The Met Receptor Tyrosine Kinase in Mammary Gland Tumorigenesis and Development

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Abstract

The Met receptor tyrosine kinase (RTK) is expressed in the mammary gland under both normal and neoplastic conditions. Overexpression of the Met receptor is found in 15-20% of human breast cancers and is correlated with shortened disease-free interval and overall survival. In order to explore the role of dysregulated Met receptor signaling on the development of mammary tumors I have characterized a transgenic mouse model that expresses either wild type or a dysregulated Met receptor in the mammary epithelium under the control of the mouse mammary tumor virus promoter/enhancer (MMTV-Met). The Met receptor variants contained a mutation that results in decreased receptor ubiquitination and prolonged receptor signaling (Y1003F) or an activating mutation that was originally observed in patients with papillary renal carcinoma (M1250T) or both mutations (YF/MT). *In vitro* and *in vivo* transformation assays demonstrated that each mutation singly is weakly transforming, however, there was an additive effect on transformation when both mutations were present. This additive effect was observed in the transgenic mice where multiparous MMTV-Met-YF/MT mice developed tumors earlier and with much greater penetrance than did mice expressing either of the single mutants. This provides the first *in vivo* model that demonstrates a role for ubiquitination in suppression of transforming activity of an RTK. MMTV-Met-YF/MT tumors displayed a range of histological phenotypes but were mainly comprised of luminal lineage cells. Notably, MMTV-Met-M1250T tumors contained cells from both the basal and luminal populations, suggesting transformation of a progenitor cell. Progenitor cell transformation in RTK transgenic mouse models is uncommon and highlights distinct signaling differences and potentially lineage specificity of the two Met mutants.

Through assays of overexpression *in vivo* and inhibition *in vitro*, Met receptor signaling has been correlated with the development of the mammary gland. To examine the effects of loss of Met receptor signaling on mammary gland development I have utilized the Cre/LoxP1 recombination system to knock-out the Met receptor from the mammary epithelium. Mammary-specific Cre recombinase efficiently excised floxed DNA as visualized by activation of a β-galactosidase reporter In $Met^{+/+}$ glands, however, few β-galactosidase positive cells are retained In the $Met^{fl/fl}$ glands and an intermediate number are retained in the $Met^{fl/+}$ glands. This indicates that Met-null cells are selected against and supports a role for Met in the development of the mammary gland.

Résumé

Le récepteur tyrosine kinase (RTK) Met est exprimé dans la glande mammaire dans des conditions normales et néoplasiques. La surexpression du récepteurs Met est retrouvée dans 15 à 20% des cancers du sein chez l'humain et est associée à des courtes périodes de rémissions et un taux de survie faible.

Dans le but d'explorer le rôle de la dérégulation de la voie Met sur le développement des tumeurs du sein, j'ai caractérisé un modèle de souris transgènique qui exprime un récepteur Met de type soit sauvage soit dérégulée dans l'épithélium mammaire sous le contrôle du promoteur MMTV (*Mouse Mammary Tumor Virus*). Les variants du récepteur Met étudiés contiennent les mutations simples ou combinées 1) Y1003F, qui provoque une diminution de l'ubiquitination et, par conséquent, une signalisation prolongée de Met et 2) M1250T, une mutation observée originellement chez des patients atteints du carcinome papillaire rénal et qui aboutit à un Met activé constitutionnellement. Des essais de transformation *in vivo* et *in vitro* ont démontré que les mutations simples sont peu transformantes alors qu'un effet additif est observé sur la transformation lorsque les deux mutations sont combinées. Cet effet additif est aussi observé au niveau des souris transgèniques. En effet, les souris MMTV-Met-YF/MT multipares développent des tumeurs plus tôt et avec une plus grande pénétrance que les souris simple transgèniques. Ceci constitue donc le premier modèle *in vivo* démontrant un rôle de l'ubiquitination dans l'activité transformante d'un RTK.

Les tumeurs MMTV-Met-YF/MT montrent un panel de phénotypes au niveau histologique mais peuvent être considérées de type luminal. Les tumeurs MMTV-Met-M1250T contiennent des cellules d'origine basale et luminale, indiquant ainsi la transformation d'une cellule progénitrice. La transformation de ce type de cellules dans les modèles de souris transgèniques pour des RTK n'est pas un phénomène commun et met en évidence des différences au niveau des cascades de signalisation ainsi qu'une possible spécificité de lignée cellulaire de la part des deux mutants de Met.

Par des essais de surexpression *in vivo* et d'inhibition *in vitro*, la signalisation via le récepteur Met a été reliée au développement de la glande mammaire. En effet, afin d'examiner les effets de la perte du récepteur Met sur le développement de cette glande, j'ai utilisé le système de recombinaison Cre/LoxP1 qui permet de dépléter l'épithélium mammaire de tous récepteurs Met. Une recombinase Cre spécifique à la glande mammaire a permi d'exciser l'ADN flanqué des séquences Lox tel qu'observé par l'activation du gène reporteur β-galactosidase dans les glandes $Met^{+/+}$. Toutefois, peu de cellules positives pour la β-galactosidase sont retenues dans les glandes $Met^{fl/fl}$ et un nombre intermédiaire est retenue dans les glandes $Met^{fl/+}$. Par conséquent, il existerait donc une sélection contre les cellules Met-null. Ces résultats suggèrent donc que Met joue un rôle dans le développement de la glande mammaire.

Dedication

This thesis is dedicated to my father, he inspired me to pursue science and later gave me motivation to become involved in both research and medicine, but most importantly, he taught me to live life to the fullest and never pass up an opportunity to have fun.

Acknowledgements

I would first like to acknowledge the guidance and support of Dr. Morag Park. She gave me the opportunity to tackle this project and take it in the direction I chose. She helped me through periods of frustration and encouraged me to explore new aspects of the model.

I would also like to thank all the members of the Park lab for their advice, assistance, and camaraderie.

I need to especially acknowledge Pascal Peschard Ph. D. for generating the constructs that became the transgenic mice that I have characterized and for his advice and assistance while sharing bench space with me for 3.5 years.

Anie Monast must also receive special thanks for her excellent work in transplantation, surgeries, genotyping, and organizing the mice.

I also need to thank David Germain for translating the abstract for me.

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I would also like to thank my family for their omnipresent support and encouragement: my sister for making me laugh and helping me put things into perspective; my mother for having good suggestions for how to deal with my difficulties and helping me remember that this is for the greater good-eventually.

Finally, I would like to thank my husband, Brandon, for all his support and time spent listening to me. Without his love, encouragement, and distraction, these past four years would have been a great deal more difficult.

Preface

Overexpression of the Met receptor tyrosine kinase is found in 15-20% of human breast cancers and is associated with a shorter disease-free interval and decreased overall survival. Our lab has been examining the role of a loss of negative regulation substitution in the Met receptor (Y1003F) that results in decreased receptor ubiquitination and prolonged signaling from the receptor. The purpose of this project was to examine the effect of mammary-specific expression of a dysregulated Met receptor that contains either the Y1003F substitution, an activating mutation (M1250T) or a combination of the two (YF/MT) and to determine if decreased receptor ubiquitination could contribute to cellular transformation. The second purpose of the project was to characterize the tumors that arose in the Met-M1250T and Met-YF/MT mice.

The Met receptor and its ligand are expressed in the mammary gland during periods of ductal development and reorganization, but the effect of loss of Met receptor signaling on the mammary gland was not known as knock-out mice are embryonic lethal. A third objective of the project was to knock-out expression of the Met receptor in the murine mammary epithelium and observe the effects on the development of the mammary ductal tree.

Contributions of Authors

1. Constructs for the transgenic mice and primers for their genotyping were generated by Pascal Peschard.

2. Manuscript 1:
   -Pascal Peschard performed the ubiquitination assay (figure 1)
   -Pascal Peschard and Monica Naujokas generated the T47D cell lines, however, I performed the immunoblots (figure 2)
   -I generated the Rat1 and EpH4 cell lines and carried out the transformation assays (figures 3, 4, table 1)
   -I selected founder lines and gathered tumor data (supplementary figure 1, Table 1)

3. Manuscript 2:
   -All figures were made from data acquired by me
   -Marisa Ponzo generated the microarray data and it was analyzed by Daniela Cernea.
   - Met sequencing was carried out by Suzanna Arcand
   -Dr. Robert Cardiff assisted in the determination of the pathological subtype of the tumors.

4. Manuscript 3:
   -All initial studies with epithelial cell infection and transplantation were carried out by me, with help from Anie Monast for the cell injections.
   -I optimized staining protocols.
   -Mammary fat pads from the MMTV-Cre/Met$^{fl/fl}$ mice were collected by Anie Monast and stained by Alysha Dedhar.
   -Nuclear fast red staining was performed by Jo-Ann Bader
   -I acquired and analysed all images.

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Chapter 1

Literature Review

1. Introduction

Over a lifetime, breast cancer will affect 1:8 women in North America, making it the second most frequent non-melanoma cancer in women, second only to lung cancer (1). The high prevalence and the beloved population affected-mothers- has brought breast cancer into the public consciousness with products marketed with a percentage of the proceeds going to breast cancer research (products marked with a pink ribbon) and resulted in a proliferation of fundraising events specific to breast cancer (CIBC Run for the Cure, Denim Day, Weekend to End Breast Cancer, Race for the Cure) as well as the entire month of October being designated as Breast Cancer Awareness Month. Thus, we strive to prevent, treat or cure breast cancer through continued research through epidemiology, patient samples, mouse models and *in vitro* molecular biologic assays.

1-1 Breast Cancer- Epidemiology

Factors associated with the development of breast cancer include lack of exercise, poor diet, smoking, drinking, exposure to ionizing radiation, genetic mutations, age at onset of menses, age at menopause, age at first full term pregnancy, obesity, and old age (2, 3). Many of these risk factors can be modified by lifestyle changes, but many, such as age of menarche, cannot.

Established, hereditary genetic defects only account for 5-10% of breast cancers (4). Two well-studied predisposing genes are *BRCA1* and *BRCA2* (*breast cancer*1 and 2) the protein products of which are involved in DNA repair. Carrying a genetic mutation in *BRCA1 or BRCA2* results in a lifetime risk of breast cancer of 60-80% thus, some of these women opt for prophylactic mastectomies (5). Carriers of *p53* mutations (LiFraumeni Syndrome) also have an increased risk of breast cancer as do those with mutations in *ATM* (ataxia-telangiectasia). Aside from these established genetic lesions or exposure to high doses of ionizing radiation, there are few factors that dramatically increase one's risk for developing breast cancer.

The mortality rates associated with breast cancer have decreased significantly in the past 20 years (1). Because of the high prevalence, screening for breast cancer

has become standard practice. The tests of self-examination and mammography are non-invasive and fairly inexpensive, and at present all women fifty years and older in Canada are encouraged to receive a mammogram at least once every two years. The increased awareness in women and the widespread use of mammography has contributed to the decrease in breast cancer-related mortality by detecting breast cancers when they are smaller and easier to resect (average decrease of 2.7% per year since 1993 in Canada (1)). As of 1997 the five year survival rate was 86% (1).

1-2 Breast Cancer-Clinical Management

Improvements in treatment have also helped lower mortality rates. Multiple studies have demonstrated that local excision (lumpectomy) of small tumors followed by radiotherapy is as effective at preventing recurrence as is mastectomy (6). Radiotherapy can be administered by external beam or, now, the more progressive brachytherapy, in which radioisotopes are temporarily implanted in the breast tissue so that high dose, localized radiation can be administered over a shorter period of time (7). Administration of cyotoxic chemotherapy has consistently resulted in improvements in disease free survival and overall survival, particularly in women younger than fifty (8). However, because of the negative side effects of chemotherapy, it is generally recommended only for women who have axillary lymph node involvement, invasive or aggressive tumors.

Various subtypes of breast cancer have now been identified that are being treated with specific chemotherapeutic adjuvant agents leading to better survival and fewer negative side effects. Nearly two thirds of breast tumors are estrogen receptor positive (9) making them susceptible to adjuvant therapy with estrogen receptor inhibitors or aromatase inhibitors. Anti-estrogens have been shown to be better tolerated than cytotoxic chemotherapy but is equally effective in halting or reversing disease progression in women with advanced, hormone receptor positive tumors (10). Twenty to thirty percent of breast tumors overexpress the Epithelial Growth Factor Receptor (EGFR) family members ErbB2 (11, 12). These tumors are now being treated with an antibody (Herceptin/trastuzumab) that binds to ErbB2 and inhibits its signaling (13) thus specifically inhibiting an important growth-stimulating pathway in

these tumors. The small molecule inhibitor gefitinib (Iressa) interferes with signaling from the EGFR and is presently in clinical trials (14). Finally, the antiangiogenic agent Avastin/bevacizumab, in combination with paxitaxel, has been shown to be effective at improving overall response in women with metastatic breast cancer (15). These targeted adjuvant agents are given in addition to the standard chemotherapy, surgery and radiation and have yielded a significant improvement in outcome associated with fewer severe side effect.

1-3 Breast Cancer-Pathology

Breast cancer is believed to develop through a series of histologically identifiable stages beginning with benign hyperplasia followed atypical hyperplasia progressing to a carcinoma in situ. Malignancy is identified by the presence of tumor cells that have invaded through the basement membrane of the duct/lobule and into the surrounding connective tissue (16). Breast cancer is classified using a tumor/lymph node/metastases (TNM) scale to indicate the presence or absence of metastases and the degree of progression of the tumor. Each of these categories is given a value from 0-3 with 0 being lowest or absent and 3 being the worst or most prevalent-in the case of metastases. The pathologist examines histological sections of the tumor or a core biopsy to determine how cytologically advanced the tumor is. The lymph nodes are excised by the surgeon and examined for the presence of breast cancer cells by the pathologist. Metastases are also determined by a cooperative effort from the pathologist and surgeon.

The most common histological phenotype, accounting for ~79% of invasive breast cancers is ductal carcinoma no special type (NST). This is a solid adenocarcinoma that lacks features to classify it as any other specific subtype. Invasive lobular carcinoma is the second most prevalent subtype, making up ~10% of breast carcinomas. These tumors typically have lost expression of E-cadherin and the cells invade the stroma in single file. Lobular carcinoma, unlike ductal carcinoma, occurs more frequently in both breasts-~20% of women with LCIS develop cancer in the second breast within 20 years (17). The other subtypes are quite uncommon, accounting for 5% or less of diagnoses. They include medullary, mucinous, tubular,

and invasive papillary carcinomas. These smaller subdivisions help design treatment plans, but the large, generic group of ductal carcinoma NST needs to be further studied and divisions within the group defined. The need for better classification is highlighted by the differences in survival between the groups. The 30 year survival for any of the defined subtypes is approximately 60% but the diagnosis of ductal carcinoma NST is associated with only a 20% survival at 30 years (16). Most likely, good prognosis subgroups are buried within the amorphous NST group but need more detailed studies in order to be identified. Although screening, diagnosis and treatment are improving, much work remains to be done in order to identify, classify and treat the many women who are afflicted now, but even more importantly, to find causes and teach prevention to avoid cancer in the future.

1-4 Mammary Gland Development

Development of the mammary gland in humans begins at approximately embryonic week 4 as the milk streak, a thickened line of ectodermal cells along the ventral surface of the embryo extending from the axilla to the inguinal region (18). By week 8 most of the milk line has regressed leaving only two epithelial discs where there will be the one set of nipples (19). The epithelial cells invade the underlying stroma, which, signalling back to the epithelial cells plays a critical role in mammary gland development (20-23). The mesenchyme underlying the future mammary gland induces the ectoderm to differentiate into mammary epithelial cells and develop into the ductal tree as demonstrated by transplantation studies (22, 24). The secondary mesenchyme, the future mammary fat pad, however, does not retain the ability to differentiate epithelial or ectodermal cells into mammary epithelial cells (25). Between the 12th and 13th weeks of development, the epithelial bud begins to branch and further invade the stroma and by the 20th week there are several ducts entering the hairless areola, however these do not yet have lumens (26). By parturition a very basic mammary tree with patent lumens has formed but there is no lobular development (27) (figure 1-1).

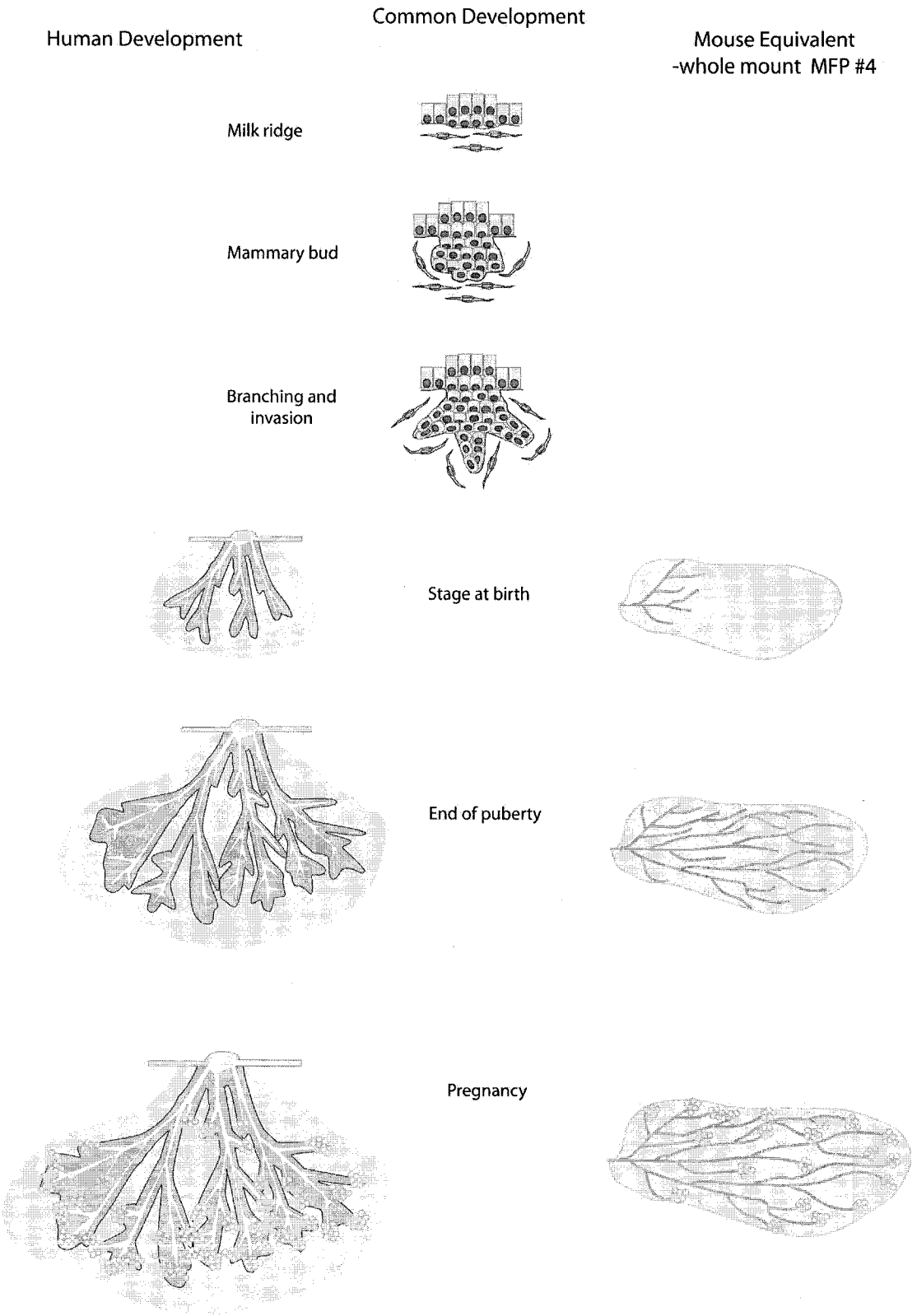

Figure 1-1 Comparative Mammary Gland Development. Epithelial cells are in pink. Dense connective tissue is dark blue. Adipose tissue is light blue. © 2006 Stephanie Petkiewicz

The mammary gland is an ideal organ for developmental studies because the majority of mammary gland development occurs after birth obviating the need to sacrifice embryos and the pregnant females as is required for study of neural (28) or renal (29) development. The mammary epithelium remains quiescent until puberty when the levels of ovarian hormones begin to rise, resulting in further proliferation of mammary epithelial cells and maturation of the gland. Ducts extend and branch distally forming clusters in lobules and the amount of fat and connective tissue in the gland increases as well, however little differentiation of epithelial cells occurs until pregnancy and lactation (19). In human males, the development of the mammary tree is the same as in females until puberty and the rudimentary ductal tree remains throughout the lifetime of the man. In mice, the primary organism used for the study of the mammary gland, the male mammary ductal system generally regresses during embryogenesis as a result of the stroma encircling and cutting off the neck of the ductal tree (30, 31).

