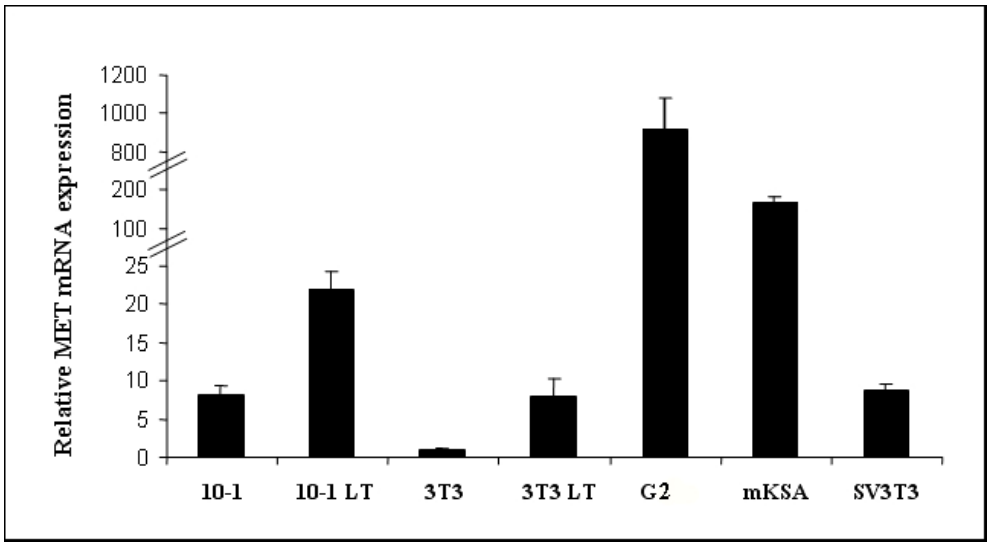


Figure 34: MET mRNA-expression in different cell lines; Expression levels were determined using total RNA from cells with MET specific primers for qRT-PCR and normalized against actin RNA. A direct correlation was found between LT and MET. Cells without T-Ag express

less MET (10-1 & 3T3) and cells with T-Ag express high amounts of MET (10-1 LT & 3T3 LT). G2 and mKSA cells express very high amounts of MET.

Expression profiling of different cell lines for MET expression with or without T-Ag strongly indicated that MET is regulated by T-Ag (Figure 34); compare 10-1 vs10-1 LT, 3T3 v/s 3T3 LT. A correlation for MET expression was found in presence of T-Ag as cells with T-Ag expressed more MET compared to the cells lacking T-Ag. A striking example was 3T3 cells with and without LT and also 10-1 cells with and without LT (Figure 35).

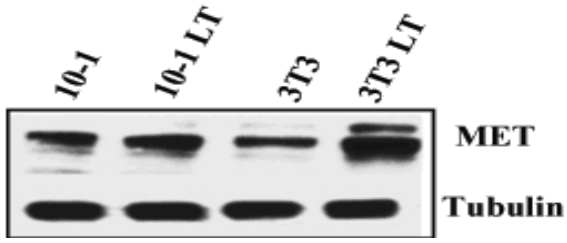


Figure 35: MET expression in BALB/c derived fibroblasts; Western blotting analysis for the expression MET in mouse fibroblasts after transducing with LT. Equal volume (50 µg) of total lysates were loaded and probed with MET-B2 antibody. Cells with LT shows increased expression of MET indicating the role of LT for increased expression of MET protein. Tubulin used as internal control.

In the case of 3T3 cell, the cells with LT (3T3 LT) showed an 8 fold increase in MET expression when compared to 3T3 cells lacking LT (Figure 34 compare bar 3 & 4). Differential expression was also seen in 10-1 cell line, but the cells with LT (10-1 LT) only showed a 3-fold increase in MET expression when compared to 10-1 cells lacking LT (Figure 34 compare bar 1 & 2).

The commonality between 3T3 and 10-1 cells are, they both are fibroblast cell lines derived from the same background BALB/c mouse in other words isogenic. The difference is in the status of p53. 3T3 cells have wtp53 and 10-1 cells are null for wtp53. Hence the MET expression level can be attributed to p53 status. This finding arise the question whether p53 as a role in MET expression.

IV.2.3 p53 acts as repressor of MET

Investigation of *MET* gene expression in the BALB/c derived fibroblast cells lines 3T3 and 10-1 cells which differ in p53 gene status ($p53^{+/+}$ and $p53^{-/-}$) with or without LT revealed the importance of p53 for *MET* gene expression. Firstly if we compare cell lines with p53 (3T3) and without p53 (10-1) we can see an 8 fold increase in 10-1 cells which lack p53 when compared to 3T3 cells which posses p53 (Figure 34 bar 1 and 3). When LT was transduced the proportional increase in MET expression is much less in 10-1 LT compared to 3T3 LT cells (Figure 34 compare bar 1 & 2 and bar 2 & 4; Figure 35). In presence of p53, LT transduced cells showed an 8 fold increase in MET expression (Figure 36, bar 3 and 4 and also Figure 34). The same phenomenon was observed in 10-1 cells also. 10-1 cells when transduced with LT showed 2 ½ fold increased MET expression (Figure 34, first 2 bars from left and Figure 35). So if we compare the total expression level of MET in 10-1 LT and 3T3 LT we can see that 2 ½ fold increase of MET expression is observed in 10-1 LT cells (Fig 34 bar 2 and 4). This can be directly attributed to the p53 level. From the literature we know that T-Ag targets the core domain of p53 and leads to its functional inactivation. Given this explanation, we could observe that in presence of p53 (3T3 cells) the proportional increase of MET expression was higher (8 fold) than in the absence of p53 (10-1 cells) which was 2 ½ fold (Figure first 4 bars from left). Comparing the levels of MET expression in 10-1, 3T3, 3T3 LT and SV3T3 cells we could further highlight the role of p53 playing as a repressor of MET. p53 downregulates MET expression, whereas absence or inactivation of p53 by T-Ag leads to transcriptional inactivation of p53 by complex formation leading to higher expression of MET (Fig 36).