1-5 Human versus Mouse Mammary Glands-Comparative Histology

Much of what is known about mammary gland development is the derived from murine studies and is assumed to be applicable to humans. However, it is important to note several differences in the structure of mammary glands between mice and humans. The most obvious difference is the number of mammary glands. The mouse has five sets of mammary glands (figure 1-2) placed along the milk line whereas in humans, the majority of the milk line regresses during embryogenesis leaving only two nipples except in the rare case of supernumerary nipples that are most commonly located in the axilla (19).

Second, humans have a much more complex ductal system than mice with several lactiferous sinuses emptying into the nipple and independent ductal trees developing from each of these main ducts, each separated by bands of connective tissue. Mice have only one duct emptying onto each nipple and there is very little connective tissue at all in their mammary glands (review (32, 33)). Human mammary ducts terminate in a highly branched, grape-like structure supported by loose connective tissue called a terminal ductal lobular unit (TDLU) (34) whereas in mice the ducts

terminate in a single bulbar structure or have minimal branching with only a thin rim of stroma separating it from the surrounding adipose (32).

The majority of the murine mammary fat pad (MFP) is adipose tissue, in closeproximity to the mammary epithelial cells, whereas in humans, there is a significant amount of connective tissue separating the fat from the epithelial cells, thereby creating a microenvironment for the epithelial cells that is different from that in the mouse (figure 1-3). This difference in stromal content has become one of the major issues for those arguing against the use of the mouse for a model of human breast cancers. Rats, however, have a more human-like mammary gland with more connective tissue, however, they have not been as well developed as a model system.

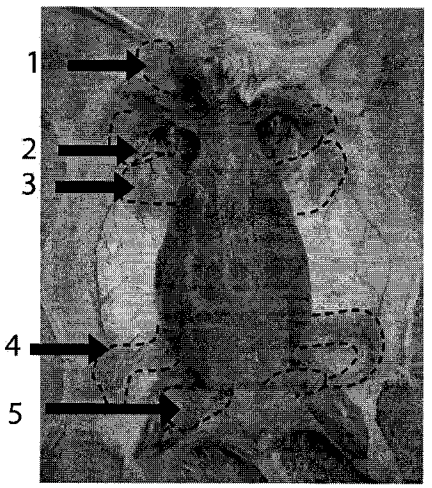

Figure 1-2 Enhanced Photo of the Five Sets of Mammary Fat Pads in a Mouse

Another important difference between mice and humans that affects mammary gland biology is the length of the estrus cycle. Depending on strain, mice cycle every 3-5 days whereas humans average a much longer 28 days. This means that the murine ductal cells are exposed to proliferative stimuli much more frequently than in humans but for a shorter duration. Histologically distinguishable changes occur in human and murine mammary epithelium through different stages of the estrus cycle (35, 36), but not all stages are present in mice due to their abbreviated cycle. In mice, following ovulation no functional corpus luteum develops (37) again

resulting in differences in hormonal effects on the mammary gland. These differences during the reproductive years are lost later in life as both mice and humans experience ovarian exhaustion, but strictly speaking, this is not a menopause in mice as they have not experienced true "menses" (38, 39). Thus, both mice and humans will experience the decreased estrogen levels and changes in the release of other neuroendocrine factors later in life that may influence tumor development.

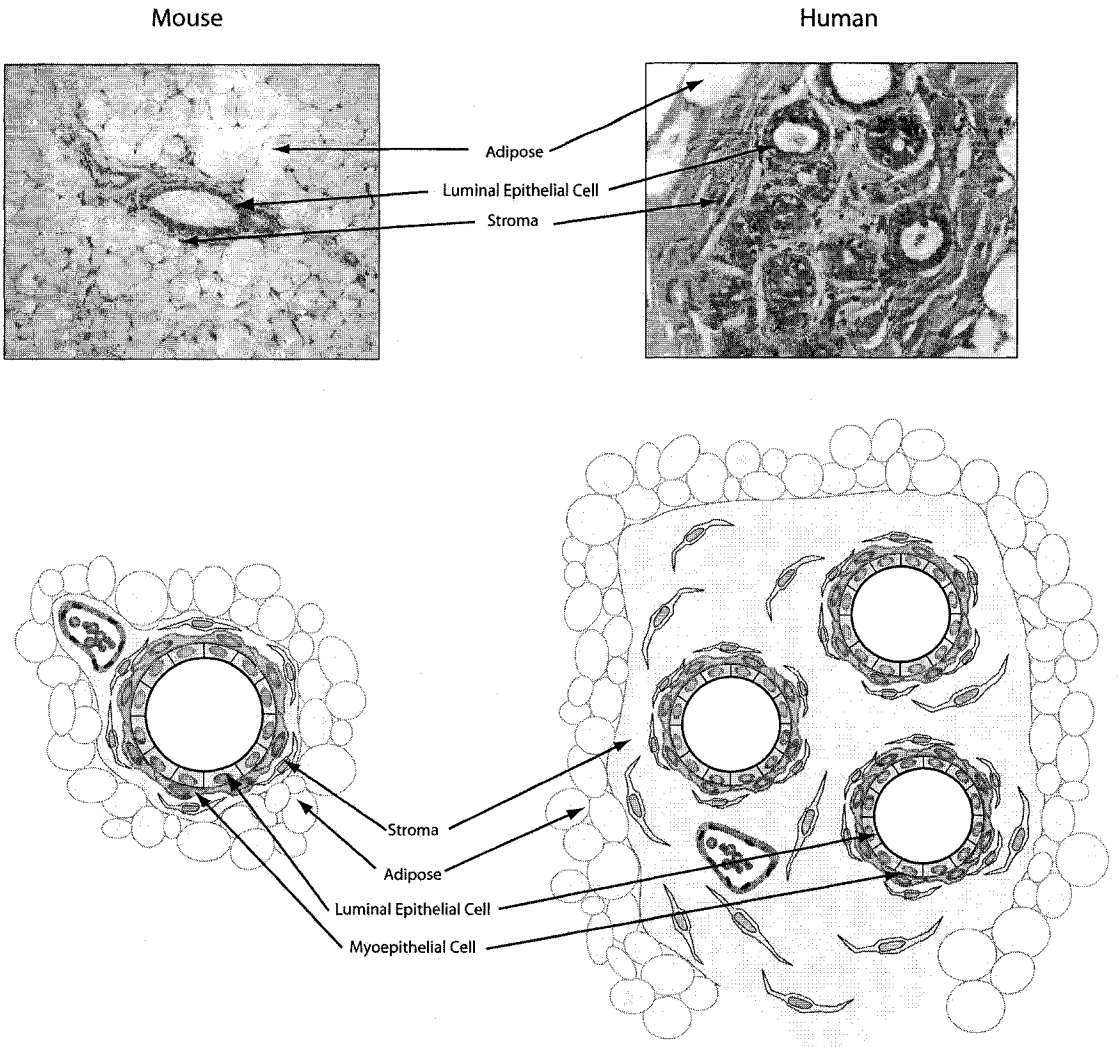

Figure 1-3 Histological Differences Between Human and Murine Mammary Glands

These differences in biology lead to another interesting aspect of using mice as a model for mammary tumorigenesis. In humans, pregnancy and lactation, particularly early in reproductive life, yield a protective effect against the development of breast cancer (40, 41). A similar protective effect of pregnancy has been found in rats (42-45) but in transgenic mouse models, pregnancy and lactation often increases the risk of developing cancer as a result of using a hormonally-responsive promoter such as the whey acidic protein (WAP) or mouse mammary tumor virus (MMTV) promoter (46). The MMT virus itself induces tumors in a similar fashion as the transgenic mice, by enhancing expression of genes near the viral integration site by hormonally-induced transcription during pregnancy and lactation, resulting in tumor initiation in multiparous mice (47). In order to decrease the confounding effects of transgene upregulation and the impact of pregnancy and lactation on breast cancer development, , various groups have generated inducible transgenic systems in which MMTV drives the expression of a transactivator that is only active in the presence of the activating compound such as tetracycline (48), ecdysone (49) or doxycycline (50). In this way, although MMTV induces high levels of expression of the transactivator during pregnancy and lactation, the gene of interest is not expressed until the activating compound is added to the water, so reproductive history can be retained as a factor contributing to or protecting against breast cancer development. Despite the differences in biology and structure, transgenic mice are useful in elucidating the role of various oncogenes in the development of mammary tumors, but new mouse models in which the gene of interest is knocked into its endogenous locus may better represent the genetic alterations in human disease.

2. Mammary Development and Stem Cells

As the majority of studies on stem cells in the mammary gland have been carried out in mice, from this point onward I will be discussing findings in mice, rather than in humans.

Years before the search for the specific cell began, the presence of a stem/precursor cell population in the mammary gland was presumed because of the

ability of the mammary epithelium to extensively proliferate during pregnancy and lactation and then to regress, but to expand again upon subsequent pregnancies. At the onset of puberty when levels of ovarian hormones rise, mammary epithelial cells are stimulated to proliferate, generating swellings at the ends of each of the ducts known as the terminal end buds (TEBs). These invasive bulbar structures migrate through the mammary fat pads away from the nipples and give rise to the cells that form the new ducts (51). The TEB is comprised of several different identifiable cell types (figure 1-4). The bulk of the cells are the "body cells" that are centrally located. Some of the body cells undergo apoptosis to create the open lumen (52) and those that remain differentiate into luminal cells (53). The "cap cells" are located on the advancing edge and are considered to be the stem cells of the TEB, as they can differentiate into contractile myoepithelial cells as well as luminal cells (51).

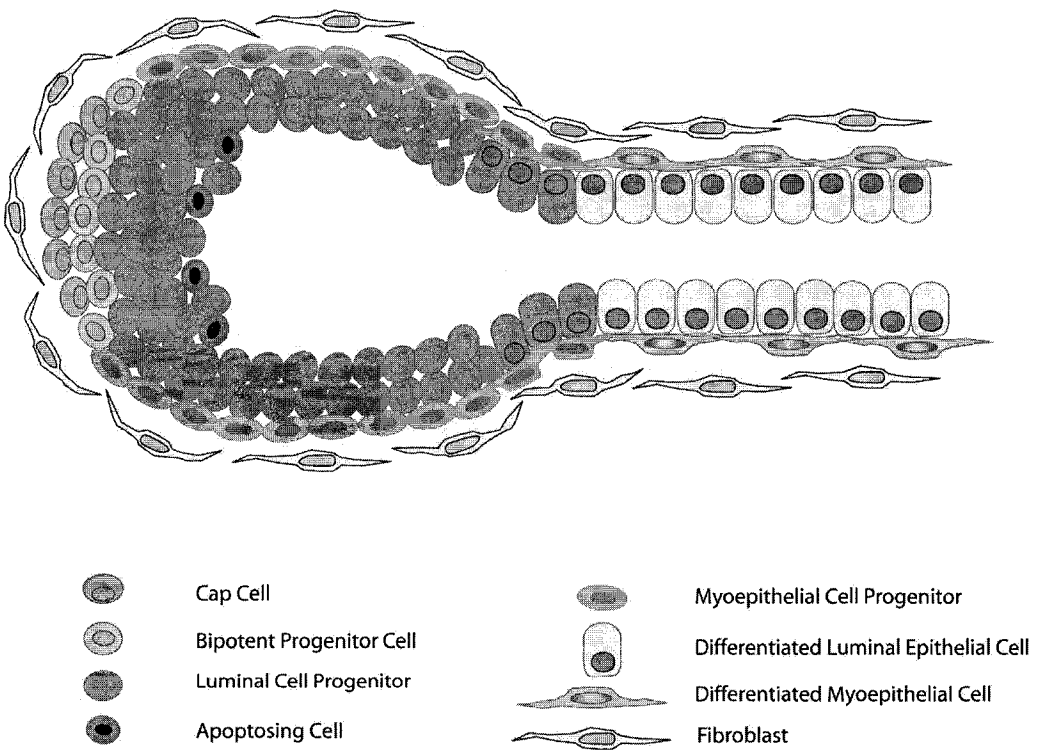

Figure 1-4 Schematic of the Terminal End Bud © 2006 Stephanie Petkiewicz

The definition of mammary stem cell requires the isolated cell to be able to self-renew and to produce cells that are capable of differentiating through several lineages. A progenitor cell is capable of forming one or more lineages but the cell's self-renewal capacity is limited. Although stem cells have the ability to self-renew, they do eventually senesce as demonstrated by transplantation studies in which mammary fragments can be serially transplanted, generating complete ductal trees until approximately seven generations (54-56). Stem cell lineages have been best defined in the hematopoietic system in which nearly all differentiated cells and the progenitor and stem cells have been identified by the presence/absence of markersidentifiable by Fluorescence Activated Cell Sorting (FACS). Mammary-derived stem cells are pleuripotent, but not totipotent, in that thus far, no one has observed isolated stem cells to differentiate into anything other than expected resident cells of the mammary gland. To be noted, however, is the ability of mammary epithelial cells to differentiate into other ectodermal derivatives in conditions of stress such as inflammation and transformation. In these situations the transdifferentiation of the glandular epithelial cells to an epidermal cyst (squamous metaplasia) may occur. This will be discussed in the **Stem Cells and Tumors** section.

It is believed that the TEB is the source of mammary gland stem cells during development (57). Following puberty, stem cells are scattered throughout the mammary ductal tree, as demonstrated by the ability of both ductal and alveolar fragments from the mammary gland to form complete ductal trees upon transplantation (55, 58-62). The location and distribution of stem cells is similar in humans, as confirmed by using dissociated cells from reduction mammoplasties. Cell populations derived from adults can be induced to differentiate into different cell types *in vitro* (63-65) and develop into basic glandular structures in xenograft models (66).

2-1 Localization and Identification of Stem Cells

The precise location and identity of the mammary stem cell has remained elusive. Ultrastructural studies have attempted to visually identify the stem cells on the basis of chromatin structure, contents of the cytoplasm and location relative to the

basement membrane (67, 68). It is generally agreed that the stem cells reside in a basal or supra-basal compartment and do not contact the lumen of the duct. Some groups have verified the presence of a stem cell by simple limiting dilution of single cells (58) and others have used FACS to isolate specific single cells that can reconstitute an entire mammary gland upon transplantation to a cleared MFP (69-71). Immunohistochemical (IHC) methods have been utilized to identify differentiated cell types of the mammary gland by their expression of specific proteins. Dulbecco et al. were the first to define the luminal versus the multipotent basal compartment of the mammary gland using IHC (53). Proteins such as cytokeratins 8, 18, 19 (CK) and mucin 1 are markers of luminal cells and cytokeratin 14 and smooth muscle actin (SMA) are markers of basal, myoepithelial cells (figure 1-5).

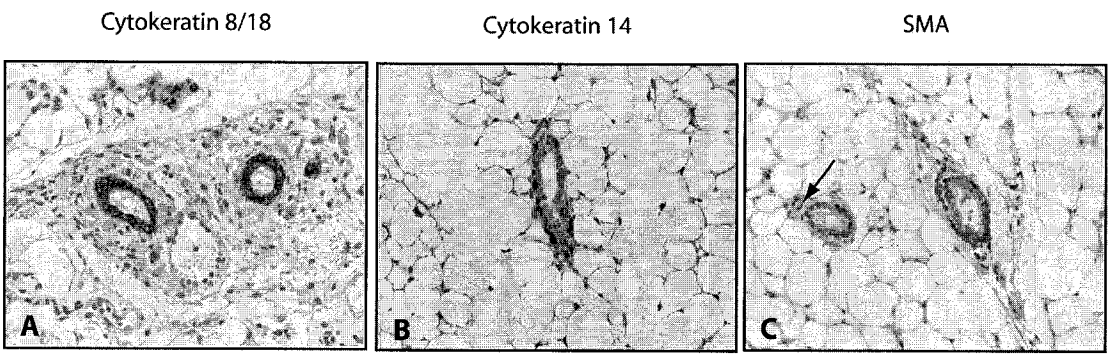

Figure 1-5 Distribution of immunohistochemical markers of cell lineage in normal virgin mammary gland. A. Cytokeratin 8/18-differentiated luminal cells. B. Cytokeratin14-myoepithelial/basal cells. C. Smooth muscle actin-fully differentiated myoepithelial cells and smooth muscle around blood vessels.

The ability of sorted primary mammary epithelial cells to change lineage in culture has been another indication of the presence of progenitor cells in mammary gland. Several groups have sorted primary cells using established markers of progenitor cells and observed changes in selected cell populations under specific culture conditions. Pechoux et al. found that cells with luminal markers could generate myoepithelial cells when switched to a culture medium that better supported the growth of myoepithelial cells, however, cells sorted for myoepithelial markers could not produce luminal cells (72). Smalley et al. found the same uni-directionality of differentiation but plated their pre-sorted cells on Matrigel and found that the

sorted luminal population could form spheres containing both luminal and myoepithelial cells whereas the plated myoepithelial cells showed no luminal differentiation (73). Unfortunately, the distinctions between cell populations made by these markers are not infallible. Two recent papers stated that their stem cell-enriched populations expressed low levels of CK18 (luminal) but high levels of CK14 (myoepithelial), thus supporting the idea that stem cells are in a suprabasal compartment but contradicting the *in vitro* data that demonstrated the terminally differentiated nature of myoepithelial cells (69, 71).