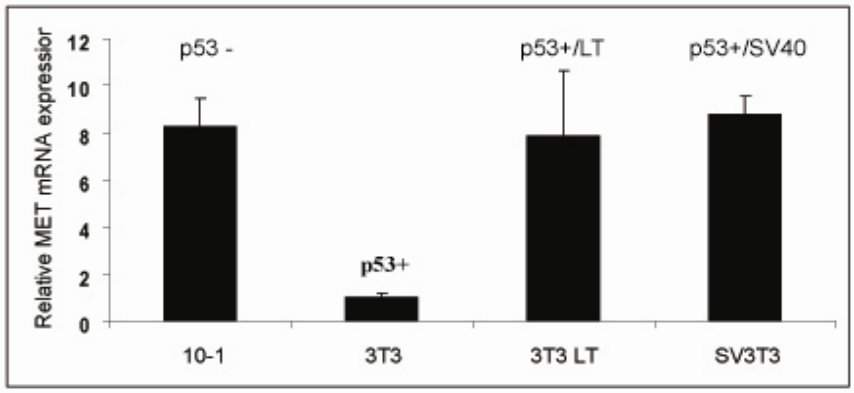


Figure 36: Downregulation of MET in the presence of wild type p53; mRNA-expression in BALB/c derived fibroblasts with and without p53 was determined using total RNA from cells with MET specific primer for qRT-PCR and normalized against actin RNA. 3T3 cells with wild type p53 show a decreased expression of MET, while cells, where p53 is absent (10-1) or inactivated (3T3 LT & SV3T3) show an increased expression of MET indicating wtp53 down regulates MET expression.

IV.2.4 wtp53 is responsible for down regulation of MET but not mutp53

The next question we wanted to ask was, whether mutp53 can also regulate MET expression. To answer this question we chose the 10-1cG9 cell line. 10-1cG9 cells express a temperature-sensitive p53 that is wild-type at 32°C and mutant conformation at 39°C respectively in the background of p53-negative 10-1 cells. cDNA was prepared taking equal volume RNA (1µg) from cells grown at 32°C and 39°C. qRT-PCR was performed to estimate *MET* mRNA expression. We observed a 3-fold increased expression of MET when p53 was in a mutant conformation suggesting that wtp53 is responsible for repression of the *MET* gene (Fig 37a). The qRT-PCR result was confirmed by Western blot analysis (Fig 37b). The data further support the postulated p53-dependent repression of the *MET* gene.

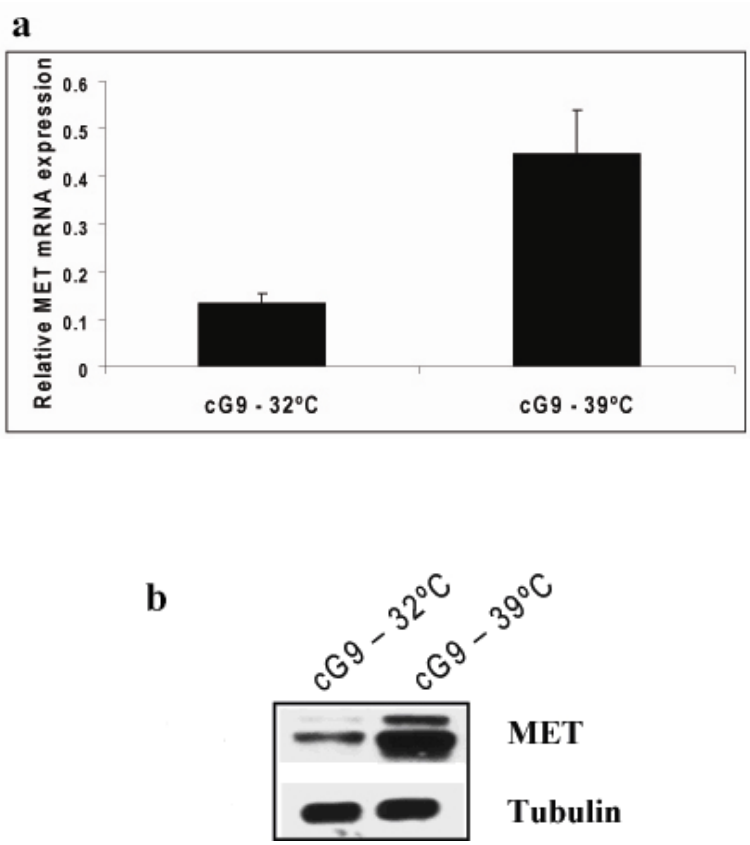


Figure 37: Wild type p53, but not mutp53 acts as *MET* repressor; 10-1cG9 cells which express a temperature-sensitive p53 that is wild-type at 32°C and mutant conformation at 39°C, respectively were grown at 32°C and 39°C. a) *MET* expression levels were determined using total RNA from cells with MET specific primers for qRT-PCR and normalized against actin RNA. The bar graph indicates a 3-fold increased expression of MET in cells with mutp53 than wtp53. b) For Western blotting total lysates were prepared from cells grown at 32°C and 39°C. Equal volumes were loaded and probed with MET-B2 antibody. The immunoblot indicates increased expression of the MET protein when cells were grown at 39°C which express mutp53 indicating wtp53 acts as repressor of MET. Tubulin was used as internal control.

IV.2.5 wtp53 binds to the *MET* gene promoter

After several leads suggesting that p53 is responsible for MET regulation we wanted to investigate whether there is a direct physical interaction of p53 with *MET* gene. Therefore, we extended these studies by performing Chromatin Immunoprecipitation (ChIP) Assay. ChIP is an important technique in the study of protein-gene interactions which allows determining the location of DNA binding sites on the genome or a particular protein of interest. Using ChIP, DNA-protein interactions are studied within the context of the cell. The experimental outline is mentioned below (Fig 38).