Initially, researchers used proliferative capacity and ability to efflux Hoechst dye as a means of identifying the mammary "side population," through FACS, a well-established technique for sorting hematopoietic stem cells (74). Several groups have sorted these side population cells and reinjected them into cleared MFPs, however, the "take" rates observed following transplantation and the variety of glandular structures obtained was not convincing of the side population being highly enriched for stem cells (70, 74, 75). The side population did, however, express variable levels of cytokeratins indicating a not fully differentiated state (74).

More recent work indicates that the side population is a coarser selection for stem/progenitor cells and more specific, more enriched populations can be isolated by FACS for a series of markers. Several groups have been paring down and refining the list of extracellular antigens (cluster of differentiation-CD) that define the stem cell. All groups begin by removing any cells that express markers of hematopoietic cells and endothelial cells, leaving the breast cell population designated lineage negative (Lin^-). CD24, also known as heat stable antigen, has been used as a marker for neural stem cells (76) and has found good application in sorting mammary cells for their ability to repopulate cleared mammary fat pads. Sleeman et al. simply sorted Lin^- cells for CD24 expression levels and found the $CD24^{low}$ population, as opposed to $CD24^{neg}$ or $CD24^{hi}$, to be enriched for stem cells (77). Shackelton et al. found that $CD24^+CD29^{hi}$ cells were the most enriched for stem cells and that a single cell from this population could form all components of a mammary gland (69). Intermediate levels of expression of CD24 was again used by Stingl et al. to isolate stem cells but he used it in conjunction with $CD49f^{hi}$, a known marker of epidermal stem cells (78).

The consistent results obtained using similar markers indicate that the search for the mammary epithelial stem cell is becoming more refined and perhaps within the next year, a consensus on the markers of these cells will be reached.

2-2 Stem Cells in Cancer

As mentioned above, stem cells persist in the mammary gland, through the lifetime of the animal, slowly dividing in self-renewal. The number of stem cells in the breast does not decrease over time, as demonstrated by the ability of mammary tissue derived from mice of different ages to regenerate a mammary gland for the same number of transplant generations (54). Because these cells are long lived, it is plausible that they could be more prone to transformation by virtue of their life-long accumulation of genetic lesions. The idea of tumors originating from a transformed stem cell has been gaining popularity and acceptance but is still debated (79-81).

The understanding that tumors contain poorly differentiated cells has been around for nearly 150 years (82). Initially it was believed that tumors contained cells that had de-differentiated, reacquiring stem cell-like characteristics as evidenced by their ability to proliferate more than a fully differentiated cell and their similar appearance to fetal tissue (82). Support for the cellular de-differentiation theory is still forthcoming. A recent paper demonstrated that culturing neuroblastoma cells in hypoxic conditions, as would be found at the center of a poorly vascularized proliferating mass, led to transcriptional changes that included downregulation of markers of lineage-specific differentiation (83). Similar results in hypoxic breast cancer were reported (84).

More recently, with the identification of stem/progenitor cells in nearly all tissues, support has been mounting for the idea that these long-lived cells could be accumulating genetic damage over the lifetime of the individual and develop into a cancer late in life. In breast cancer this hypothesis appeared plausible in light of the fact that women who have breast fed, thereby inducing differentiation and subsequent apoptosis of a large number of epithelial cells, have a reduced risk of developing breast cancer. It was initially believed that the cellular differentiation in pregnancy and lactation and thet wave of apoptosis at weaning eliminated a large proportion of

the cells that might harbor deleterious mutations. As convenient as this seemed, recent reports from the laboratory of Kay-Uwe Wagner have revealed that a population of cells, despite expressing markers of lactational differentiation, persist in the gland after involution and proliferate upon subsequent rounds of pregnancy and lactation (59, 85, 86). This indicates that a population of cells with stem/progenitor characteristics differentiate but do not die upon involution. These findings, then, do not support the large-scale differentiation and apoptosis theory for the breast cancer protective effect of pregnancy and lactation.

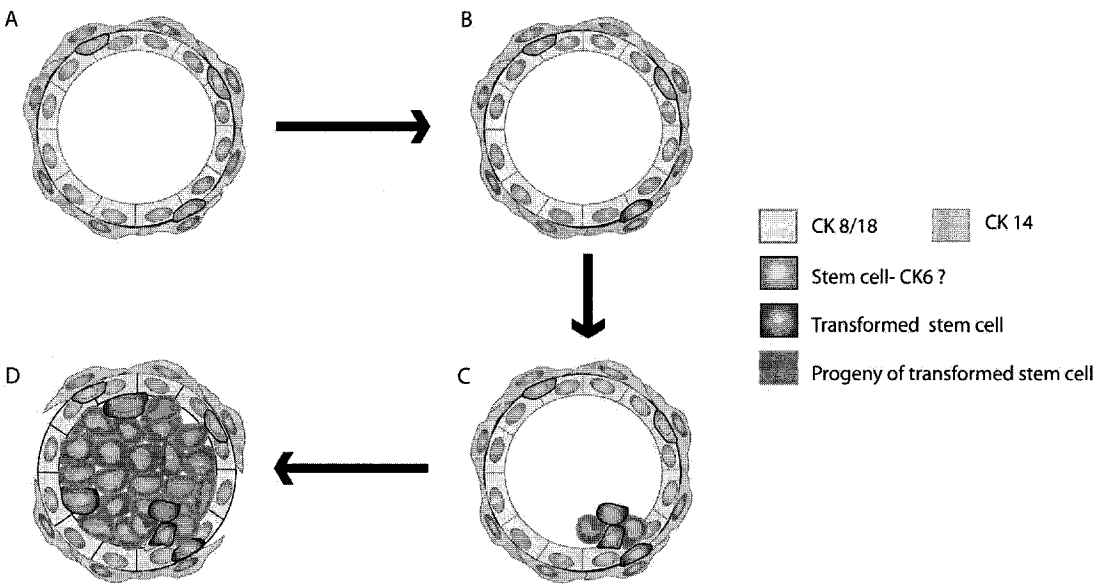

Figure 1-6 Tumor Stem Cell Theory. A. Normal mammary stem cells reside in a suprabasal location along the ducts or in TLDUs. B. Stem cells may acquire genetic hits over the lifetime of the animal leading to transformation. C. The transformed stem cell will self-renew as well as generate differentiated progeny. D. Expansion of the transformed cell population to carcinoma in situ. © 2006 Stephanie Petkiewicz

Several other hypotheses have been proposed to explain the decreased risk of breast cancer following an early first pregnancy. One hypothesis suggests that changes in the levels of expression of hormone receptors in the mammary epithelium that are observed following pregnancy render the cells less responsive to the mitogenic effects of estrogen and progesterone (87). Other groups have demonstrated

that there are permanent transcriptional-level changes in the epithelial population as visualized by clustered microarray profiles in the epithelial cells following pregnancy or treatment with pregnancy-levels of estrogens (in rats) resulting in resistance to transformation (88-90). Additionally, it has been shown that pregnancy or pregnancy-level hormones increases the sensitivity of p53 to DNA damage (91). In conclusion, the protection resulting from pregnancy and lactation is not as simple as elimination of initiated cells, but rather there appears to be some permanent change in all cells of the mammary epithelium, including the stem cell population.

2-3 Hematopoietic Cells as a Model for Breast Cancer Stem Cells

A great deal of support for the theory of tumor stem cells comes from work in the field of hematopoiesis. Many studies have demonstrated that tumors (leukemias) result from the transformation of a stem cell or early progenitor cell and result in tumors containing both progenitors and more differentiated progeny (92). The stem cell field is well developed in the hematopoietic system with identifiable pluripotent stem cells that give rise to both the myelocytic and lymphoid multipotent progenitor cells. From those two progenitor cells there are several more stages of differentiation that result in more progenitor cells and finally the full range of differentiated blood cells (figure 1-7). Each of the cells at the different stages of maturity can be identified by expression or lack of expression of a range of membrane-bound proteins and can be isolated by FACS. Some of the established hematopoietic stem cell markers such as stem cell antigen-1 (SCA-1), or the examination of CD antigens has been used to identify mammary stem cells (93).

Transformation of stem cells followed by increased proliferation and tumor growth is a well-established phenomenon in the hematopoietic system in the development of leukemias. One of the best studied leukemias with obvious stem cell transformation is chronic myelogenous leukemia (CML). The most common genetic aberration in CML is a chromosomal translocation of chromosomes 9 and 22 generating what is known as the Philadelphia Chromosome (Ph) (94) and the resulting fusion product bcr-abl (breakpoint cluster region-abelson kinase) (95, 96). The presence of a specific genetic lesion in the source cell and all its progeny, but not

in unaffected cells, of an individual implies that the leukemia began by transformation of a single cell -the tumor stem cell. Determining the cancer's clonality can be accomplished by examining cells for the presence of Ph as well as examining X-chromosomal inactivation in heterozygous females. Studies have shown that the leukemic cells all carry Ph and the same X-chromosome inactivation (when able to be studied) and the translocation is also present in differentiated, functional, myeloid, but typically not lymphoid, cells (97-99). Thus, the translocation has occurred in a progenitor population that gives rise to the myeloid lineages (figure1-7).

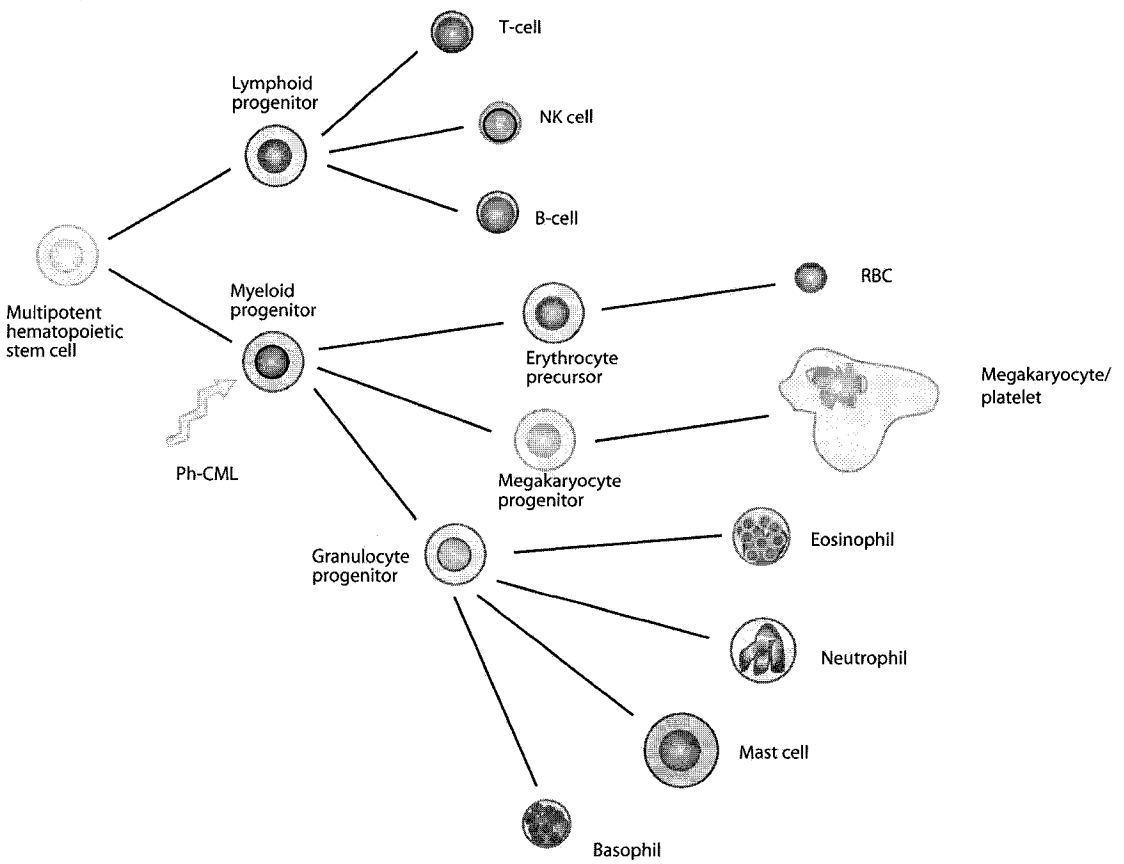

Figure 1-7 Hematopoietic Cell Lineages. Stem cells become progenitor cells and finally fully differentiated cells.

As indicated by the moniker *chronic* myelogenous leukemia, the disease progresses slowly with an overabundance of myeloid cells. However, the presence of

bcr-abl predisposes the cells to genomic instability (100-103) and progression to an acute phase called blast crisis (104, 105). When this occurs, there is hyperproliferation of a progenitor cell that, because of its failure to differentiate, overwhelms the individual with immature, non-functional cells leading to death of the individual. This series of steps towards uncontrolled tumor growth again, supports the notion that cancers consist of transformed stem/progenitor cells that continue to acquire mutations during tumor progression and generate the multiple cell types often found in breast tumors.

2-4 Evidence of Stem Cells in Tumors

The majority of a tumor mass is comprised of cells that are differentiated and not able to proliferate nor generate a tumor upon transplantation (106, 107). Studies of hematopoietic cells from leukemic patients have demonstrated that only those cells that expressed stem cell markers were able to cause the development of leukemia in transplanted mice (108, 109). Studies using human breast cancer samples have shown that following sorting of the cells for stem cell markers, that only specific populations with these markers are able to initiate tumor formation upon transplantation (106).

Support for the presence of stem cells in solid tumors has come from several studies in which populations with stem cell-like properties have been isolated from solid tumors. Al-Hajj et al. transplanted human breast tumor cells into nude mice and the resulting tumors were examined for lineage markers. Subpopulations of cells from the human tumors that were sorted by FACS for defined stem cell population markers consistently formed heterogenous tumors upon reinjection whereas those cells sorted for the more differentiated cell types did not form tumors (106). Hemmati et al. isolated from brain tumors cell populations that had characteristics similar to those of proliferating neuronal stem cells (110). Similarly, cells expressing stem cell markers and showing the ability to proliferate in culture were isolated from primary lung tumors (111). Another group isolated clones, representing a small fraction of the total cells of the tumor, from human brain tumors, that had the ability

to proliferate in suspension and differentiate into several neuronal lineages when placed in appropriate culture conditions (112).

2-5 Cancer Stem Cells-Immunohistochemical Markers

The identity and location of stem cells in solid tumor samples is being pursued using immunohistochemical markers. As mentioned in the **Stem Cells and Mammary Development** section, it is believed that stem cells reside in a basal or a supra-basal location. Thus markers of cells that reside in basal locations in normal mature or developing mammary tissue are useful markers for stem cells in tumor samples. Expression profiles of the intermediate filament cytokeratin (CK) was found to be a good classifier of different cell types. Expression of the high molecular weight CKs (4, 5, 14, 17) that are also expressed in stratified epithelium was found to be in the basal portion of the ducts and thus these became known as the basal cytokeratins (113). CK5 and CK14 are also considered to be markers of myoepithelial cells, but as myoepithelial cells also reside in a basal location, it is difficult to distinguish a progenitor cell from a myoepithelial cell by location alone. Cytokeratin 6 is a marker of hyperproliferative cells in the epithelium (114) and has been shown to be a basal cell marker that is not commonly found in the adult mammary gland (115). Various reports have indicated that CK6, in the absence of expression of other markers of differentiation, is found only in developing mammary gland (116, 117), alternatively, one report claimed that CK6 is never the sole cytokeratin expressed by a mammary cell (115). One study of multiple strains of mice from Jackson Labs claimed to have never observed expression of CK6 expression at any stage of mammary gland development and even strengthened their observation by negative results from RT-PCR (118). Unlike the confused findings on markers of basal cells, the cytokeratin expression profile of luminal mammary epithelial cells is agreed upon. Luminal cells express the simple epithelial markers of (CK7, 8, 18, 19) (119).

Confusion in terminology has arisen because the term "basal" is no longer restricted to meaning cells with a basal localization. As stem cells are located in the basal or suprabasal location, the term "basal" is applied to a population of cells that

may have progenitor or stem cell qualities. These two definitions of basal are not yet interchangeable as there is no absolute consensus on the markers of progenitor/stem cells in the breast. For example, CK5 and CK14 are considered to be markers of myoepithelial cells, however, the expression of these markers is not restricted to fully differentiated myoepithelial cells (120, 121) and as myoepithelial cells reside in a basal location, it is difficult to distinguish a progenitor cell from a myoepithelial cell (122). Additionally, p63, a marker of myoepithelial cells (123-126) is also a marker of proliferative cells (127) which would reside in a basal location, again adding to the difficulty distinguishing between basal and myoepithelial cells. The confusion of lineage specificity does not extend to markers of luminal cells (CK7, 8, 18, 19), however, where these markers are always restricted to cells of luminal differentiation and location (122, 128).

Thus, a model has been proposed for epithelial cell differentiation and timing of expression of specific markers which suggest that the progenitor population expresses CK5 but then progresses to co-expression of CK5/CK8/18 or CK5/SMA then terminally differentiates to either luminal or myoepithelial lineages respectively (121, 129). This model is plausible, if one considers the state of co-expression as not fully committed, in light of the finding that a small percentage of cells sorted for luminal markers (CK8/18) lose expression of CK8/18 in culture conditions favorable for myoepithelial cells and differentiate into cells expressing myoepithelial markers including SMA (72).

2-6 Cancer Stem Cells- The Basal Tumor Phenotype

As early as 1983, immunohistochemical makers were utilized to classify breast cancers by the presence or absence of basal markers (130). In 1987 Dairkee et al. published the initial study linking expression of basal cytokeratins with poor prognosis in mammary tumors (131). Subsequently, multiple studies have been carried out on large numbers of breast tumors to identify the percentages of tumors that express basal cytokeratins and how these IHC-determined profiles relate to prognosis (132-134). Several studies have focused on CK6 expression, which is found in a select group of mammary carcinomas and have suggested that CK6

positive tumors have a more undifferentiated (basal) phenotype (119, 135-137). The same conclusions have been made using antibodies against CK14 and CK5, all of which can form keratin dimers (CK5/14, CK5/6) (138).

Using these markers of basal/progentor cells, pathologists now frequently classify breast tumors into basal and luminal subtypes. Basal tumors are those that contain cells expressing basal/progenitor markers whereas luminal tumors contain a higher proportion of cells expressing the high molecular weight luminal markers. These distinct groups of tumors are three of five groups (luminal A, luminal B, basal, ErbB2-overexpressing, normal-like) that were initially delineated by transcriptional profiling and supported by expression of various markers of differentiation (139-141). These groupings reliably separate tumors by prognosis with luminal A tumors being of the best prognosis and basal and ErbB2-overexpressing tumors having the worst prognosis (139). Similar microarray-driven tumor subtype groupings and their associations with prognosis have been confirmed by other research groups using different tumor banks (142, 143). Tumors with *BRCA1* mutations all fall into the basal subtype (138, 140, 144) as do many of the p53 mutation carriers (137, 139, 145, 146), thus associating the basal phenotype with genomic instability. Reports by Sorlie et al. have stated that the basal subtype consistently clusters as a distinct entity and they suggest that perhaps basal tumors are a completely different disease entity and perhaps their standard treatment should be reviewed (139, 140).

The range of markers being used to determine the basal subtype is expanding, partially because identification of patients with tumors with stem cell characteristics is desirable as it correlates with a poor prognosis. It is much more convenient and inexpensive to be able to identify these patients by IHC than by microarray analysis. With an increasing number of reports being published, it is becoming clear that perhaps there may not be a definitive list of markers, but rather any one tumor may contain cells expressing a certain percentage of markers commonly associated with the basal subtype. Tumors classified as basal by microarray studies or by expression of basal cytokeratins by IHC have also been shown to more frequently than the other tumor groups display the following features: overexpression of the epidermal growth factor receptor (EGFR) (145, 146), have p53 mutation or p53 nuclear localization

(137, 139, 145, 146), stain positively for p63 (144), be hormone receptor negative (136, 146) and underexpress ErbB2 (136, 137, 144, 146).

2-7 Cancer Stem Cells-Impact on Therapy

The importance of identifying patients with a basal/stem cell tumor phenotype is demonstrated by follow-up data on patients with basal tumors that reveals a much shorter survival time and more frequent relapse (139, 140). Basal cells may have characteristics that cause them to be more resistant to chemotherapy. In reconsidering the qualities of stem cells, they may include side population cells, that have the ability to efflux Hoechst dye. Hoechst dye efflux is possible if cells express one of the ATP-binding cassette superfamily (ABC) transporters. The ABC transporters are also known as multidrug resistance proteins (MDRPs) that can function to pump out cytotoxic drugs (147-150). At present, no one has done IHC for MDRPs on breast tumor samples, but when planning a chemotherapeutic regimen it might be useful to know which women have higher expression of MDRPs and may be refractory to chemotherapy. Interestingly, Yano et al. used *in situ* hybridization to localize MDRPs to the basal layer of the skin (mammary is also ectodermal derivative), thus supporting the belief that MDRPs may be expressed in basal/stem cell populations (151). In addition to being chemo-resistant, it has recently been demonstrated that progenitor cells are radio-resistant as well. Following irradiation of either whole mice or cultured primary cells, the fraction of SP or Sca-1+ cells increased compared with pre-irradiation (152).

As tumors are heterogeneous, basal/stem cells may represent only a small fraction of the cells generating the mass whereas their more differentiated progeny could form the majority of the tumor; thus, the differentiated cells may be killed off by chemotherapy or raditation therapy but the initiating stem cells or transformed progenitors could escape, remaining behind and resulting in a later recurrence. Additionally, if stem cells are a more slowly dividing population and the daughter cells are the more highly proliferative group that makes up the majority of the tumor mass then cytotoxic drugs that affect dividing cells would most likely miss the stem cells, again increasing the risk of recurrence (figure1-8).

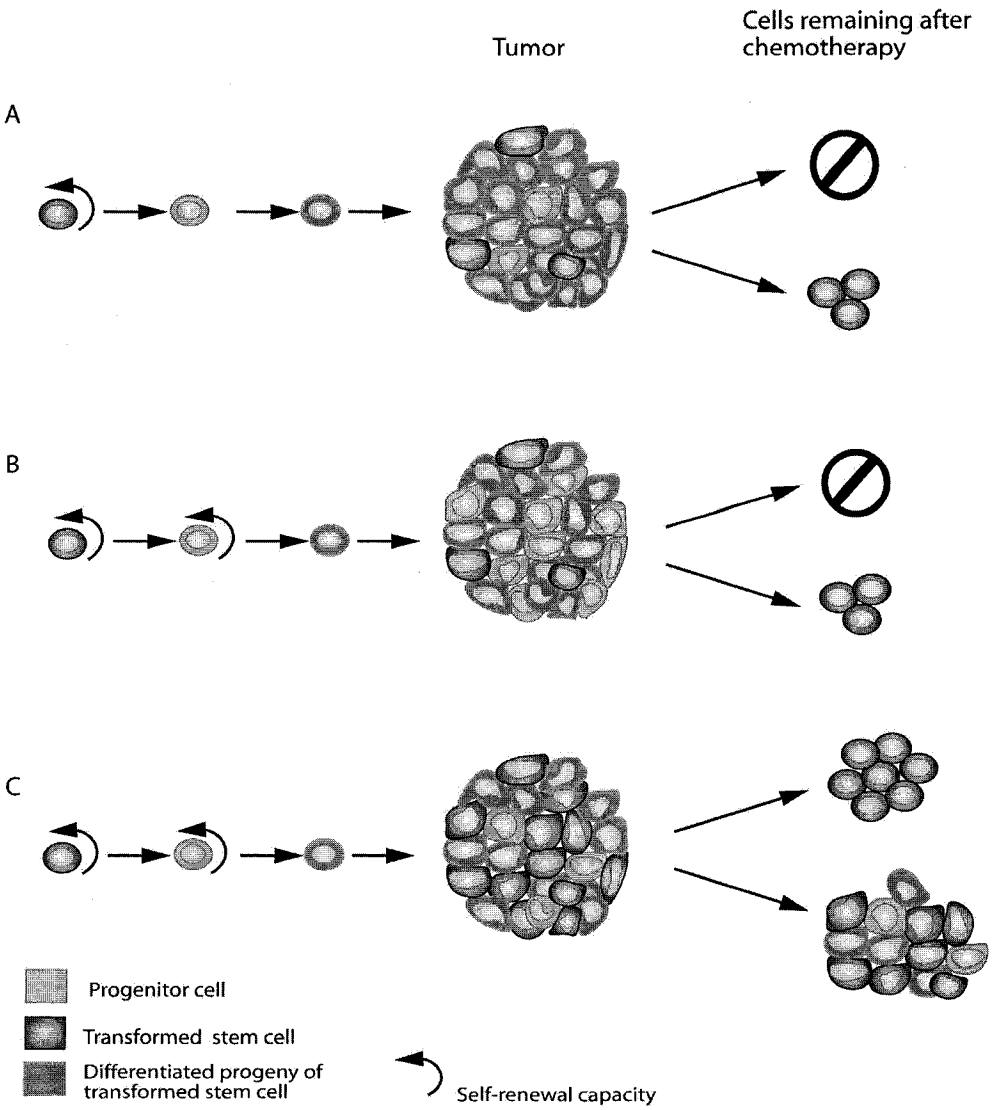

Figure 1-8 Cancer Stem Cell/Basal Tumor. Top arrow indicates results following chemotherapy that targets stem cells; bottom arrow is non-stem cell targeting. A. Only stem cell has self-renewal capacity and majority of tumor is comprised of differentiated cells. B. Both stem and progenitor cell have self-renewal capacity generating highly heterogeneous tumors but progenitors proliferate faster than stem cells. C. Both stem and progenitor cells self-renew, but stem cells have specific characteristics that made them more resistant to chemotherapy. © 2006 Stephanie Petkiewicz

Chemotherapeutic resistance of transformed progenitor cells has been demonstrated in CML patients. The kinase inhibitor imatinib targets bcr-abl and treatment results in CML "cure" in most patients. However, when the bone marrow is examined, Ph positive cells are still present although clinically patients are in cyotological remission (153, 154). These Ph positive cells frequently contain new

mutations in the bcr-abl coding sequence allowing them to escape imatinib-mediated death (155) or they may be resistant as a result of increased expression of one of the MDRPs that are highly expressed in hematopoietic progenitor cells (156).

3. Epithelial-Mesenchymal Transition

The examination of tumors for epithelial-mesenchymal transition (EMT) is another area of breast cancer research that requires the defining of markers of specific cell types. Although the definition of an EMT is still an area of debate, it is generally agreed that it is a process by which cells expressing markers of terminal epithelial differentiation and displaying an epithelial morphology with cell-cell adhesion lose the cell-cell adhesions, acquire a more fibroblastic morphology, lose markers of epithelial differentiation and gain expression of markers of mesenchymal cells. This process is well-described in embyrogenesis and wound healing, during which processes cells delaminate from a cohesive structure and migrate to other locations. In development, the EMT is frequently followed by an MET (Mesenchymal-Epithelial Transition) and the reacquisition of epithelial characteristics and stable cell-cell adhesions. Two examples of EMT in embroygenesis are the stage of gastrulation during which time ectodermal cells invaginate to form the mesoderm and the second is migration of neural crest cells away from the neural plate to form the myriad neural crest derivatives including melanocytes and ganglia in the intestine. One caviat to using gastrulation as an example of EMT is that during embryogenesis most cells are not terminally differentiated thus it may not be a true EMT. During wound healing, EMT can result in pathological conditions, especially in the kidney where EMT, in the context of chronic renal disease results in renal tubular cells differentiating into stroma-producing fibroblasts and renal failure (157, 158).

In tumors, EMT is considered to be marked by cells losing expression of cell-cell adhesion proteins, especially E-cadherin, developing a spindle cell morphology, losing markers of polarity, and gaining expression of fibroblastic markers such as vimentin and S100A4 (figure 1-9). Additionally, it is generally believed that with these characteristics, the tumor cells become more invasive thus increasing the risk of

metastasis. Several model systems have demonstrated that expressing negative regulators of E-cadherin or generating cells that, in culture, have lost markers of polarity results in a increased frequency of metastases in experimental systems compared with the parental cells (159-161). In the breast, E-cadherin is expressed throughout development by the luminal cells, but the cap cells and myoepithelial cells express P-cadherin (162, 163). Thus, loss of expression of E-cadherin could indicate a change in differentiation of luminal epithelial cells.

The spindle-cell morphology raises some important issues for pathologists who have, in the past, relied on cellular morphology and context to identify cell types and tumor phenotypes. Breast tumors that contain spindle cells were given a number of different names, indicating uncertainty of the cell of origin. They have been known as spindle cell tumors, myoepitheliomas, carcinosarcomas, metaplastic carcinomas or carcinomas with a desmoplasia. As the use of IHC to identify the constituents of a tumor has become more prevalent, myoepitheliomas have been identified as their own rare subgrouping (164-167). Metaplastic tumors are those in which specialized epithelial cells from one tissue differentiate into other derivatives of the same cellular lineage, as is observed in the development of keratin pearls from ductal epithelial cells in squamous metaplasia of the breast. The carcinosarcomas and and desmoplastic carcinomas are identified by the presence of cells expressing either luminal cell markers or markers of fibroblasts, but not within the same cell. The use of vimentin as a marker of EMT in breast tumors is confounded by the low levels of expression of vimentin by myoepithelial cells (168). High levels of expression of vimentin is found in a small fraction of breast cancer samples (169-171). A recent paper by Korsching et al. has suggested that the presence of vimentin-positive cells indicates the expansion of a stem or progenitor cell population rather than an EMT (171). Also of note is that one group, when sorting stem cells, identified vimentin as one of the markers more highly expressed in the stem cell group (70). Thus, the distinction between a true EMT tumor and a tumor containing a large number of stem cells has become blurred with further investigations into lineage markers.

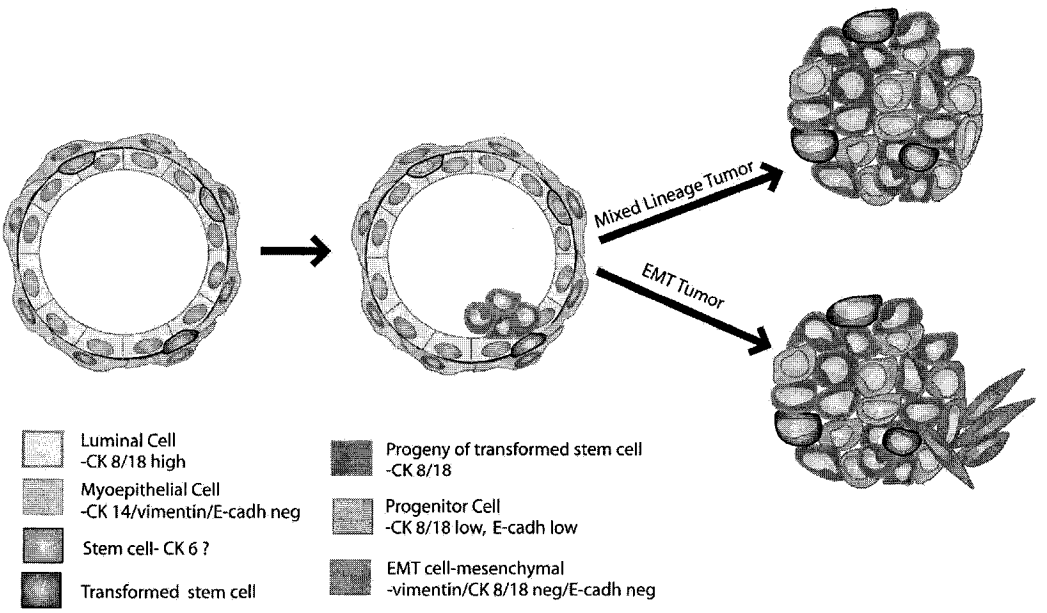

Figure 1-9 Basal Tumor vs. EMT © 2006 Stephanie Petkiewicz

4. Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTK) form a group of related transmembrane proteins with an extracellular ligand-binding domain and an intracellular tyrosine kinase domain. Signals downstream from RTKs contribute to myriad cellular functions including growth, differentiation, motility, invasion, proliferation and survival. RTKs are activated by ligand binding which induces a conformational change in the intracellular domain. The conformational change is followed by phosphorylation of tyrosine residues in the kinase domain, activation of the kinase domain and increased accessibility of both the ATP- and substrate-binding sites. These changes allow phosphorylation of the receptor itself, thereby providing specific tyrosine residues that act as docking sites to recruit downstream signaling molecules that propagate the signal intracellularly (review (172)).

The first RTK to be identified was the epidermal growth factor receptor (EGFR), isolated as a protein that could bind epidermal growth factor (EGF) and displayed kinase activity (173). EGFR was initially identified as an oncoprotein from the avian erythroblastosis virus that could transform both hematopoietic cells and

fibroblasts (174). The association with aberrant RTK signaling and cellular transformation has persisted and strengthened with time. Other RTKs including the c-Kit receptor (175), colony-stimulating factor-1 receptor (176), c-Sea (177), and the c-Ros receptor (178) were also identified from oncogenic retroviruses. At present there are 58 known genes for receptor tyrosine kinases and 30 of those are associated with human tumors (172).

4-1 HGF/Met Receptor Tyrosine Kinase-Background

The Met receptor tyrosine kinase was originally identified as an oncogenic fusion protein Tpr-Met (Translocated promoter region-Met) resulting from a chromosomal translocation in an osteosarcoma cell line treated with the carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (179-183). It was determined that the MET sequence located on chromosome 7 band 7q21-q31 coded for a tyrosine kinase (184, 185) but it was only later understood that the native MET sequence coded for a receptor tyrosine kinase rather than a cytoplasmic protein, like Tpr-Met (186-188). The ligand for the receptor was identified independently by two groups who named it scatter factor (SF) for its ability to induce cell scatter in a two-dimensional culture system (189) or hepatocyte growth factor (HGF) for its mitogenic effects on hepatocytes (190-192). The identified ligands were determined to be the same molecule (193-195) and here will be referred to simply as HGF. Knock-out mouse studies indicate that HGF and Met form an exclusive, specific ligand/receptor pair because the phenotypes of both the Met and the HGF knock-outs are nearly identical (196-198).

As a receptor tyrosine kinase (RTK), Met is a transmembrane protein that binds ligand with its extracellular domain resulting in receptor dimerization and activation of the kinase domain. Met is the prototypical member of a family of RTKs that also includes mammalian Ron/STK (Recepteur D'origine Nantais/Stem cell derived Tyrosine Kinase) (199, 200) and avian c-Sea (177). All three contain an extracellular ligand binding domain and an intracellular tyrosine kinase domain (figure 1-10). Several other DNA sequences have been identified that code for proteins with structural similarity to the extracellular domain of Met but with no

enzymatic activity in the cytoplasmic domain (201). The ligand for Ron is macrophage stimulating protein (MSP) which has a structure that is highly similar to that of HGF (202-204). In chickens, c-Sea, the cellular homologue of the oncogene v-Sea (177) may be the orthologue of Ron as it was found to bind and be activated by chicken-MSP (205) and no orthologues of c-Sea have been found in mammals nor have other Ron orthologues been found in birds. In pufferfish, however, a Met orthologue has been identified, as have potential orthologues of c-Sea and Ron, making it the only organism known to express all three family members (206).

Met is synthesized as a 150 kDa precursor polypeptide that becomes heavily glycosylated to generate a 170 kDa precursor protein that is further glycosylated and cleaved at a furin site (207, 208) to form a 50 kDa extracellular α-chain and a 140 kDa transmembrane β-chain that contains the intracellular kinase domain (209, 210). The two chains are joined at the C-terminus of the α-chain and N-terminus of the β-chain by a disulphide bond. The extracellular ligand-binding domain requires both the α- and β-chains to generate the sema domain (211-213), a domain that is critical for binding of HGF. Crystal structure studies using HGF fragments have indicated that Met binds to dimerized HGF resulting in receptor dimerization (214, 215).

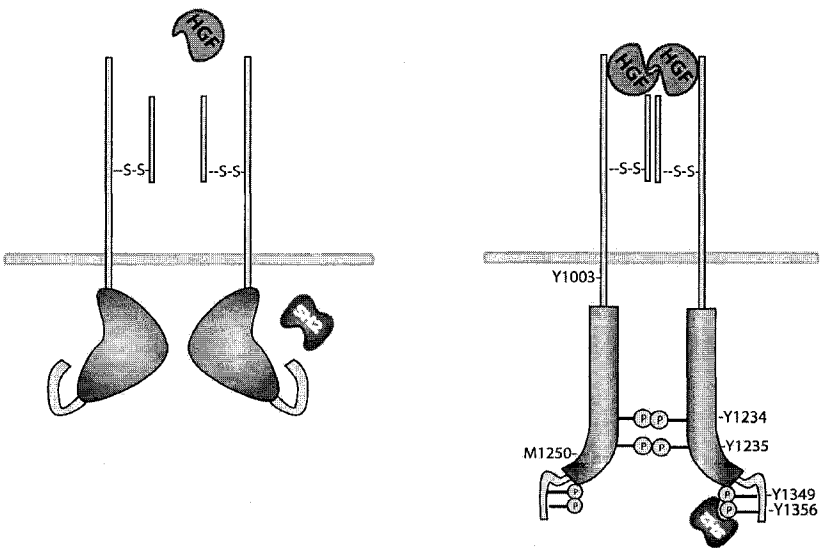

Figure 1-10 **Met Receptor Dimerization**

Met is expressed by epithelial cells in a number of tissues including: liver (216, 217), kidney (216, 217), pancreas (218, 219), intestine (216), esophagus (220), stomach (220), lung (217) breast (221, 222) and tooth germ (223). It is also expressed in endothelial cells (224), hematopoietic lineage cells (225-227) and melanocytes (228, 229). Its role in embyrogenesis is apparent by the embryonic lethality of the Met knock-out mice. The Met-null mice die at approximately embryonic day 14.5 and have defects in placental and liver development as well as failure of muscle cell precursors to migrate into the tongue, diaphragm, and limbs (196).