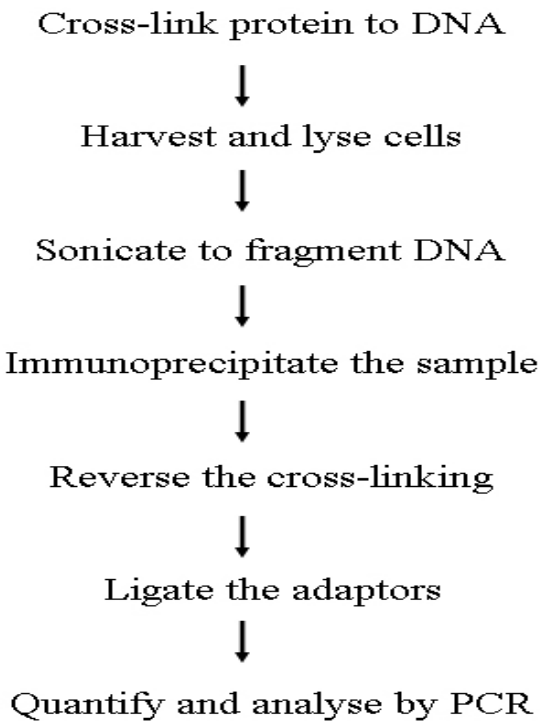


Figure 38: Chromatin Immunoprecipitation protocol outline.

By in-silico analysis using the computer program Pat Search we could identify a putative p53 binding site within region +233 to +264 of the MET promoter with the sequence:

+ 233 ggggaggtgcaaactagaattgagcttgtcg 264+

Different oligos were designed encompassing the whole promoter region upstream from -1390bp to downstream +560bp of *MET* gene.

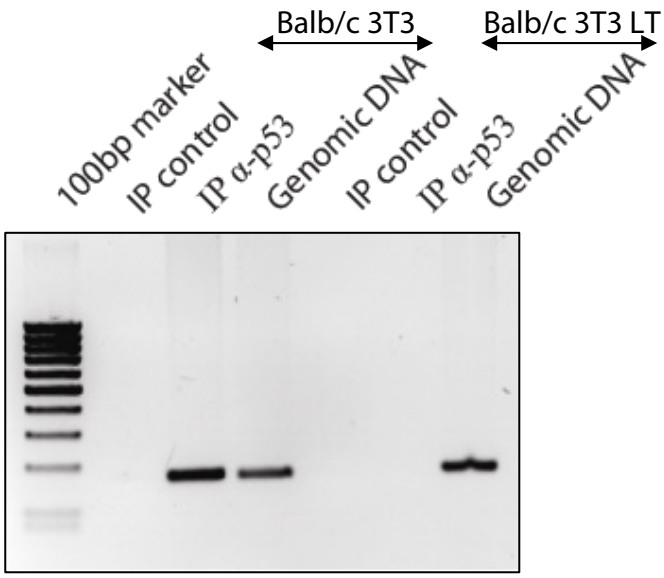


Figure 39: p53 binds to the *MET* promoter; Chromatin immunoprecipitation assay in BALB/c 3T3 and BALB/c 3T3 LT cells. Chromatin complexes were cross linked *in vivo* with formaldehyde. The p53-associated DNA fragments were immunoprecipitated (IP) with goat polyclonal antibody against p53. DNA samples were isolated before IP (lane labelled 'Genomic DNA'), after specific IP (lane labelled 'IP α-p53', for chromatin immunoprecipitation) and negative control where no antibody was added (IP control). PCR was performed with flanking primers amplifying a putative p53 binding site on the *MET* promoter. Clear enrichment is seen in BALB/c 3T3 cells whereas no amplification is seen in BALB/c 3T3 LT cells.

The cell lines of choice to perform ChIP were BALB/c 3T3 and BALB/c 3T3 LT. The cells were grown to 80% confluency. 10^8 cells were taken per experiment. For details see Methods section. The immunoprecipitated DNA was enriched and PCR was performed with *MET* promoter specific primers. Clear enrichment was seen in BALB/c 3T3 cells indicating that p53 binds to the *MET* promoter (Fig 39). ChIP with 3T3 LT cells did not show any binding of p53 in *MET* promoter. As shown in the above figure we were able to detect physical association of p53 with MET.

From the literature we know that LT targets p53 and functionally inactivates p53. This interaction of LT with p53 blocks p53 dependent gene expression. This is due to the fact that LT binding blocks DNA binding by p53, so that it cannot associate with its target promoters. Hence we could see an increased expression MET up to 8 fold in case of BALB/c 3T3 LT cells compared to BALB/c 3T3 cells clearly indicating that p53 negatively regulates MET expression.

IV.2.6 Knockdown of MET in mKSA cells by Lentivirus-delivered small hairpin RNA inhibits growth in soft agar

SV40 is shown to induce transformation of many different types of cultured cells, including both rodent and human cells. From our previous experiments mentioned above we have observed that SV40 T antigen induces MET expression. From the literature we also know that in SV40-positive malignant mesothelioma cells, MET was activated. MET receptor activation was associated with S-phase entry (Cacciotti et al., 2001). Until now this is the only study directly correlating between SV40 and MET. mKSA cell line which is transformed by SV40 is a proven experimental model for murine pulmonary metastasis (Watts et al., 1997). Knowing all these facts of a SV40 transformed cell line such as mKSA, we wanted to test whether knock down of *MET* gene can still have transforming activity. A key feature of transformed cells is their ability to form colonies in soft agar. Hence we wanted to knock down *MET* gene in mKSA cells which expressed MET abundantly and grow them in soft agar to investigate the transforming and invasive features of these cells.

Soft agar assay assesses the ability of cells to grow in the absence of anchorage. Anchorage independent growth is one of the hallmarks of transformation, which is considered the most accurate and stringent *in vitro* assay for detecting malignant transformation of cells. Typically, cells are put in an agarose suspension and the formation of colonies is monitored. The proliferation of cells in this semisolid culture media is monitored for 2-3 weeks before sizable colonies appear. The colonies are counted manually. Standard soft agar assays are usually performed in 100mm or 60 mm dishes. For our experiments we used 60mm dishes.