4-2 HGF

As mentioned above, HGF was discovered by two different groups calling it either scatter factor (SF) (189) or hepatocyte growth factor (HGF) (190-192) depending on the biology observed following treatment of different cell types. HGF is produced by stromal cells in a number of tissues including breast (221, 222), liver (230), kidney (231, 232), stomach (233), lung (234), dental papillae (235), and uterus (236). HGF is also produced by a number of blood cells including platelets (190), macrophages (237), monocytes (226), and endothelial cells (237).

HGF is a heterodimeric protein consisting of a 69 kDa α-chain and a 34 kDa β-chain joined by a disulphide bond (238). Like Met, HGF is synthesized as a single chain precursor that is cleaved in order to generate the heterodimer. There are several serine proteases that are capable of cleaving and activating HGF including hepatocyte growth factor converting enzyme (239), thrombin (240), and both urokinase type and tissue type plasminogen activators (241, 242). Uncleaved HGF is stored in the extracellular matrix by binding to heparin (191, 243) and glucosamino glycans such as heparan sulfate (244) and dermatan sulfate (245). This low-affinity binding (246) to the extracellular proteins limits the diffusion of HGF, however, there have been reports of liver injury inducing increased HGF expression in the lung (237) and high serum concentrations of HGF being associated with various pathological conditions (247-250). HGF can also be released by the breakdown of heparin by heparin hydrolases that are produced by platelets, lymphocytes and mast cells

following tissue injury (251). Tissue injury also upregulates production of hepatocyte growth factor activator (HGFA). HGFA has a weak affinity for heparin that is increased during injury, thus putting the activator and the inactive HGF in proximity to each other and allowing for the release of HGF from surrounding matrix (252).

Upon cleavage, the α-chains fold into four kringle domains that are involved in the interaction with heparin and the binding to Met. There is a naturally occurring splice variant of HGF (NK2) that retains the first two of kringle domains and the N-terminus. It can act as weak Met agonist, inducing scatter of human mammary epithelial cells (253) and exerting a mitogenic effect on lung cells in culture (254) or it can behave as an antagonist by competing with full length HGF for Met binding (255). The mitogenic signal stimulated by HGF or NK fragments is enhanced by binding to heparin (254, 256). A synthetic splice variant, NK1, contains only the N-terminus and the first kringle domain. *In vitro* studies using NK1 have shown it to have both agonistic activity (254, 257) and antagonistic activity (258), depending on the cell type treated. The NK4 fragment is solely a Met antagonist, most likely due to competition for Met binding but not inducing an HGF-like signal (259).

4-3 HGF/Met Receptor Functions *In Vivo*

HGF/Met plays a critical role in embryogenesis as demonstrated by the embryonic lethality of the HGF and Met knock-out mice (196-198). Met and HGF functions are required for proper placental development; knock-out animals have a smaller labyrinthine region of the placenta resulting from a decreased number of trophoblasts and a poorly developed vascular bed (198). Intrauterine injections of HGF to HGF-null mice can rescue the embryonic lethality, but the pups die immediately after birth as a result of still incomplete development of the diaphragm (260). HGF/Met signaling is also required for the migration of muscle cell precursors into the limb buds and tongue as seen by their absence from these locations in the Met-null mouse (196). Interestingly, overexpression of HGF under a ubiquitously expressed promoter induces aberrant muscle cell migration into the central nervous system leading to paralysis of the transgenic mice (229). Met signaling is also involved in the development of the liver (196, 197, 261, 262), kidney (261, 263),

intestine (261, 262, 264), pancreas (262, 265), breast (221, 266, 267) and lung (261, 268). Note that the majority of these tissues require the development of a branched tubular network, a function associated with HGF/Met signaling (269).

HGF/Met signaling plays an important role in tissue repair and HGF production is frequently upregulated in injured tissues. One way in which Met signaling contributes to tissue repair is through the induction of angiogenesis. HGF acts directly on endothelial cells inducing them to migrate, invade, proliferate and organize into tubular structures *in vitro* (224, 270-273). *In vivo* studies have also demonstrated a role for HGF in the development of new blood vessels; implantation of HGF pellets into the cornea or subcutaneous tissue induces the extension of existing blood vessels into previously avascular areas (274, 275). Both Met and HGF are expressed by endothelial cells (224, 237) and there is cooperation in the induction of angiogenesis with the potent angiogenic factor vascular endothelial growth factor (VEGF). HGF has been shown to increase the expression of VEGF from smooth muscle cells (271) that encircle blood vessels and from keratinocytes, indicating HGF's role in healing skin wounds (276). The importance of Met/VEGF cooperativity in angiogenesis was highlighted by a study in which VEGF pellets were inserted into the cornea, inducing vascular development; however, co-treatment with the Met-antagonist NK4 inhibited VEGF-mediated angiogenesis (277).

Met also has a general role in cell mitogenesis and migration following injury. Multiple studies have shown that partial hepatectomy, liver disease, or treatment with a liver-damaging agent results in increased expression of HGF from the liver (191, 192, 243). Transgenic mice that overexpress HGF in the liver recover from partial hepatectomy much faster than their nontransgenic controls (278). Additionally, studies of liver-specific knock-out of HGF demonstrated that HGF production is required for proper liver regeneration (279). Serum levels of HGF are also seen to rise following damage to kidney (280), lung (281) or heart (282). HGF/Met signaling can even prevent tissue damage, as in the condition of chronic renal disease. Levels of TGF-β rise in chronic renal disease while levels of HGF fall (283), leading to development of fibrosis that impairs kidney function, however treatment of kidney tissue with HGF results in decreased fibrosis and increased proliferation of renal

tubular cells (284), making HGF an excellent option for treating chronic renal disease.

4-4 HGF/Met Receptor Functions *In Vitro*

HGF/Met signaling is pleiotropic *in vitro* acting as a mitogen, motogen, and morphogen. HGF was initially described a growth factor for primary hepatocytes (192, 285), however, the mitogenic effects of HGF/Met signaling are present in a number of other cell types including keratinocytes, renal tubular cells and colonic epithelial cells (286-289). As indicated by the appellation scatter factor, HGF/SF is well-known for its ability to induce primary epithelial cells or cell lines to scatter in a two dimensional culture system (189, 290, 291). Scatter is the result of coordinated temporary breakdown of adherens junctions (292) and reorganization of the cytoskeleton (293, 294). Supporting the role of HGF in angiogenesis, endothelial cells in culture can be induced to migrate following HGF treatment (224, 291). The HGF-mediated effect of inducing cell migration is seen in invasion assays as well. HGF treatment of cells can stimulate break-down of the matrix by inducing production of proteinases (295, 296) leading to cellular invasion as measured by a modified Boyden chamber assay (297-300).

Met signaling also can induce endothelial cells, as well as several types of epithelial cells, to undergo a morphogenic program of tubulogenesis in a three-dimensional culture resulting in the formation of a branched tubular structure with a hollow lumen (301-306). Madin-darby canine kidney (MDCK) cells have been used extensively to study Met's ability to induce tube formation. Tubulogenesis is a complex process that involves cell proliferation, migration and invasion to advance the cells away from a primary hollow cyst, apoptosis for lumen formation and polarization with apical surfaces of the epithelial cells facing inward (269, 307).

Met is commonly identified as a factor that induces EMT *in vitro*, however, this is not strictly correct because the Met-induced changes are transient. The scatter induced in two dimensions by HGF treatment is an EM-like transition in which the cell-cell adhesions are only temporarily lost and adhesion proteins are internalized (292). A dramatic EM-like transition is observed in a three-dimensional culture

system when MDCK cells expressing an activated ErbB2 receptor are treated with HGF. MDCK-ErbB2 cells form tubular structures in the absence of HGF but with the addition of HGF, the cells dissociate and invade the matrix. These behavioral changes are accompanied by internalization of E-cadherin and thus loss of cell-cell adhesions (299). As the definition of EMT requires that there be loss of epithelial markers, these morphogenic changes do not qualify as E-cadherin was not completely lost nor was the expression of fibroblastic markers examined. Thus, with the increasingly stringent definition for EMT, it may be time to reconsider HGF/Met signaling as an inducer of EMT.

4-5 Met Signaling

Met signaling is induced by receptor dimerization and phosphorylation of tyrosine residues (188) in the kinase domain, thereby activating the kinase (308, 309). Two tyrosines in the c-terminus (1349, 1356) serve as multisubstrate binding sites where the protein complexes assemble that generate the downstream signals (309, 310). The docking protein Gab1 (Grb2-associated binder 1), binds Met indirectly through Grb2 at Y1356 and directly at Y1349 (311), and was shown to be the major phosphorylated protein in HGF-treated epithelial cells that are undergoing tubular morphogenesis (312). Additionally, Gab1 binding and function is required for the induction of branching morphogenesis (313, 314). The importance of Gab1 downstream from Met is demonstrated by the Gab1 knock-out mouse that displays a phenotype nearly identical to that of the Met or HGF knock-out mice (315). Tyrosine 1356 serves as a docking site for a number of other downstream signaling proteins such as Grb2 (314, 316, 317), Shc (317), p85 subunit of PI3K (318), SHIP (319), SHP-2 (320), phospholipase Cγ1 (316) and Stat3 (321).

Following HGF stimulation there is robust activation of the mitogen-activated protein kinase (MAPK) and PI3K pathways as seen downstream of a number of other RTKs (322). The MAPK pathway is associated with the mitogenic signal downstream from RTKs whereas Akt phosphorylation, as observed following HGF stimulation, is generally downstream from PI3K and associated with cell survival (323, 324). Dissection of the pathways activated and required for Met-mediated

scatter or morphogenesis reveals that the signals involved in HGF-mediated tubulogenesis cannot be restricted to one linear route. Inhibition of either the MAPK or PI3K pathways impairs Met-induced cell scatter (292, 325), invasion and morphogenic effects (326). Cytoskeletal rearrangments are mediated in part by Met's activation of Ras, Rac and Cdc42 (293, 327); and Rap1 activation through the adaptor protein Crk also plays a role in cell scatter and invasion (300, 328, 329).

4-6 Met Receptor Activation and Downregulation

Dysregulation of RTK signaling can occur through a variety of means including genomic amplification, stabilization of mRNA or protein, activating mutations, chromosomal translocation, loss of negative regulation or production of both ligand and receptor within one cell to generate an autocrine loop. Genomic amplification resulting in Met overexpression has been noted in gastric (330) and esophageal (331) cancers. Overexpression leading to high concentrations of Met at the plasma membrane can result in ligand-independent receptor dimerization and activation (332). Chromosomal translocation is another method of generating dyregulated RTK signals. The original oncogenic form of Met resulted from chromosomal translocation (179, 181) that replaced the extracellular portion of the Met receptor with a leucine zipper dimerization motif lead to constitutive dimerization and activation of the kinase domains (333). Spontaneous expression of Tpr-Met in tumors and cell lines has been examined and various groups have stated both that they have (334-336) and have not (337, 338) detected Tpr-Met in a number of tumors, in particular gastric cancer. Generation of an autocrine loop has been observed in a number of tumors expressing both HGF and Met (339). Changes in Met mRNA or protein stability in the context of disease has not yet been studied.

Loss of negative regulation is a developing area of tumor biology. With the loss of negative regulation, RTKs are not degraded as efficiently as wild type receptors and following activation, may display prolonged signaling. Tpr-Met, although dimerized and activated by the addition of the leucine zipper (333), has further enhanced oncogenic activity as a result of the loss of a binding site for a ubiquitin ligase (340-342). The critical tyrosine residue, Y1003, is located in the

juxtamembrane domain. When phosphorylated, Y1003 functions as a Grb2-independent binding site for the ubiquitin ligase Cbl and a Y→F substitution at that site results in decreased receptor ubiquitination and prolonged receptor phosphorylation and activation of the MAPK/ERK pathway (343). Adding back the Cbl-binding motif to Tpr-Met or a Tpr-Met targeted to the membrane reduces its transforming ability [(341) Mak, H et al. *submitted*]. Mutations in the juxtamembrane domain of Met have been found in lung cancer (344) as well as deletion of exon 14 which removes a large portion of the juxtamembrane domain that includes the Cbl-binding motif (345).

Activating mutations have also been found in the Met receptor in the context of cancer. The best characterized activating mutation in the Met receptor is a M→T mutation in the P+1 loop of the kinase domain. This mutation was originally discovered in patients with hereditary and sporadic papillary renal carcinoma (346). Studies have shown that cells expressing a Met receptor carrying this mutation are transformed in both *in vitro* (347-350) and *in vivo* assays (350, 351). A homologous mutation is present in the RTK Ret in patients with multiple endocrine neoplasia 2B (MEN2B) (352), thus this particular site in the kinase domain is critical for maintaining appropriate, regulated Met receptor signaling. Both structure modeling studies (353) and biochemical assays have indicated that the presence of this mutation in Met results in decreased substrate specificity which includes the acquiring the ability to bind Stat3 (347, 354). Several studies have demonstrated that Met M1250T has increased kinase activity as shown by phosphorylation of an exogenous substrate (347, 349) however, studies in our lab have not shown this to be the case [Musallam L, unpublished results].

4-7 Met Receptor Tyrosine Kinase-Role in Cancer

The Met receptor, as the Tpr-Met fusion protein, was originally identified as a transforming factor from a carcinogen-treated osteosarcoma cell line (179), thus, from the beginning, dyregulated Met receptor signals have been associated with cellular transformation. The Tpr-Met fusion protein has been shown to induce anchorage-independent growth (341, 355) and focus formation in fibroblasts (316, 356) as well

as tumor formation following subcutaneous injection of Tpr-Met-expressing cells into nude mice (355, 357). Met receptor dysregulation is found in human cancers, in particular in human papillary renal carcinoma where the same kinase-domain mutations were found in both spontaneous and hereditary cancers (346, 358-360). Met receptor mutations or deletions have been found in a number of other human tumors including childhood hepatocelluar carcinomas (361), mesotheliomas (362), gastric (363), ovarian (364), oropharyngeal (365), and lung cancer (345). Overexpression of Met has been noted in a number of solid cancers such as esophageal (331), colorectal (366, 367), stomach (330, 368), mesothelioma (369), head and neck cancers (370-375), thyroid (376-378), lung (379), bladder (380) and breast (381-385). Several cases of esophageal adenocarcinoma (331) and scirrhous gastric cancer (330) were found to have amplification of the Met locus with concurrent overexpression of Met receptor noted in the esophageal cancers.

Tumor growth is dependent on angiogensis and as mentioned above, Met also plays a role in angiogenesis. Studies of sarcomas (386) and mesotheliomas (369) have shown a correlation between Met expression and microvessel density. The increased vascular density may aid tumor growth by preventing hypoxia and necrosis but it may also play a role in metastasis by providing more routes for tumor cell shedding into blood vessels. Additionally, HGF has been shown to promote formation of lymphatic vessels (387, 388) which could also provide a route for metastatic spread of tumors.

Met signaling in tumors may also promote the formation of metastases. A number of animal models of metastasis have demonstrated that increased levels of expression of HGF can facilitate the formation of distal metastases. Tail vein injection of Tpr-Met-transfected or murine Met-transfected NIH3T3 cells that endogenously express HGF both resulted in 100% of the injected mice developing lung metastases whereas only 18% of control developed lung metastases (389). A similar experiment using transfected leiomyosarcoma cells expressing both HGF and Met demonstrated that the autocrine loop promoted formation of metastases following both tail vein injection and subcutaneous tumor development (390). Serum HGF levels have been found to be elevated in breast (248, 391-393), lung (394), and liver

cancers (395, 396), and particularly in breast, higher serum HGF levels correlate with more advanced disease and more frequent metastases (391-393). To model the state of high serum HGF and to observe its effect on tumor growth and metastasis, Yu and Merlino injected melanoma cells expressing high levels of Met into the tail vein of transgenic mice that overexpress HGF and found significantly more lung metastases developed in the transgenics than in the non-transgenic mice, demonstrating the role of HGF in promoting the development of metastases in tumors that express Met (397).

5. Met Receptor Functions in Normal Mammary Gland

HGF and Met are both expressed in the normal mammary gland and are involved in the development of the epithelial ductal tree. Studies in rat (222) and mouse (221) have shown that levels of both receptor and ligand are highest during adolescence, early pregnancy and involution-periods of ductal development and glandular reorganization- but expression is nearly undetectable during late pregnancy and lactation-periods of cellular differentiation. In normal glands, Met expression is limited to the mammary epithelial cells (305) whereas HGF is expressed mainly by the stromal cells (398). Several *in vitro* and *in vivo* experiments have examined the role of the HGF/Met signaling in the mammary gland. Early studies of the effect of HGF on various epithelial cell types revealed that HGF treatment of breast epithelial cell lines in three-dimensional culture leads to the formation of a branched tubular structure (301, 304). In contrast, under similar culture conditions neuregulin, an EGFR ligand, induces the formation of alveolar structures (267). Whole, excised mammary fat pads from young mice developed highly branched ductal trees when treated with HGF whereas the untreated glands had a poorly developed ductal system and blocking HGF with antisense oligonucleotides prohibited ductal outgrowth (399). Increased ductal branching was observed when mammary epithelial cells overexpressing HGF were transplanted into cleared mammary fat pads (266). Additionally, a transgenic mouse overexpressing HGF under the control of the mammary-specific whey acidic protein promoter developed an overly branched

ductal tree (400). All of these studies support a role for HGF/Met signaling in the development of a branched ductal system in the mammary gland.

5-1 Met/HGF in Human Breast Cancer

Met is overexpressed in ~20% of breast cancers (381-383). Studies of samples from both patients with lymph node metastases and those without lymph node metastases have shown that high levels of Met expression in the primary tumors predicts a shorter disease-free survival and a higher risk of recurrence (381, 384, 401, 402). A microarray study of breast cell lines found Met overexpression in those lines that grouped with the Basal subtype of breast cancer, which, again, is associated with poor prognosis (403). Changes in ligand levels are also seen in breast cancer patients; high serum levels of HGF have been correlated with a shorter disease-free interval following surgery in breast cancer patients (404) and a higher tumor/lymph node/metastasis (TNM) score in women with invasive breast cancers (248, 391-393).

5-2 Met Receptor Related Mouse Models of Breast Cancer

The role of HGF/Met receptor signaling in the development or progression of breast cancers has been supported by several transgenic mouse models that develop mammary adenocarcinomas. Mice generated utilizing the ubiquitously expressed metallothionein (MT) promoter demonstrate the sensitivity of breast tissue to dysregulated Met signaling. The first Met transgenic generated was the MT-Tpr-Met mouse. These mice developed a number of different of tumors but the most common was mammary adenocarcinoma in parous mice at approximately 381 days old with a penetrance of 42% (405). The MT-HGF mouse developed various carcinomas and a few sarcomas; the most prevalent tumor, again, was the mammary adenocarcinoma, which developed in 41% of the females older than 6 months, regardless of whether or not they had bred (406). Interestingly, more than 50% of MT-HGF mice died by 6 months of age as a result of intestinal obstruction, progressive renal failure or paralysis that resulted from striated muscle growing ectopically in the central nervous system (229).

Other MT-HGF-related mice have been studied. The synthetic HGF splice variant NK1 was put under the MT promoter and was found to have a phenotype nearly identical to the MT-HGF mouse (407). NK1 contains only the N domain first kringle domain of HGF (258) and can act as a Met receptor antagonist (258) or a partial agonist (254, 257) depending on the cell type examined. Human mammary epithelial cells had demonstrated a partial Met receptor agonistic activity however, it antagonizes HGF activity, potentially by competing for binding (214, 257). MT-NK1 transgenics displayed precocious mammary alveolar development and a susceptibility to mammary adenocarcinomas as well as melanomas and hepatocellular carcinomas (407). More recently, Gallego et al. generated mice with mammary-specific expression of HGF using the Whey Acidic Protein (WAP) promoter (400). Nulliparous WAP-HGF females developed hyperplastic ductal trees with lobular outgrowths from the ducts as well as an increased number of fibroblasts surrounding the ducts. Parous mice lactated normally but the abnormal ductal tree was evident again at involution. Within ten months of continuous breeding 49/55 mice developed mammary tumors, many of them multifocal and some with lung metastases. Additionally, 10/50 virgin females developed mammary tumors before 1 year of age. The histology of these tumors was better described than in the other models and it was noted that frequently the tumors were a combination of glandular adenocarcinoma and squamous metaplasia (400).

The first full-length Met receptor transgenic mouse was generated to examine the effects of two Met receptor mutants that were isolated from patients with papillary renal carcinoma. One MT-Met founder mouse from each strain developed a mammary adenocarcinoma with distal metastases (351). Graveel et al. generated Met receptor knock-in mice in which they inserted a panel of mutations found in papillary renal carcinoma into the endogenous locus (408). These mice developed a range of tumors-mostly sarcomas and lymphomas-often with long latency. Only the wt Met and the Met-M1250T mutant mice developed carcinomas but no mammary carcinomas were noted (408). The failure to develop mammary tumors may be related to the strain of mice utilized for initial experiments. The Met knock-in lines have now been back-crossed from a mixed background to the FVB/N background and

in the FVB/Ns, mammary tumors develop with high frequency (C. Graveel, personal communication). A third Met receptor transgenic utilized a tetracycline responsive promoter to induce wt Met receptor overexpression in the mammary gland (409). None of the wt Met transgenics displayed any abnormal mammary phenotype, however transplantation of primary mammary epithelial cells infected with a retrovirus expressing tetracycline-on wt Met resulted in the formation of "nonprogressing nodules." When Myc was co-expressed with wt Met, adenocarcinomas developed that contained an unusually high number of progenitor cells (409). Thus, it appears that wt Met is not sufficient to induce mammary tumorigenesis, however, HGF overexpression is.

6. Non-Met RTK Transgenic Mice

Many subfamilies of RTKs have been used to generate transgenic mice with mammary gland phenotypes. The best characterized models of RTK-driven breast cancer are the MMTV-ErbB2 mice. As will be discussed under **Pathway Pathology** the tumors that arise are consistently solid, nodular adenocarcinomas with large nuclei containing open chromatin (410). Expression of an activated ErbB2 receptor under MMTV results in mammary tumors at approximately 90 days of age and 100% penetrance in multiparous animals (411). Overexpression of the related receptor EGFR under the control of MMTV or the β-lactoglobulin promoter also leads to the development of mammary adenocarcinomas in 17-33% of multiparous mice but latency and histological details are not given (412).

The colony stimulating factor-1 receptor (CSF-1R) and ligand (CSF-1) transgenics also develop mammary tumors and highlight the importance of stromal-epithelial interactions in the development of mammary tumors (413). Although the name CSF-1 indicates a role in hematopoiesis, and it plays an important role in attracting and simulating macrophages, CSF-1 also plays an important role in the development and functioning of the mammary gland as demonstrated by CSF-1 knock-out mice. CSF-1 null mice display incomplete growth of the mammary ductal tree accompanied by precocious lobuloalveolar development but lactational failure

(414). Approximately 50% of MMTV-CSF-1 or -CSF-1R mice developed mammary adenocarcinomas at over 12 months old and the tumors show large numbers of macrophages in and around the tumors, possibly contributing to tumor growth (413).

The Met family protein Ron has also been used in transgenic experiments, in particular in the lung where targeted overexpression induces tumor formation (415, 416). In the mammary gland, loss of Ron signaling does not affect mammary development or function (417), but when mice expressing a kinase-dead Ron are crossed with the MMTV- polyoma Middle T (PyMT) mice there is an increase in tumor latency and decrease in tumor burden. The Ron-deficient/PyMT tumors also had decreased vascularity, decreased proliferation and increase apoptosis contrasted with PyMT-alone, demonstrating that Ron expression contributes to PyMT-initiated tumor development (418). Recently, MMTV-Ron-wt and Ron-M1231T (a mutation analogous to the M1250T mutation in Met) mice have been described. By 390 days, all mice had developed mammary tumors and 91% of MMTV-Ron-M1231T mice were shown to have lung and/or liver metastases, again supporting the role of Ron in mammary tumorigenesis and induction of an invasive program (419).

All of these models support the notion of RTK signalling playing a critical role in development and maintenance of the mammary gland. Overexpression or knock-out of an RTK can lead to developmental defects as seen with the CSF-1 knock-out or increased frequency of tumors as with the dystregulated ErbB2 transgenics.

7. Tumor-Stroma Interactions

The majority of transgenic animals target expression of the gene of interest to the mammary epithelium, however, it is critical that we not neglect the role of the surrounding stroma. The stroma contains stores of growth factors, such as HGF, that bind to proteoglycans and can be easily released *en masse* (420). Fibroblasts residing in the stroma produce growth factors for nearby epithelial cells as well as for cells of the immune system that are recruited during inflammation, injury and tissue remodelling (421). Genetic lesions accumulate in fibroblasts as well as epithelial

cells, and senescent fibroblasts secrete more growth factors than those of younger animals (422, 423). Dysregulation of the growth-promoting function of the stroma and the changes that occur in the stroma over time can influence tumor development as demonstrated by transplant studies in which irradiated, cleared MFPs induce otherwise non-tumorigenic cells to form tumors (424) or when pre-neoplastic cells are co-injected with senescent fibroblasts form tumors faster and with greater frequency than the same cells injected with presenescent fibroblasts (425).

7-1 Myofibroblasts

The tumor-stroma interactions are reciprocal with the tumor exerting an influence on the stroma as well. Tumor induced changes in the stroma are revealed by studies that utilize breast-derived cancer-associated fibroblasts (CAFs) rather than normal tissue fibroblasts to enhance "take" rates in transplantation and to induce non-tumorigenic cells to form tumors on transplantation (66, 426). CAFs, also known as myofibroblasts, are considered to be "activated" fibroblasts, identifiable by their expression of smooth muscle actin. Myofibroblasts are a component of desmoplasia-the stromal reaction to tumor consisting of fibroblasts, extracellular matrix, and inflammatory cells (427). Myofibroblasts are also present during normal wound healing and the expression of SMA permits them to be contractile and aid wound closure (428). They also promote wound healing, and tumor growth, by producing a range of growth factors to stimulate epithelial and endothelial growth but also to recruit inflammatory cells (429). Recently, it has been shown that an important role of myofibroblasts is the production of ECM-degrading proteins, potentially aiding tumor cell invasion and metastasis (430-432).

The origin of the myofibroblast is controversial, although most seem to be derived from growth factor-activated local fibroblasts (428). Others have shown, however, that cells from circulation (433-435), and endothelial cells (436) can also contribute to the population of myofibroblasts in either cancer or tissue damage. EMT may also be a source of myofibroblasts because although tumor cell transition to myofibroblast has not been definitively demonstrated, the change from epithelial cell to SMA-expressing fibroblastic cell is well-documented in renal fibrosis (157).

Some support for tumor EMT-derived stromal cells has come from a study that found non-random X-chromosome inactivation in tumors and adjacent fibroblasts, indicating that the fibroblasts may have a common origin with the tumor cells (437).

7-2 Immune cells

The role of inflammatory cells in tumor development has been highlighted by two recent papers. Colony stimulating factor-1 (CSF-1) is a potent growth factor for macrophages and signals through the RTK CSF-1R. Nowicki et al. crossed the potent mammary tumor model, MMTV-PyMT, with CSF-1 null mice and found that although tumors in the PyMT/CSF-$1^{-/-}$ mice arose with the same latency and frequency as in MMTV-PyMT-alone the tumors in the PyMT/CSF-1 null mice did not progress to invasive, metastatic tumors as frequently as the MMTV-PyMT-alone mice. Upon histological examination, one obvious difference between the two groups of mice was the decreased number of macrophages present in the PyMT/CSF-$1^{-/-}$ tumors (438) highlighting the role that growth factor- and MMP-producing macrophages may have on tumor progression. In a second paper highlighting the importance of inflammatory cells in tumor development, mice deficient in mature B and T lymphocytes, expressing a transgene that predisposes them to squamous cell carcinoma, had decreased progression to fulminate cancer whereas those with competent immune systems had progressing lesions. Transfer of competent B and T cells to the immunodeficient mice resulted in increased frequency of tumor progression in the previously resistant mice demonstrating the role the immune system plays in tumor development (439).

The role of the stroma has been largely neglected, possibly because the range of cell types and their varying functions makes too complicated a system to manipulate. However, from these above examples, it is clear that it plays an important role in the initiation and progression of cancers.

8. Mouse Models of Mammary Tumorigenesis

Studying human tumors to find patterns in gene expression or cell differentiation or overexpression of specific proteins has yielded a wealth of information on tumor biology. However, as tumor samples have generally progressed to a point at which they can be detected and require intervention, researchers are usually examining an end point, rather than what initiated the tumor. Finding commonalities amongst tumors in tumor banks is also made more difficult by humans being a large, outbred population, resulting in a great deal of uncontrollable and unmeasurable variability, which may confound or mask similarities between samples. In order to examine initiating factors in breast cancer and to control for the vast number of genetic modifiers in an outbred population, science has utilized murine models.

There are a number of ways in which to introduce a genetic changes into the mammary gland (figure 1-11). Transgenic mice have been in use since 1982 when Palmiter et al. injected a cDNA for rat growth hormone under the control of the metallothionein (MT) promoter into the pronucleus of *in vitro* fertilized mouse eggs. The resulting pups grew significantly larger than their non-transgenic littermates indicating the presence of a stable genetic change (440). Since then, multiple studies have utilized the non-specific MT promoter to drive tumorigenesis in a variety of organs.

Specifically targeting expression of an oncogene to the mammary epithelium became possible through the use of tissue-specific promoters such as the whey acidic protein (WAP) (441), mouse mammary tumor virus (MMTV) (442), and cytokeratin14 (443). Targeting expression to the epithelium allowed researchers to observe the effects of an oncogene on the mammary epithelium in the context of normal, non-expressing stroma.

At approximately the same time that tissue-specific promoters were being developed, knock-out mice were being used to investigate the loss of function of a tumor suppressor on development of mammary tumors. The use of knock-out mice is exemplified by a paper from 1995 where Varmus and colleagues crossed their

MMTV-wnt-1 transgenics with mice missing one allele of p53 and observed a decreased latency to tumor formation (444).

The next step towards better controlling transgene expression was through the use of inducible systems in tissue-specific transgenic mice (445). Transgene expression was controlled by dietary modification (adding tetracycline to the water

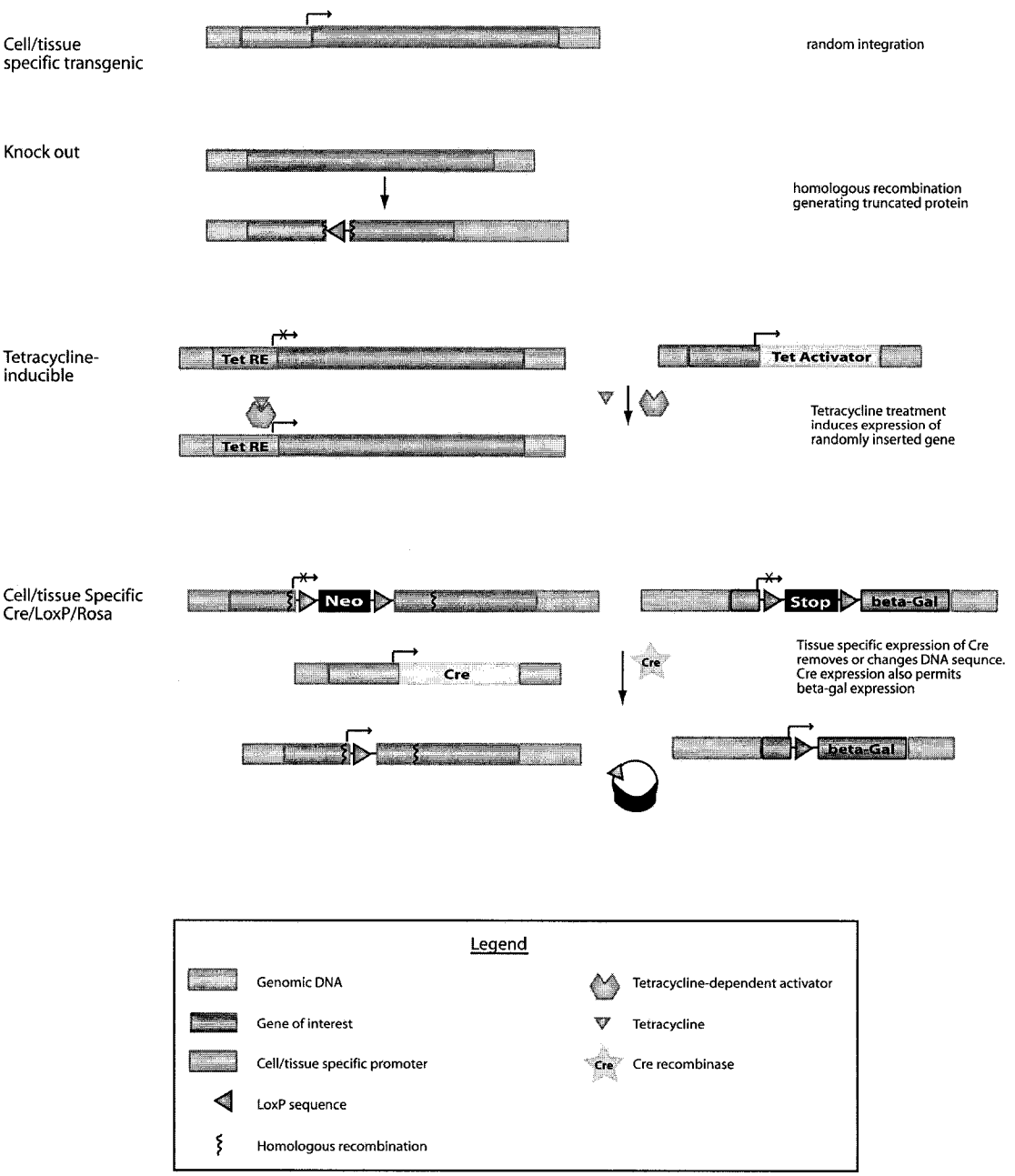

Figure 1-11 Strategies for Generating Genetically Modified Mice

allows function of the tetracycline response element ie. tet-on), researchers were able to turn on expression at various points in development or at different ages and even turn off expression once a tumor had developed thereby investigating the dependence of a tumor on the initiating event (446).

The next breakthrough in mouse model development was the use of targeted knock-in mice. Following homologous recombination, altered DNA sequences are inserted into the endogenous locus, replacing the wild type sequences. This allows for examination of the role of specific mutations in proteins being expressed under the control of their endogenous promoters with all the associated transcriptional modifiers (447).

Combining transgenic technology with knock-outs and inducible systems resulted in the development of Cre-*loxP* knock-in or knock-out mice. These mice permit induction or cessation of a signal at a specified time. Cre-recombinase is a bacteriophage P1 DNA recombinase that splices out DNA located between *loxP* sequences. The activity of the Cre recombinase can be controlled by placing *Cre* under a mammary-specific promoter or tet-on/off system. This system allows for deletion of sequences in the gene of interest yielding a conditional knock-out; alternatively removal of stop sequences preceding the gene of interest resulting in a knock-in. The Cre-*loxP* system, in combination with a line of transgenic reporter mice that express β-galactosidase (GTRosa26) (448, 449), has been used extensively by Kay-Uwe Wagner's group to examine various knock-out systems and to track cell fates through different stages of mammary gland development and tumorigenesis (59, 85, 450). The addition of the GTRosa26 reporter allows for identification of cells that have expressed Cre recombinase by activating the β-galactosidase which, in the presence of x-gal substrate, turns cells a dark blue color.

9. Strengths and Failings of Mouse Models

As mentioned in the **Mammary Development** section, there are differences in the structure of mammary gland and in some aspects of reproduction between

humans and mice, but mice are a convenient, controllable system with a short lifespan and thus a shorter latency to tumor formation. The rat mammary gland is more similar to that of the human, however, rats live longer and require more space, making them less desirable for large studies.

The FVB/N inbred line of mice is commonly used for studies of mammary tumorigenesis. This strain was derived in the 1930s from an outbred Swiss colony and established in the 1970s at the National Institutes of Health (NIH). The mice were found to be sensitive to the Friend leukemia Virus B strain and inbreeding was selected for on the basis of the $Fv1^{+}$ allele, thus the name FVB/N (451). These mice are convenient for the generation of transgenic mice for the study of mammary tumorigenesis as they have large pronuclei and are susceptible to mammary tumors. Another advantage of using the FVB/N strain is that they do not methylate the MMTV promoter, thereby shutting down expression of the transgene, to the same extent as other inbred lines do (452, 453). Unfortunately, their susceptibility to mammary tumors is detrimental to their use as a model for breast cancer because they have a high rate of spontaneous mammary tumors. A paper by Nieto et al. describes the frequent finding of mammary hyperplasias in post-lactational mice in the sample collection of the University of California, Davis Mutant Mouse Pathology Laboratory (454). Additionally, older mice from colonies at specific research institutes were found to frequently develop pituitary adenomas (455). The pituitary lesions led to lactating mammary adenocarcinomas in 67% of the multiparous mice, presenting a potential confounder for mammary tumors that arise in mice older than 18 months of age (455).