To perform soft agar assay we selected mKSA cell line. mKSA cell line is mouse kidney epithelial cells which are transformed by SV40. The plasmid construct containing a MET specific shRNA sequence cloned into the lentiviral vector pGIPZ was purchased from OpenBiosystems. This vector features CMV promoter, bicistronic and codes for eGFP via IRES. The sequence of shRNA is as follows

shMET 70 – 4053bp 5' CGCTACTTATGTGAATGTAAA 3' - 4073bp

Lentiviral particles were produced by transfecting 293T cells using Polyethylenimine. Viral particles were harvested, filtered, concentrated and infected repeatedly at 24 and 48 hours time intervals on mKSA cells. Then the cells were subjected to Fluorescence Activated Cell Sorting (FACS) for Enhanced Green Fluorescent Protein (eGFP) expression. 10% cells were positive for eGFP. More than 10^8 cells were subjected to FACS to have enough cells to perform soft agar assay and to isolate RNA for qRT-PCR and also to prepare total cell lysates for Western blot analysis.

Knockdown of MET expression in mKSA cells was successful. Up to 90% downregulation of MET mRNA expression was observed when compared with FACS sorted non-infected mKSA cells (Fig 40a). Western blot analysis was also performed to confirm the loss of MET protein expression (Fig 40b).

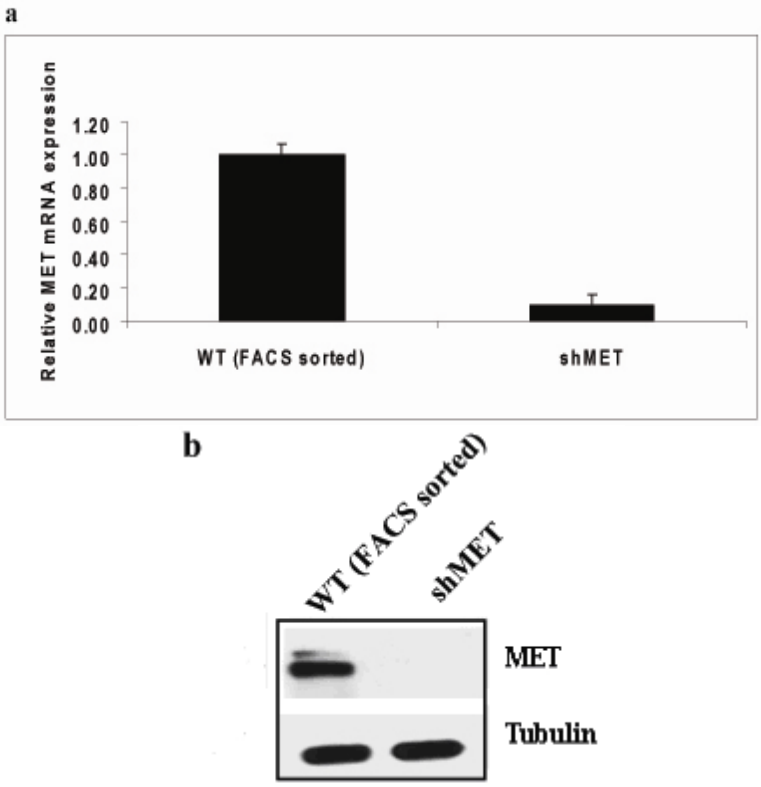


Figure 40: Knockdown of MET in mKSA cells; Validation of lentivirus delivered MET shRNA for silencing MET expression. RNA and total cell lysates were prepared from FACS sorted mKSA cells transduced with MET shRNA and non transduced wild type cells. a) Expression level was determined using total RNA from cells with MET specific primers for qRT-PCR and normalized against actin RNA. The bar graph shows a 90% decreased expression of MET in mKSA cells transduced with MET shRNA when compared wild type cells. b) For Western blotting equal volume (50 µg) of lysate was loaded and probed with MET-B2 antibody. The immunoblot indicates no expression of MET in mKSA cells transduced with MET shRNA. Tubulin was used as internal control.

After knocking down *MET* gene in mKSA cells these cells were subjected to soft agar assay. The detailed protocol for soft agar assay is mentioned in Methods section. 10^4 cells were plated in 5cm Petri dishes. The experiment was done in quadruplicates. As positive control non-infected cells were subjected to FACS sorting and then subjected to soft agar assay. From the same cells RNA for qRT-PCR and total cell lysate for Western blot analysis was prepared.

mKSA cells in which MET expression was silenced lost their capacity to grow in anchorage-independent manner in soft agar. The non-infected wild type cells formed colonies (Fig 41). These results clearly demonstrate the role of MET in SV40 induced cell transformation.

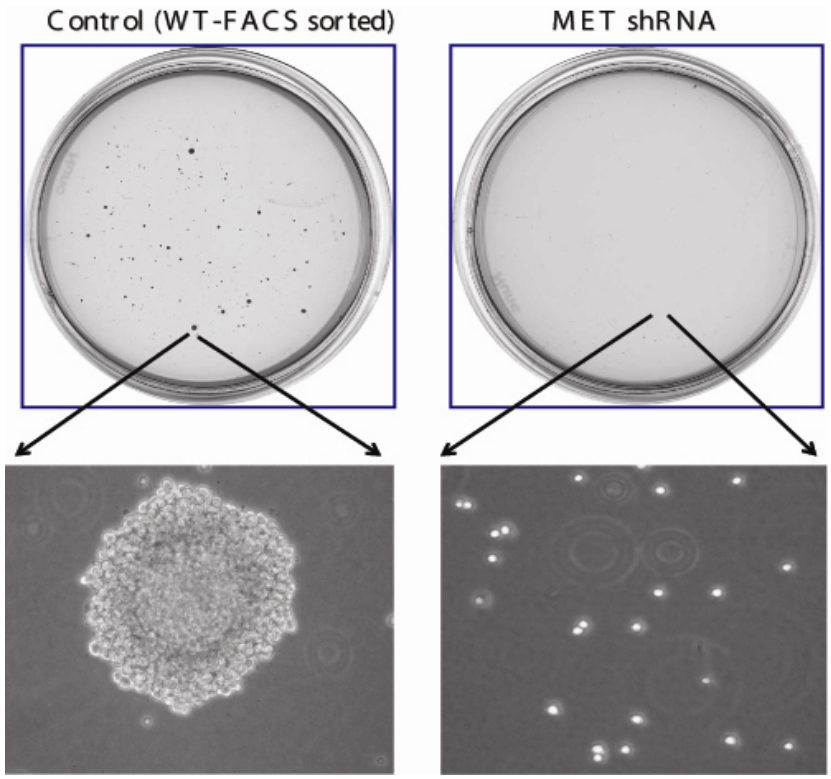


Figure 41: MET knockdown results in impairment of cell ability to grow in an anchorage-independent manner; mKSA cells were transduced with lentivirus encoding MET shRNA. The transduced cells were subjected to FACS and then subjected to a soft agar assay. 10^4 cells were plated in 5cm Petri dishes. As positive control non-infected wild type cells were subjected to FACS and plated just like the transduced cells. Cells were grown in soft agar for 2 weeks and then stained with 0.005% crystal violet dye. The colonies were photographed and the plates were scanned. The lower panel shows the grown colony and cells photographed with 10X magnification; the upper panel shows the scanned Petri plate.

V. DISCUSSION

V.1 Inactivation of WAP promoter in cell culture conditions

The role of WAP has been described previously for the generation of milk in mouse, rat, rabbit, camel, pig (Baranyi et al., 1995; Beg et al., 1986; Hennighausen and Sippel, 1982; Hobbs et al., 1982; Simpson et al., 1998). WAP-T mice generated in our lab carry the SV40 early gene region under the control of the murine *WAP* promoter. The *WAP* promoter is hormonally and developmentally regulated by lactotrophic hormones like estrogen, prolactin, hydrocortisone, insulin (Burdon et al., 1991; Pittius et al., 1988). The expression of the transgene T-Ag can be induced by mating and is directed to epithelial cells of terminal ductal lobular units like mammary epithelial cells of the differentiating and lactating gland. Hence, the *WAP* promoter has been successfully used for the generation of mouse models for breast cancer (Husler et al., 1998; Tzeng et al., 1993).

WAP gene expression is understood to be limited to mammary-gland epithelial cells and its regulation is tightly controlled by complex interactions between lactogenic hormones, extracellular matrix and intercellular interactions (Hobbs et al., 1982). WAP-T1 mouse model generated in our lab was found to be a good mouse model for analysing mammary carcinogenesis. In spite of having a successive *in vivo* WAP based mouse models it still remains a challenging project to develop an *in vitro* WAP induced primary culture cell lines derived from the WAP animal model. WAP induced cell lines have been established by cloning WAP promoter and expressing in established cell lines like T47D a human mammary carcinoma cell line (Lipnik et al., 2005). Our primary goal was to establish a cell culture model to understand SV40 induced tumorigenesis where the expression of T antigen driven by *WAP* promoter could be turned on and off using lactotrophic hormones in primary mammary epithelial cells from WAPT mouse tumors. We made a modest attempt to activate WAP expression in primary cell culture systems. Various media like RPMI, DMEM, DME F12, and QEM were used. QEM with 5 % FCS was standard medium for primary mammary epithelial cells because it yielded the best results. Silencing of the *WAP* promoter was initiated by the shut down of transcription of the *WAP* gene itself. No mRNA transcript was found after 12 hours post plating of fresh tumor mammary epithelial cells and also in explants plated with matrigel embedment (Figure 8, 9). Inactivation of the *WAP* promoter in cell culture

conditions can be attributed to many reasons. Given the complexity of the regulation of the *WAP* promoter and its tight regulation, activation of WAP may not work under *in vitro* conditions. *WAP* gene expression also depends mainly on the synergistic action of prolactin and glucocorticoids. The microenvironment that exists *in vivo* is difficult to recapitulate in cell culture conditions even though attempts were made to grow the cells in 3D cultures to recapitulate the *in vivo* scenario and supplementing with lactotrophic hormones like insulin, prolactin, β estradiol, hydrocortisone and dexamethasone for induction. Since our transgene in under control WAP promoter silencing of WAP lead to inactivation of T-Ag. Nevertheless, the expression of T-Ag was observed in few cells from the primary culture preparation from mice numbered WAP-T1#55, WAP-T1#88, WAP-T1#50, WAP-T1#81, and WAP-T1 #100 in few passages. This may be attributed to the fact that T-Ag is a stable protein and we thus could observe the expression of T-Ag in few cells for two passages. To summarise our attempts to activate WAP in primary mammary epithelial cells from WAPT1 mouse tumors thereby to establish a cell culture model to understand SV40 induced tumorigenesis failed. Tzeng et al also tried to establish T antigen positive primary mammary epithelial cell culture from their WAP-SV-T/t mouse model. They noticed that all T/t-antigen positive mammary epithelial cells exhibited a spontaneous apoptosis in cell culture (Tzeng et al., 1998).The exact mechanism by which WAP inhibitory factor shuts off WAP mRNA synthesis or accumulation require additional studies.

V.2 Subcellular location of MET is membranous

In case of multi-cellular organisms, individual cells are often linked with each other via cell-cell contact to form three-dimensionally structured tissues or organs. Various proteins are involved in the formation of these tight and compact cell-cell adhesions, suppressing free cell movement. Some proteins are attached only to the membrane surface, while others have one region buried within the membrane and domains on one or both sides of it. Cell-cell signaling or interactions are generally carried out by the protein domains which are bound on these extracellular membrane surfaces. Domains lying along the cytosolic face of the membrane have a wide range of functions, from anchoring cytoskeletal proteins to the membrane to triggering intracellular signaling pathways. Membrane proteins are classified into two classes viz., integral and

peripheral based on the nature of the membrane-protein interactions. Integral proteins have one or more segments that are embedded in the phospholipid bilayer. Most of the integral proteins span the entire phospholipid bilayer. These transmembrane proteins contain one or more membrane-spanning domains as well as domains, from four to several hundred residues long, extending into the aqueous medium on each side of the bilayer. These cell-cell contacts play a vital role in intracellular signaling, although the molecular mechanisms of these signaling pathways are not fully understood. MET is an integral protein which is disulfide linked α-β heterodimeric receptor tyrosine kinase. Its 50 kDa α-subunit is completely extracellular. The large part of 140 kDa β-subunit spans externally and its tyrosine kinase domain lies internally.