The pathology of mammary tumors generated in transgenic mice can be quite similar to those seen in humans. Firstly, location of the cell of origin for most mammary tumors in both species is believed to be the same. Spontaneous tumors that arise in inbred mouse lines develop from cells in the lobulo-alveolar units, the murine equivalent of the human terminal ductal lobular units (456), initially as hyperplastic alveolar nodules (457). These cells are the most proliferatively active cells in the ductal tree, responding to changes in hormone levels through estrus cycles and various stages of development (32). In humans, it is believed that most adenomas

also originate in cells of the terminal ductal lobular unit (458, 459) and begin as atypical hyperplasias or ductal carcinoma in situ. Despite originating in the same cells, the "spontaneous" murine tumors that result from infection with the mouse mammary tumor virus do not resemble human tumors (32). Transgenic mouse models however, such as the ErbB2 models or the E-cadherin knock-outs, do develop tumors that, aside from stromal differences, appear quite similar to human tumors that overexpress ErbB2 or lack expression of E-cadherin (460, 461). The human-like murine tumors progress through the same stages of development as do human tumors: from hyperplasia to carcinoma in situ to invasive tumors with loss of myoepithelial cells around the tumor (462). It is possible, however, to generate tumors in transgenics that do not resemble human tumors, possibly by being initiated by a transforming event that is not common in humans (463) as seen in the wnt-pathway transgenic tumors (464).

The promoter used to drive expression of a transgene or Cre-recombinase is of utmost importance in determining the tumor characteristics or if a tumor develops at all because it controls when and in which cells the potentially transforming event takes place. The WAP promoter is active in differentiated, milk-producing cells, so only after initiation of pregnancy is expression under this promoter induced, thus little expression is present during critical phases of development (465, 466). MMTV is regulated by steroid hormones so expression of genes under MMTV is enhanced during pregnancy and lactation when levels of ovarian hormones are high but is also active during the critical stages of development during puberty (467-470). Either the MMTV or WAP promoter is sufficient to drive expression in the mammary epithelium and does not express in the stroma thereby allowing examination of the role of various transgenes in an otherwise normal context.

One failing of murine mammary tumors is that nearly all tumors are estrogen receptor negative (471). Even as early as 1896, physicians had realized the dependence of breast tumors on estrogen and treated premenopausal women with advanced breast disease with oophorectomy (472). By microarray studies, a primary division of tumor samples is by hormone receptor status with estrogen receptor positive and negative tumors associated with differences in prognosis (141, 473-476).

Although in the absence of anti-estrogen therapy estrogen receptor positivity is not consistently associated with longer survival, (477, 478), it is a predictive factor for response to hormone therapy thereby bettering outcomes and prolonging disease-free survival. Estrogen receptor positive tumors remain partially dependent upon estrogen for growth stimulatory effects and thus can be treated with anti-estrogens such as tamoxifen and fulvestrant, and aromatase inhibitors that decrease the amount of estrogen being synthesized. By generating mice with estrogen receptor negative tumors, we are losing an important aspect of the growth factor signalling pathways within tumors and a factor that affects the treatment of human breast tumors.

Overall, great advances have been made in generating mice with more human-like genetic alterations. Each model system allows a slightly different question to be asked regarding the role of a transgene in tumorigenesis. The progression of model systems accompanied by more prevalent use of immunohistochemical techniques to investigate tumor phenotypes and better training of pathologists to make comparisons between murine and human tumors will allow more transferable knowledge to be gleaned from transgenic models.

10. Pathway Pathology

The concept of pathway pathology has been championed by Dr. Robert Cardiff M.D./Ph.D. at the University of California, Davis. The term refers to observing similar histological phenotypes amongst tumors generated by the same oncogene or oncogenes affecting the same signalling pathways (410, 460). Transgenic mice expressing Myc, Ras, or ErbB2 in the mammary gland are three commonly described, and well-studied, mouse models of breast cancer that demonstrate consistent phenotypes. Myc-induced tumors retain glandular structures with some strands of stroma and are comprised of large cells with bluish staining cytoplasm, pleomorphic nuclei, condensed chromatin and prominent nucleoli (410). Ras-induced tumors have a solid, nodular pattern but are oriented around blood vessels resulting in palisading nuclei and a papillary appearance. Ras tumor cells are smaller than those of the myc-tumors and have reddish cytoplasm and round nuclei

(410). Several mammary-directed ErbB2 transgenics with slight differences in the transgene and different integration sites have been generated (411, 479, 480), but all tumors from these models have a similar phenotype (410). The ErbB2 tumors are solid, nodular adenocarcinomas with zonal differentiation with more differentiated cells towards the center of the nodule and less differentiated, proliferating cells towards the outer edge. The tumors are comprised of cells with pale pink cytoplasm; large, regular nuclei, an open chromatin pattern and a small nucleolus (410). These three models display rather homogenous, solid tumors, but not all models are so simple. The wnt-pathway transgenics develop heterogeneous tumors that contain luminal, myoepithelial and progenitor cells (464). Transgenic mice for wnt-1 (481), wnt10b (482), β-catenin (481) and mutant β-catenin (483) all develop adenocarcinomas that frequently contain areas of squamous metaplasia. The presence of increased numbers of luminal, myoepithelial and progenitor cells is striking because ras and ErbB2 transgenic tumors are limited to differentiated luminal cell lineage (484). These observations suggest that not all transgenes are capable of enhancing stem cell proliferation and may be part of the reason that ras and ErbB2 tumors have a more limited range of tumor phenotypes whereas wnt tumors are more varied.

List of Abbreviations

ABC-ATP-Binding Cassette
ABC-Avidin-Biotin Conguated
CAF-Cancer-Associated Fibroblast
Cbl-Casitas B-lineage Lymphoma
CD-Cluster of Differentiation
CML-Chronic Myelogenous Leukemia
EGFR- Epidermal Growth Factor Receptor
EMT-Epithelial-Mesenchymal Transition
ErbB2-Erythroblastic leukemia viral oncogene homologue2
FACS- Fluorescence Activated Cell Sorting
FVB/N-Friend's leukemia Virus B strain ($Fv1^{+}$)-NIH-mouse strain
Gab1-Grb2-Associated Binder 1
HGF- Hepatocyte Growth Factor
IHC-Immunohistochemistry
Lin^{-}-Lineage negative
MAPK-Mitogen Activated Protein Kinase
MDRP-Multidrug Resistance Protein
MEN-Multiple Endocrine Neoplasia
MFP-Mammary Fat Pad
MSP-Macrophage Stimulating Protein
MT-Metallothionein
Ph-Philadelphia chromosome
Ron-Recepteur D'Origine Nantais
RTK-Receptor Tyrosine Kinase
Sea-Sarcoma, Erythroblastosis, Anemai
SF-Scatter Factor
TEB-Terminal End Bud
TDLU-Terminal Ductal Lobular Unit
WAP-Whey Acidic Protein
VEGF-Vascular Endothelial Growth Factor

References

1. Reis, L. A. G., Eisner, M.P., Kosary, C.L., Hankey, B.F., Miller, B.A., Clegg, B.A., Mariotto, A., Feuer, E.J., Edwards, B.K., (eds). SEER Cancer Statistics Review, 1975-2002. Bethesda: National Cancer Institute, 2005.
2. Colditz, G. A. and Hankinson, S. E. The Nurses' Health Study: lifestyle and health among women. Nat Rev Cancer, *5:* 388-396, 2005.
3. Dumitrescu, R. G. and Cotarla, I. Understanding breast cancer risk -- where do we stand in 2005? J Cell Mol Med, *9:* 208-221, 2005.
4. Hodgson, S. V., Morrison, P. J., and Irving, M. Breast cancer genetics: unsolved questions and open perspectives in an expanding clinical practice. Am J Med Genet C Semin Med Genet, *129:* 56-64, 2004.
5. Domchek, S. M. and Weber, B. L. Clinical management of BRCA1 and BRCA2 mutation carriers. Oncogene, *25:* 5825-5831, 2006.
6. Mauriac, L., Luporsi, E., Cutuli, B., Fourquet, A., Garbay, J. R., Giard, S., Spyratos, F., Sigal-Zafrani, B., Dilhuydy, J. M., Acharian, V., Balu-Maestro, C., Blanc-Vincent, M. P., Cohen-Solal, C., De Lafontan, B., Dilhuydy, M. H., Duquesne, B., Gilles, R., Lesur, A., Shen, N., Cany, L., Dagousset, I., Gaspard, M. H., Hoarau, H., Hubert, A., Monira, M. H., Perrie, N., and Romieu, G. Summary version of the Standards, Options and Recommendations for nonmetastatic breast cancer (updated January 2001). Br J Cancer, *89 Suppl 1:* S17-31, 2003.
7. Pawlik, T. M., Buchholz, T. A., and Kuerer, H. M. The biologic rationale for and emerging role of accelerated partial breast irradiation for breast cancer. J Am Coll Surg, *199:* 479-492, 2004.
8. Polychemotherapy for early breast cancer: an overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet, *352:* 930-942, 1998.
9. Katzenellenbogen, B. S. and Frasor, J. Therapeutic targeting in the estrogen receptor hormonal pathway. Semin Oncol, *31:* 28-38, 2004.
10. Buzdar, A. U. Endocrine therapy in the treatment of metastatic breast cancer. Semin Oncol, *28:* 291-304, 2001.
11. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science, *235:* 177-182, 1987.
12. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science, *244:* 707-712, 1989.
13. Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J., and Norton, L. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med, *344:* 783-792, 2001.

14. Wakeling, A. E. Inhibitors of growth factor signalling. Endocr Relat Cancer, *12 Suppl 1:* S183-187, 2005.
15. Miller, K. D., Chap, L. I., Holmes, F. A., Cobleigh, M. A., Marcom, P. K., Fehrenbacher, L., Dickler, M., Overmoyer, B. A., Reimann, J. D., Sing, A. P., Langmuir, V., and Rugo, H. S. Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. J Clin Oncol, *23:* 792-799, 2005.
16. Kumar, V. Robbins and Cotran Pathologic Basis of Disease, 7th edition. Philadelphia: Elsevier Saunders, 2005.
17. Rosen, P. P. and Oberman, H. A. Tumors of the Mammary Gland. Atlas of Tumor Pathology, Third Series, Fascicle 7. Washington, D.C.: Armed Forces Institute of Pathology, 1993.
18. Hovey, R. C., Trott, J. F., and Vonderhaar, B. K. Establishing a framework for the functional mammary gland: from endocrinology to morphology. J Mammary Gland Biol Neoplasia, *7:* 17-38, 2002.
19. Isaacs, J. H. E. Textbook of Breast Diseases. St. Louis: Mosby-Year Book Inc., 1992.
20. Silberstein, G. B. Tumour-stromal interactions. Role of the stroma in mammary development. Breast Cancer Res, *3:* 218-223, 2001.
21. Hennighausen, L. and Robinson, G. W. Signaling pathways in mammary gland development. Dev Cell, *1:* 467-475, 2001.
22. Kratochwil, K. Organ specificity in mesenchymal induction demonstrated in the embryonic development of the mammary gland of the mouse. Dev Biol, *20:* 46-71, 1969.
23. Cunha, G. R., Young, P., Christov, K., Guzman, R., Nandi, S., Talamantes, F., and Thordarson, G. Mammary phenotypic expression induced in epidermal cells by embryonic mammary mesenchyme. Acta Anat (Basel), *152:* 195-204, 1995.
24. Sakakura, T., Nishizuka, Y., and Dawe, C. J. Mesenchyme-dependent morphogenesis and epithelium-specific cytodifferentiation in mouse mammary gland. Science, *194:* 1439-1441, 1976.
25. Howard, B. and Ashworth, A. Signalling pathways implicated in early mammary gland morphogenesis and breast cancer. PLoS Genetics, *2:* 1121-1130, 2006.
26. Howard, B. A. and Gusterson, B. A. Human breast development. J Mammary Gland Biol Neoplasia, *5:* 119-137, 2000.
27. Naccarato, A. G., Viacava, P., Vignati, S., Fanelli, G., Bonadio, A. G., Montruccoli, G., and Bevilacqua, G. Bio-morphological events in the development of the human female mammary gland from fetal age to puberty. Virchows Arch, *436:* 431-438, 2000.
28. Copp, A. J. Neurulation in the cranial region--normal and abnormal. J Anat, *207:* 623-635, 2005.
29. Schmidt-Ott, K. M., Lan, D., Hirsh, B. J., and Barasch, J. Dissecting stages of mesenchymal-to-epithelial conversion during kidney development. Nephron Physiol, *104:* p56-60, 2006.

30. Wysolmerski, J. J., Van Houten J.N. Normal Mammary Development and Disorders of Breast Development and Function. *In:* M. Molitch (ed.), www.endotext.org, website. South Dartmouth: mdtext.com, inc., 2002.
31. Kratochwil, K. In vitro analysis of the hormonal basis for the sexual dimorphism in the embryonic development of the mouse mammary gland. J Embryol Exp Morphol, *25:* 141-153, 1971.
32. Cardiff, R. D. and Wellings, S. R. The comparative pathology of human and mouse mammary glands. J Mammary Gland Biol Neoplasia, *4:* 105-122, 1999.
33. Parmar, H. and Cunha, G. R. Epithelial-stromal interactions in the mouse and human mammary gland in vivo. Endocr Relat Cancer, *11:* 437-458, 2004.
34. Russo, J., Gusterson, B. A., Rogers, A. E., Russo, I. H., Wellings, S. R., and van Zwieten, M. J. Comparative study of human and rat mammary tumorigenesis. Lab Invest, *62:* 244-278, 1990.
35. Ramakrishnan, R., Khan, S. A., and Badve, S. Morphological changes in breast tissue with menstrual cycle. Mod Pathol, *15:* 1348-1356, 2002.
36. Robinson, G. W., McKnight, R. A., Smith, G. H., and Hennighausen, L. Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. Development, *121:* 2079-2090, 1995.
37. DeLeon, D. D., Zelinski-Wooten, M. B., and Barkley, M. S. Hormonal basis of variation in oestrous cyclicity in selected strains of mice. J Reprod Fertil, *89:* 117-126, 1990.
38. Wise, P. M., Smith, M. J., Dubal, D. B., Wilson, M. E., Krajnak, K. M., and Rosewell, K. L. Neuroendocrine influences and repercussions of the menopause. Endocr Rev, *20:* 243-248, 1999.
39. Faddy, M. J., Gosden, R. G., and Edwards, R. G. Ovarian follicle dynamics in mice: a comparative study of three inbred strains and an F1 hybrid. J Endocrinol, *96:* 23-33, 1983.
40. Rosner, B., Colditz, G. A., and Willett, W. C. Reproductive risk factors in a prospective study of breast cancer: the Nurses' Health Study. Am J Epidemiol, *139:* 819-835, 1994.
41. Henderson, B. E., Ross, R. K., and Pike, M. C. Toward the primary prevention of cancer. Science, *254:* 1131-1138, 1991.
42. Sinha, D. K., Pazik, J. E., and Dao, T. L. Prevention of mammary carcinogenesis in rats by pregnancy: effect of full-term and interrupted pregnancy. Br J Cancer, *57:* 390-394, 1988.
43. Yang, J., Yoshizawa, K., Nandi, S., and Tsubura, A. Protective effects of pregnancy and lactation against N-methyl-N-nitrosourea-induced mammary carcinomas in female Lewis rats. Carcinogenesis, *20:* 623-628, 1999.
44. Russo, J. and Russo, I. H. Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. Cancer Res, *40:* 2677-2687, 1980.
45. Russo, J. and Russo, I. H. Susceptibility of the mammary gland to carcinogenesis. II. Pregnancy interruption as a risk factor in tumor incidence. Am J Pathol, *100:* 497-512, 1980.

46. Cardiff, R. D. Validity of mouse mammary tumour models for human breast cancer: comparative pathology. Microsc Res Tech, *52:* 224-230, 2001.
47. Callahan, R. and Smith, G. H. MMTV-induced mammary tumorigenesis: gene discovery, progression to malignancy and cellular pathways. Oncogene, *19:* 992-1001, 2000.
48. Hennighausen, L., Wall, R. J., Tillmann, U., Li, M., and Furth, P. A. Conditional gene expression in secretory tissues and skin of transgenic mice using the MMTV-LTR and the tetracycline responsive system. J Cell Biochem, *59:* 463-472, 1995.
49. Albanese, C., Reutens, A. T., Bouzahzah, B., Fu, M., D'Amico, M., Link, T., Nicholson, R., Depinho, R. A., and Pestell, R. G. Sustained mammary gland-directed, ponasterone A-inducible expression in transgenic mice. Faseb J, *14:* 877-884, 2000.
50. Gunther, E. J., Belka, G. K., Wertheim, G. B., Wang, J., Hartman, J. L., Boxer, R. B., and Chodosh, L. A. A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. Faseb J, *16:* 283-292, 2002.
51. Williams, J. M. and Daniel, C. W. Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Dev Biol, *97:* 274-290, 1983.
52. Humphreys, R. C., Krajewska, M., Krnacik, S., Jaeger, R., Weiher, H., Krajewski, S., Reed, J. C., and Rosen, J. M. Apoptosis in the terminal endbud of the murine mammary gland: a mechanism of ductal morphogenesis. Development, *122:* 4013-4022, 1996.
53. Dulbecco, R., Allen, W. R., Bologna, M., and Bowman, M. Marker evolution during the development of the rat mammary gland: stem cells identified by markers and the role of myoepithelial cells. Cancer Res, *46:* 2449-2456, 1986.
54. Young, L. J., Medina, D., DeOme, K. B., and Daniel, C. W. The influence of host and tissue age on life span and growth rate of serially transplanted mouse mammary gland. Exp Gerontol, *6:* 49-56, 1971.
55. Daniel, C. W., De Ome, K. B., Young, J. T., Blair, P. B., and Faulkin, L. J., Jr. The in vivo life span of normal and preneoplastic mouse mammary glands: a serial transplantation study. Proc Natl Acad Sci U S A, *61:* 53-60, 1968.
56. Daniel, C. W. and Young, L. J. Influence of cell division on an aging process. Life span of mouse mammary epithelium during serial propagation in vivo. Exp Cell Res, *65:* 27-32, 1971.
57. Kenney, N. J., Smith, G. H., Lawrence, E., Barrett, J. C., and Salomon, D. S. Identification of Stem Cell Units in the Terminal End Bud and Duct of the Mouse Mammary Gland. J Biomed Biotechnol, *1:* 133-143, 2001.
58. Kordon, E. C. and Smith, G. H. An entire functional mammary gland may comprise the progeny from a single cell. Development, *125:* 1921-1930, 1998.
59. Boulanger, C. A., Wagner, K. U., and Smith, G. H. Parity-induced mouse mammary epithelial cells are pluripotent, self-renewing and sensitive to TGF-beta1 expression. Oncogene, *24:* 552-560, 2005.
60. Ormerod, E. J. and Rudland, P. S. Regeneration of mammary glands in vivo from isolated mammary ducts. J Embryol Exp Morphol, *96:* 229-243, 1986.