One of the standard techniques for localisation of proteins, immunofluorescence staining, was performed to localise MET protein in primary WAPT1 mouse tumor mammary epithelial cells in culture, and in established cell lines like A431, mKSA, AML12 and G2. The availability of several monoclonal and polyclonal antibodies against MET gave us the choice to test with more than one antibody. MET is known to be a membranous protein. The immunofluorescence staining pattern of WAPT1#75 tumorigenic mouse primary mammary epithelial cells by polyclonal C28 antibody (Figure 11) was intriguing, as it indicates that MET might be translocated from the cytoplasmic membrane to the nucleus during passage zero to five. This observation raised the question, whether MET is also a member of the growing list of membrane proteins like ErB4, Notch, APP, E-Cadherin, and so on, which were known as membrane proteins, but get translocated into nucleus and play role in gene expression. Use of more than one antibody to confirm the localisation of MET in the nucleus in different cell lines like A431, AML12, mKSA provided more confusion than clarity. The results of immunofluorescence staining of A431, a human cell line in which MET is expressed highly, suggested that MET is localised both in cytoplasm and nucleus when stained with B2, CVD13, N16 antibodies (Figure 12, 16, 18) and only in nucleus with C28 antibody (Figure 14) which was contradictory when compared to antibody datasheet since MET was shown to localize only in cytoplasm. But Western blotting results differed from immunofluorescence staining results. The B2 antibody in Western blotting failed to show the presence of MET in either cytoplasmic or nuclear fraction, whereas antibodies C28, N16 and CVD13 confirmed the presence of MET in both nuclear and cytoplasmic fractions. We could also see cleaved forms of MET in nuclear fractions when probed with C28, CVD13 and N16 antibodies (Figure 22a).The possible

explanation may be that B2, a monoclonal antibody raised against mouse MET which according to manufacturer's guidelines, can also detect to a lesser extent human MET, but was not able to detect SDS denatured human MET protein in Western blotting. Hence in a human cell like A431 existence of nuclear MET was observed.

The results were indifferent when we tested for the presence of nuclear MET in AML12 and mKSA which are mouse cell lines, which expresses high levels of MET protein. The B2 antibody as expected showed cytoplasmic staining in immunofluorescence staining (Figure 20), which was also confirmed by Western blotting (Figure 21). With the N16 antibody we could see membranous staining (Figure 20), but no full length MET and only cleaved form of MET on the Western blot (Figure 21). The C28 and CVD13 antibodies showed predominantly nuclear staining (Figure 20), in particular the C28 antibody. Again no full length MET was detected (Figure 21) but only the cleaved form of MET (Figure 21). The reason is AML12 and mKSA are murine cell lines and C28, CVD13 and N16 antibodies are raised against human MET. These antibodies still detected MET in immunofluorescence staining which might be the cleaved form of MET as detected in Western blotting (Figure 21). This observation may once again indicated towards the point that denatured protein lost its epitope for these antibodies while performing Western blotting but not for immunofluorescence staining in which the epitope is still accessible for the antibody for detection of MET. Existence of nuclear MET and translocation of MET into nucleus has been reported recently by two independent groups (Gomes et al., 2008; Pozner-Moulis et al., 2006). Gomes et al shows that MET as a whole protein translocates to the nucleus to initiate calcium signalling in SkHep1 cells. Pozner-Moulis et al report that MET undergoes cleavage resulting in a 60kDa cytoplasmic fragment which enters the nucleus in A431 and HEK293 cells. This led us to consider that what we observe when stained with C28 antibody is a cleaved form of MET. RACE PCR and quantitative PCR performed on WAPT1 # 08 TMECs passage 40 which expressed exclusively nuclear MET revealed no smaller isoforms. To conclusively prove whether the different staining pattern produced by the C28 antibody is true or not we decided to silence MET expression in these cells using shRNA. qRT- PCR indicated up to 90% reduction of MET but Western blotting results were contradictory. When the immunoblot was probed with B2 we could see significant down regulation of full length MET but there was no change is the expression level of cleaved MET when probed with C28 and N16 antibodies (Figure 27) indicating that what we observed as MET protein was not MET. C28 and N16

antibody in immunofluorescence staining was recognising something non-specific in mouse cells. Hence we decided to raise our own custom antibodies against MET. Custom antibodies N1, N2 & C1, C2 that should recognise N terminal and C terminal epitopes respectively, gave more nonspecific bands on Western blotting. These custom antibodies were also not able to prove the existence of nuclear MET conclusively. The commonality of both Gomes and Pozner et al., studies is the use of human cell lines, where we could also show the presence of MET both in cytoplasm and nucleus in A431 cell line using C28 antibody (Figure 14, 15). In summary, our goal was to prove the existence of nuclear MET in mouse cell line in order to study the role of MET during tumorigenesis in our WAP-T mouse model which shows *MET* gene amplification. Our attempts failed to prove conclusively the existence of nuclear MET or translocation of MET into nucleus in a mouse cell line.

V.3 wtp53 is a new regulator of *MET* gene

The major biological outputs of p53 pathway in response to various cell-physiologic stresses are inhibition of cell proliferation, induction of apoptosis and senescence. Studies have demonstrated that p53 functions as a transcription activator and also as a repressor and regulates a number of target genes at the transcriptional level. Transcription repression of downstream target genes by p53 is thought to occur via one of a few mechanisms (Ho and Benchimol, 2003). p53 may directly bind to p53 consensus to repress promoter activity or it may act through interference of the basal transcriptional machinery via the binding of p53 to the TATA-binding protein (Farmer et al., 1996) or the binding of other transcriptional factors. However, a number of the genes are transcriptionally repressed by p53. Downstream target genes that are negatively regulated by p53 include genes regulating apoptosis (Hoffman et al., 2002) and the G2/M of the cell cycle (Chun and Jin, 2003; Innocente et al., 1999; Taylor and Stark, 2001; Zhao et al., 2000). MET is the protein tyrosine kinase cell surface receptor for HGF and transmits its multiple signals such as induction of cell growth, differentiation, and the apoptotic/antiapoptotic processes. MET also plays an important role in tumor growth and progression. Studies of *MET* gene expression regulation are important in understanding its biological functions in normal and abnormal tissue growth. The main reason for the phenomenal recent interest in p53 is the finding that a high proportion of human cancers (up to 50 %) contain mutations of the p53 gene

likewise, the events during tumorigenesis where *MET* gene amplification with subsequent protein expression which drives to metastasis is also of phenomenal interest to cancer biologists.

Here for the first time we report a direct role of p53 as a negative repressor of MET. The direct role of p53 in controlling the expression of MET was found out in an indirect way. The first evidence came by co-staining T-Ag and MET in G2 cells. It was interesting to observe that the MET expression was directly proportional to T-Ag expression (Figure 34) indicating a role for T-Ag in regulating MET expression.

Till date there has been only one study conducted to show the interaction between SV40 and MET. Cacciotti et al., have shown that SV40 replication in human mesothelial cells induces HGF/MET receptor activation. In malignant mesothelioma derived cell lines expression of SV40 T-Ag lead to constitutive phosphorylation of MET receptor (Cacciotti et al., 2001). Expression profiling of MET in various cell lines with or without T-Ag further concluded that T-Ag has a role to play for the expression of MET in these cell lines (Figure 34). It is of much interest to note that mouse fibroblasts with p53-null background and wtp53 when transduced with LT, the level of MET expression increased 3-8 folds respectively further indicating that LT has a role to play in the regulation of MET (Figure 35). This phenomenon can be best explained from the action of T-Ag which leads to transcriptional inactivation of p53 by complex formation, thus it cannot associate with its target promoters (Bargonetti et al., 1992; Lilyestrom et al., 2006). This particular experiment gave us the first hints to look at the p53 status. There are reports in the literature suggesting that T-Ag/p53 complex has growth stimulatory activities also. Maurizio et al., reported that in human cells, T-Ag/p53 complexes regulate transcription of the insulin-like growth factor I (IGF-I) gene by binding to the IGF-I promoter together with pRB and p300. We assumed that this may be the phenomena in our case too since p53-null background 10-1 cells showed 3 fold increase in MET expression whereas 3T3 cells with wtp53 cells showed a significant 8 fold increase in MET expression. From one more experimental observation we could also confirm that the absence or inactivation of p53 leads to high expression of MET (Figure 36). Totally all these data suggested that p53 acts a repressor of *MET* gene. Expression of temperature sensitive p53 in cG9 cells confirmed that wild type p53 acts as repressor of MET but not mutant p53 (Figure 37). Finally, ChIP provided the proof of mechanistic action p53 as a repressor of MET. p53 mediated repressor activity in the mouse *MET* promoter was mapped to the region +233 to +264 by *in silico* analysis. This

p53 potential binding site was confirmed by performing ChIP analysis (Figure 39). Through a series of experiments we conclude that p53 acts as a negative repressor of MET. The biological significance of p53-mediated repression of *MET* gene in tumorigenesis warrants further investigation. In the present study, we took a stepwise approach to show that p53 represses the expression of MET. Altogether, the data suggest that the *MET* gene amplification and subsequent upregulation of MET expression may be due to mutp53 which may be directly responsible for the increased tumorigenesis in our WAPT mouse model for breast carcinoma which is advocated by of SV40 LT. Further study will be necessary to define the mechanisms by which further mechanisms, wtp53 represses MET expression and to determine if evidence of this interaction can be shown, and also to explore the functional implications in more details.

V.4 MET plays key role in SV40 T-antigen mediated cellular transformation

T antigen is known as one of the most powerful oncoproteins capable of transforming different kind of cell types. The transforming activity of T-Ag is due in large part to its perturbation of the pRB (Dyson et al., 1989a) and p53 (Mietz et al., 1992) tumor suppressor proteins. In addition, T-Ag binds to several other cellular factors, including the transcriptional co-activators p300 and CBP, which may contribute to its transformation function. Several other features of T-Ag that appear to contribute to its full transformation potential are yet to be completely understood. Study of T-Ag therefore continues to provide new insights into the mechanism of cellular transformation. The consequences of a transformed cell are cell-autonomous proliferation, block of differentiation, immortalization, escape from apoptosis and acquisition of invasive ability. During the past years, we have gained sufficient knowledge about the genes involved in the first steps of cellular transformation, but our understanding of the biochemical mechanisms are still lacking. Research from past few decades has provided reasonable experimental evidences indicating that specific extracellular factors can sustain the invasive growth program. Among them, a essential role is played by MET (Comoglio and Trusolino, 2002). In our study we have provided the first evidence that T-Ag need MET for transforming a cell.

In this study, we have used lentivirus based shRNA delivery system to target the *MET* oncogene in an established SV40 transformed mKSA cell line, in which like naturally occurring tumors, express high levels of endogenous MET protein. Here we show that MET silencing resulted in suppression of the invasive growth program *in vitro,* as demonstrated by cell' inability to grow and form anchorage-independent colonies. This impairment is due to both decreased proliferation and increased propensity to undergo apoptosis. Compared to the majority of the known oncoproteins, SV40 T-Ag is sufficient alone to transform a numerous cell types in culture and in transgenic animals (Adams and Cory, 1991; Livingston and Bradley, 1987). By the way, SV40 mutants through genetic analyses have shown that SV40 T-Ag possesses at least three transforming domains with variable, cell-type-dependent transformation potential (Kierstead and Tevethia, 1993; Symonds et al., 1993). Nevertheless, specificity cannot be necessarily assigned to these domains. Instead endogenous factors differentially expressed in particular cells might be variably complementing different SV40 T-Ag mutants for the multiple SV40 T-Ag functions related to transformation. Furthermore, even when SV40 T-Ag is wild type, its transforming potential might be influenced by the cellular milieu. In any case our experiments throw light on importance of MET pathway which plays significant role and act as a prerequisite for the SV40 T-Ag transforming potential. For the first time we show that SV40 T-Ag essentially needs MET for transformation of a cell. Through our experiments we can speculate that there is synergistic action taking place to induce increased tumorigenesis where p53 is being inactivated by SV40 T-Ag and subsequent activation of MET has a multitude action for an increased evidences of tumorigenesis in our WAP-T mouse model.

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VIII APPENDIX

ABBREVIATIONS

°C	Degree celcius
µ	Micro
µM	micro molar
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin Immunoprecipitation
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide Triphosphates
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
EpCAM	Epithelial cell adhesion molecule
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal calf serum
G	g-force
GFP	Green fluorescent protein
h	Hours
HCl	Hydrochloric acid
HEPES	2-(4-(2-Hydroxyethyl)-piperzino)-ethansulfonic acid
HRP	Horse Raddish Peroxidase
Ig	Immunoglobulin
IgG	Immunoglobulin subclass G
IPTG	Isopropyl-ß-D-thiogalactoside
IRES	Internal ribosome entry site
kDa	Kilo Dalton
LB	Luria Bertani
LT	Large tumor antigen
MECs	Mammary epithelial cells
MET	Mesenchymal epithelial transition factor
mg	Milli gram
mM	Milli molar
mRNA	Messenger RNA
mut	Mutant
ng	Nano gram
nt	Nucleotides
ODx	Optical density at x
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehye
PMSF	Phenyl methyl sulfonyl fluoride

RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcriptase- PCR
SDS-PAGE	Sodium dodecyl sulphate poly acrylamide gel electrophoresis
SV40	Simian vacuolating virus 40
T-Ag	tumor antigen
TE	Tris EDTA
TEMED	N,N,N',N'-Tetramethylenediamine
TMECs	Tumor mammary epithelial cells
UTR	Untranslated region
v/v	Volume per volume
V	Volts
w/v	Weight per volume
WAP	Whey acidic protein
wt	Wild type
µg	micro gram

Primers used for ChIP experiment

Name	Sequence 5' → 3'
MET-P1	GCAGGCCTCCAAAACTGTAA
MET-P2	CAGAGCGAAGACAAGGAAGG
MET-P3	CCTTCCTTGTCTTCGCTCTG
MET-P4	CATTTCCCTGCAAGGATACAA
MET-P5	AACTCCTGGGGTTCTTCCAG
MET-P6	GCGTGGACAGAGATCTAGGC
MET-P7	GCGCCTAGATCTCTGTCCAC
MET-P8	AGTTTCCCTCTCCCCAGCTA
MET-P9	TAGCTGGGGAGAGGGAAACT
MET-P10	GATGGGGACTGGCACCAC
MET-P11	GGTGCCAGTCCCCATCTC
MET-P12	GTGACTCAGCCGGGCAAC
MET-P13	GCTGACTCGCTGGAGAAGG
MET-P14	ACGCGGCTGGAGTTTGTA
MET-P15*	AGCCCCTCTGCTTTCTTTGT
MET-P16*	GCCGTCAACAACACTCCTTC

* Primers encompassing putative p53 binding site

Primers used to check the splice variants of *MET* gene

Name	Sequence 5' → 3'
A1	ACCGCTGACTCGCTGGAGAA
A2	TGATGGCCGTGTAGGACGAC
B1	CGTCCTACACGGCCATCATA

Name	Sequence 5' → 3'
B2	CTTCAGCCGTCTCACCGATA
C1	ATCGGTGAGACGGCTGAAGG
C2	CTGACATACGCGGCTTGGAG
D1	CCAAGCCGCGTATGTCAGTA
D2	CCTGCATGAAGCGACCTTCT
E1	ACGTCAGAAGGTCGCTTCAT
E2	TCTCGTTGCCAAGCAGAACT
F1	TTCCAATCCTGCAGTCAGTG
F2	CACTGTATTGCGTCGTCTCT
G1	AGAGACGACGCAATACAGTG
G2	AGGAGTAGTGCAGCAGATGA
H1	GAGATCATCTGCTGCACTAC
H2	GACTGCTTGCTTCCACTCTA
I1	CTCTGGAGCTGTGTTGTGTA
I2	GTCTCCACTTGTCAGGATAG
J1	GAATGGAGCATGCAGACAAG
J2	TTCACTCCTCAGGCAGATTC
K1	GAATCTGCCTGAGGAGTGAA
K2	TTCTGCCTTGCAACAGGTAG
L1	GTTCACCACCAAGTCAGATG
L2	GGATGGCAACAGAGAAGGAT
M1	GTCCACGTGAACGCTACTTA
M2	CGCTGAGATAGGAGTGTTGT
N1	TGCGGTAACTGCACCACTAT
N2	GTTCACGACTGCAACACAAC
O1	GCAACCTGCAGCTACAACAC
O2	TGAGCCAACACTCCTTGCTA
P1	GGAGTGTTGGCTCACACCTA
P2	TCCTCCTGAACGAGAGTGTC
Q1	TCGTTCAGGAGGAGGCTTCT
Q2	GTGCACTGGCAATCAGTACA
R1	CATTACGTCACCAGGACTTG
R2	GGTGTCTTCCACAGCTCTTA
S1	GTGGTGGAGCATATCAGAAC
S2	GCAGGTATAGGCAGTGACAA

Primers used for quantitative RT-PCR

Name	Sequence 5' → 3'
Actin-F	GAAATCGTGCGTGACATCAAAG
Actin-R	TGTAGTTTCATGGATGCCACAG
MET-F	CCAGAGCTGGTCCAAGCAGTTCAG
MET-R	GGGATGGCTGAAGTCTTTCAT
T- Antigen-F	TATGTCAGCAGAGCCTGTAGAACCAAAC
T- Antigen-R	GAGAAAGGTAGAAGACCCCAAG
WAP-F	GTTGCCTCATCAGCCTTGTTC
WAP-R	CTGAAGGGTTATCACTGGCAC

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