61. Smith, G. H. and Medina, D. A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. J Cell Sci, *90 (Pt 1):* 173-183, 1988.
62. Smith, G. H. Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype. Breast Cancer Res Treat, *39:* 21-31, 1996.
63. Stingl, J., Eaves, C. J., Zandieh, I., and Emerman, J. T. Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. Breast Cancer Res Treat, *67:* 93-109, 2001.
64. Stingl, J., Eaves, C. J., Kuusk, U., and Emerman, J. T. Phenotypic and functional characterization in vitro of a multipotent epithelial cell present in the normal adult human breast. Differentiation, *63:* 201-213, 1998.
65. Dontu, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J., and Wicha, M. S. In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev, *17:* 1253-1270, 2003.
66. Kuperwasser, C., Chavarria, T., Wu, M., Magrane, G., Gray, J. W., Carey, L., Richardson, A., and Weinberg, R. A. Reconstruction of functionally normal and malignant human breast tissues in mice. Proc Natl Acad Sci U S A, *101:* 4966-4971, 2004.
67. Chepko, G. and Smith, G. H. Three division-competent, structurally-distinct cell populations contribute to murine mammary epithelial renewal. Tissue Cell, *29:* 239-253, 1997.
68. Chepko, G. and Dickson, R. B. Ultrastructure of the putative stem cell niche in rat mammary epithelium. Tissue Cell, *35:* 83-93, 2003.
69. Shackleton, M., Vaillant, F., Simpson, K. J., Stingl, J., Smyth, G. K., Asselin-Labat, M. L., Wu, L., Lindeman, G. J., and Visvader, J. E. Generation of a functional mammary gland from a single stem cell. Nature, *439:* 84-88, 2006.
70. Alvi, A. J., Clayton, H., Joshi, C., Enver, T., Ashworth, A., Vivanco, M. M., Dale, T. C., and Smalley, M. J. Functional and molecular characterisation of mammary side population cells. Breast Cancer Res, *5:* R1-8, 2003.
71. Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H. I., and Eaves, C. J. Purification and unique properties of mammary epithelial stem cells. Nature, *439:* 993-997, 2006.
72. Pechoux, C., Gudjonsson, T., Ronnov-Jessen, L., Bissell, M. J., and Petersen, O. W. Human mammary luminal epithelial cells contain progenitors to myoepithelial cells. Dev Biol, *206:* 88-99, 1999.
73. Smalley, M. J., Titley, J., Paterson, H., Perusinghe, N., Clarke, C., and O'Hare, M. J. Differentiation of separated mouse mammary luminal epithelial and myoepithelial cells cultured on EHS matrix analyzed by indirect immunofluorescence of cytoskeletal antigens. J Histochem Cytochem, *47:* 1513-1524, 1999.
74. Welm, B. E., Tepera, S. B., Venezia, T., Graubert, T. A., Rosen, J. M., and Goodell, M. A. Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. Dev Biol, *245:* 42-56, 2002.

75. Liu, B. Y., McDermott, S. P., Khwaja, S. S., and Alexander, C. M. The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci U S A, *101:* 4158-4163, 2004.
76. Rietze, R. L., Valcanis, H., Brooker, G. F., Thomas, T., Voss, A. K., and Bartlett, P. F. Purification of a pluripotent neural stem cell from the adult mouse brain. Nature, *412:* 736-739, 2001.
77. Sleeman, K. E., Kendrick, H., Ashworth, A., Isacke, C. M., and Smalley, M. J. CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer Res, *8:* R7, 2006.
78. Li, A., Simmons, P. J., and Kaur, P. Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. Proc Natl Acad Sci U S A, *95:* 3902-3907, 1998.
79. Hill, R. P. Identifying cancer stem cells in solid tumors: case not proven. Cancer Res, *66:* 1891-1895; discussion 1890, 2006.
80. Wicha, M. S., Liu, S., and Dontu, G. Cancer stem cells: an old idea--a paradigm shift. Cancer Res, *66:* 1883-1890; discussion 1895-1886, 2006.
81. Soltysova, A., Altanerova, V., and Altaner, C. Cancer stem cells. Neoplasma, *52:* 435-440, 2005.
82. Sell, S. Stem cell origin of cancer and differentiation therapy. Crit Rev Oncol Hematol, *51:* 1-28, 2004.
83. Axelson, H., Fredlund, E., Ovenberger, M., Landberg, G., and Pahlman, S. Hypoxia-induced dedifferentiation of tumor cells--a mechanism behind heterogeneity and aggressiveness of solid tumors. Semin Cell Dev Biol, *16:* 554-563, 2005.
84. Helczynska, K., Kronblad, A., Jogi, A., Nilsson, E., Beckman, S., Landberg, G., and Pahlman, S. Hypoxia promotes a dedifferentiated phenotype in ductal breast carcinoma in situ. Cancer Res, *63:* 1441-1444, 2003.
85. Wagner, K. U., Boulanger, C. A., Henry, M. D., Sgagias, M., Hennighausen, L., and Smith, G. H. An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. Development, *129:* 1377-1386, 2002.
86. Wagner, K. U. and Smith, G. H. Pregnancy and stem cell behavior. J Mammary Gland Biol Neoplasia, *10:* 25-36, 2005.
87. Welsch, C. W. and Nagasawa, H. Prolactin and murine mammary tumorigenesis: a review. Cancer Res, *37:* 951-963, 1977.
88. Russo, J., Balogh, G. A., Heulings, R., Mailo, D. A., Moral, R., Russo, P. A., Sheriff, F., Vanegas, J., and Russo, I. H. Molecular basis of pregnancy-induced breast cancer protection. Eur J Cancer Prev, *15:* 306-342, 2006.
89. Blakely, C. M., Stoddard, A. J., Belka, G. K., Dugan, K. D., Notarfrancesco, K. L., Moody, S. E., D'Cruz, C. M., and Chodosh, L. A. Hormone-induced protection against mammary tumorigenesis is conserved in multiple rat strains and identifies a core gene expression signature induced by pregnancy. Cancer Res, *66:* 6421-6431, 2006.
90. Uehara, N., Unami, A., Kiyozuka, Y., Shikata, N., Oishi, Y., and Tsubura, A. Parous mammary glands exhibit distinct alterations in gene expression and

proliferation responsiveness to carcinogenic stimuli in Lewis rats. Oncol Rep, *15:* 903-911, 2006.

91. Sivaraman, L., Conneely, O. M., Medina, D., and O'Malley, B. W. p53 is a potential mediator of pregnancy and hormone-induced resistance to mammary carcinogenesis. Proc Natl Acad Sci U S A, *98:* 12379-12384, 2001.
92. Marley, S. B. and Gordon, M. Y. Chronic myeloid leukaemia: stem cell derived but progenitor cell driven. Clin Sci (Lond), *109:* 13-25, 2005.
93. Janeway, C. A., Travers, P., Walport, M., and Capra, J. D. Immunobiology: The immune system in health and disease, 4th edition. New York: Elsevier Science Ltd./Garland Publishing, 1999.
94. Rowley, J. D. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature, *243:* 290-293, 1973.
95. Bartram, C. R., de Klein, A., Hagemeijer, A., van Agthoven, T., Geurts van Kessel, A., Bootsma, D., Grosveld, G., Ferguson-Smith, M. A., Davies, T., Stone, M., and et al. Translocation of c-ab1 oncogene correlates with the presence of a Philadelphia chromosome in chronic myelocytic leukaemia. Nature, *306:* 277-280, 1983.
96. Heisterkamp, N., Stephenson, J. R., Groffen, J., Hansen, P. F., de Klein, A., Bartram, C. R., and Grosveld, G. Localization of the c-ab1 oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. Nature, *306:* 239-242, 1983.
97. Fialkow, P. J., Gartler, S. M., and Yoshida, A. Clonal origin of chronic myelocytic leukemia in man. Proc Natl Acad Sci U S A, *58:* 1468-1471, 1967.
98. Nitta, M., Kato, Y., Strife, A., Wachter, M., Fried, J., Perez, A., Jhanwar, S., Duigou-Osterndorf, R., Chaganti, R. S., and Clarkson, B. Incidence of involvement of the B and T lymphocyte lineages in chronic myelogenous leukemia. Blood, *66:* 1053-1061, 1985.
99. Ferraris, A. M., Canepa, L., Melani, C., Miglino, M., Broccia, G., and Gaetani, G. F. Clonal B lymphocytes lack bcr rearrangement in Ph-positive chronic myelogenous leukaemia. Br J Haematol, *73:* 48-50, 1989.
100. Kelman, Z., Prokocimer, M., Peller, S., Kahn, Y., Rechavi, G., Manor, Y., Cohen, A., and Rotter, V. Rearrangements in the p53 gene in Philadelphia chromosome positive chronic myelogenous leukemia. Blood, *74:* 2318-2324, 1989.
101. Rowley, J. D. and Testa, J. R. Chromosome abnormalities in malignant hematologic diseases. Adv Cancer Res, *36:* 103-148, 1982.
102. Laneuville, P., Sun, G., Timm, M., and Vekemans, M. Clonal evolution in a myeloid cell line transformed to interleukin-3 independent growth by retroviral transduction and expression of p210bcr/abl. Blood, *80:* 1788-1797, 1992.
103. Salloukh, H. F. and Laneuville, P. Increase in mutant frequencies in mice expressing the BCR-ABL activated tyrosine kinase. Leukemia, *14:* 1401-1404, 2000.
104. Alimena, G., De Cuia, M. R., Diverio, D., Gastaldi, R., and Nanni, M. The karyotype of blastic crisis. Cancer Genet Cytogenet, *26:* 39-50, 1987.

105. Honda, H., Ushijima, T., Wakazono, K., Oda, H., Tanaka, Y., Aizawa, S., Ishikawa, T., Yazaki, Y., and Hirai, H. Acquired loss of p53 induces blastic transformation in p210(bcr/abl)-expressing hematopoietic cells: a transgenic study for blast crisis of human CML. Blood, *95:* 1144-1150, 2000.
106. Al-Hajj, M., Wicha, M. S., Benito-Hernandez, A., Morrison, S. J., and Clarke, M. F. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A, *100:* 3983-3988, 2003.
107. Ponti, D., Costa, A., Zaffaroni, N., Pratesi, G., Petrangolini, G., Coradini, D., Pilotti, S., Pierotti, M. A., and Daidone, M. G. Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. Cancer Res, *65:* 5506-5511, 2005.
108. Lewis, I. D., McDiarmid, L. A., Samels, L. M., To, L. B., and Hughes, T. P. Establishment of a reproducible model of chronic-phase chronic myeloid leukemia in NOD/SCID mice using blood-derived mononuclear or CD34+ cells. Blood, *91:* 630-640, 1998.
109. Dick, J. E. Normal and leukemic human stem cells assayed in SCID mice. Semin Immunol, *8:* 197-206, 1996.
110. Hemmati, H. D., Nakano, I., Lazareff, J. A., Masterman-Smith, M., Geschwind, D. H., Bronner-Fraser, M., and Kornblum, H. I. Cancerous stem cells can arise from pediatric brain tumors. Proc Natl Acad Sci U S A, *100:* 15178-15183, 2003.
111. Kim, C. F., Jackson, E. L., Woolfenden, A. E., Lawrence, S., Babar, I., Vogel, S., Crowley, D., Bronson, R. T., and Jacks, T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell, *121:* 823-835, 2005.
112. Singh, S. K., Clarke, I. D., Terasaki, M., Bonn, V. E., Hawkins, C., Squire, J., and Dirks, P. B. Identification of a cancer stem cell in human brain tumors. Cancer Res, *63:* 5821-5828, 2003.
113. Nagle, R. B., Bocker, W., Davis, J. R., Heid, H. W., Kaufmann, M., Lucas, D. O., and Jarasch, E. D. Characterization of breast carcinomas by two monoclonal antibodies distinguishing myoepithelial from luminal epithelial cells. J Histochem Cytochem, *34:* 869-881, 1986.
114. Weiss, R. A., Eichner, R., and Sun, T. T. Monoclonal antibody analysis of keratin expression in epidermal diseases: a 48- and 56-kdalton keratin as molecular markers for hyperproliferative keratinocytes. J Cell Biol, *98:* 1397-1406, 1984.
115. Clarke, C. L., Sandle, J., Parry, S. C., Reis-Filho, J. S., O'Hare, M. J., and Lakhani, S. R. Cytokeratin 5/6 in normal human breast: lack of evidence for a stem cell phenotype. J Pathol, *204:* 147-152, 2004.
116. Smith, G. H., Mehrel, T., and Roop, D. R. Differential keratin gene expression in developing, differentiating, preneoplastic, and neoplastic mouse mammary epithelium. Cell Growth Differ, *1:* 161-170, 1990.
117. Sapino, A., Macri, L., Gugliotta, P., Pacchioni, D., Liu, Y. J., Medina, D., and Bussolati, G. Immunophenotypic properties and estrogen dependency of budding cell structures in the developing mouse mammary gland. Differentiation, *55:* 13-18, 1993.

118. Mikaelian, I., Hovick, M., Silva, K. A., Burzenski, L. M., Shultz, L. D., Ackert-Bicknell, C. L., Cox, G. A., and Sundberg, J. P. Expression of terminal differentiation proteins defines stages of mouse mammary gland development. Vet Pathol, *43:* 36-49, 2006.
119. Putti, T. C., Pinder, S. E., Elston, C. W., Lee, A. H., and Ellis, I. O. Breast pathology practice: most common problems in a consultation service. Histopathology, *47:* 445-457, 2005.
120. Otterbach, F., Bankfalvi, A., Bergner, S., Decker, T., Krech, R., and Boecker, W. Cytokeratin 5/6 immunohistochemistry assists the differential diagnosis of atypical proliferations of the breast. Histopathology, *37:* 232-240, 2000.
121. Boecker, W. and Buerger, H. Evidence of progenitor cells of glandular and myoepithelial cell lineages in the human adult female breast epithelium: a new progenitor (adult stem) cell concept. Cell Prolif, *36 Suppl 1:* 73-84, 2003.
122. Gusterson, B. A., Ross, D. T., Heath, V. J., and Stein, T. Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. Breast Cancer Res, *7:* 143-148, 2005.
123. Barbareschi, M., Pecciarini, L., Cangi, M. G., Macri, E., Rizzo, A., Viale, G., and Doglioni, C. p63, a p53 homologue, is a selective nuclear marker of myoepithelial cells of the human breast. Am J Surg Pathol, *25:* 1054-1060, 2001.
124. Nylander, K., Vojtesek, B., Nenutil, R., Lindgren, B., Roos, G., Zhanxiang, W., Sjostrom, B., Dahlqvist, A., and Coates, P. J. Differential expression of p63 isoforms in normal tissues and neoplastic cells. J Pathol, *198:* 417-427, 2002.
125. Wang, X., Mori, I., Tang, W., Nakamura, M., Nakamura, Y., Sato, M., Sakurai, T., and Kakudo, K. p63 expression in normal, hyperplastic and malignant breast tissues. Breast Cancer, *9:* 216-219, 2002.
126. Ribeiro-Silva, A., Zambelli Ramalho, L. N., Britto Garcia, S., and Zucoloto, S. The relationship between p63 and p53 expression in normal and neoplastic breast tissue. Arch Pathol Lab Med, *127:* 336-340, 2003.
127. McKeon, F. p63 and the epithelial stem cell: more than status quo? Genes Dev, *18:* 465-469, 2004.
128. Page, M. J., Amess, B., Townsend, R. R., Parekh, R., Herath, A., Brusten, L., Zvelebil, M. J., Stein, R. C., Waterfield, M. D., Davies, S. C., and O'Hare, M. J. Proteomic definition of normal human luminal and myoepithelial breast cells purified from reduction mammoplasties. Proc Natl Acad Sci U S A, *96:* 12589-12594, 1999.
129. Bocker, W., Moll, R., Poremba, C., Holland, R., Van Diest, P. J., Dervan, P., Burger, H., Wai, D., Ina Diallo, R., Brandt, B., Herbst, H., Schmidt, A., Lerch, M. M., and Buchwallow, I. B. Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept. Lab Invest, *82:* 737-746, 2002.
130. Moll, R., Krepler, R., and Franke, W. W. Complex cytokeratin polypeptide patterns observed in certain human carcinomas. Differentiation, *23:* 256-269, 1983.

131. Dairkee, S. H., Mayall, B. H., Smith, H. S., and Hackett, A. J. Monoclonal marker that predicts early recurrence of breast cancer. Lancet, *1:* 514, 1987.
132. Abd El-Rehim, D. M., Pinder, S. E., Paish, C. E., Bell, J., Blamey, R. W., Robertson, J. F., Nicholson, R. I., and Ellis, I. O. Expression of luminal and basal cytokeratins in human breast carcinoma. J Pathol, *203:* 661-671, 2004.
133. Malzahn, K., Mitze, M., Thoenes, M., and Moll, R. Biological and prognostic significance of stratified epithelial cytokeratins in infiltrating ductal breast carcinomas. Virchows Arch, *433:* 119-129, 1998.
134. van de Rijn, M., Perou, C. M., Tibshirani, R., Haas, P., Kallioniemi, O., Kononen, J., Torhorst, J., Sauter, G., Zuber, M., Kochli, O. R., Mross, F., Dieterich, H., Seitz, R., Ross, D., Botstein, D., and Brown, P. Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. Am J Pathol, *161:* 1991-1996, 2002.
135. Korsching, E., Packeisen, J., Agelopoulos, K., Eisenacher, M., Voss, R., Isola, J., van Diest, P. J., Brandt, B., Boecker, W., and Buerger, H. Cytogenetic alterations and cytokeratin expression patterns in breast cancer: integrating a new model of breast differentiation into cytogenetic pathways of breast carcinogenesis. Lab Invest, *82:* 1525-1533, 2002.
136. Nielsen, T. O., Hsu, F. D., Jensen, K., Cheang, M., Karaca, G., Hu, Z., Hernandez-Boussard, T., Livasy, C., Cowan, D., Dressler, L., Akslen, L. A., Ragaz, J., Gown, A. M., Gilks, C. B., van de Rijn, M., and Perou, C. M. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res, *10:* 5367-5374, 2004.
137. Jacquemier, J., Padovani, L., Rabayrol, L., Lakhani, S. R., Penault-Llorca, F., Denoux, Y., Fiche, M., Figueiro, P., Maisongrosse, V., Ledoussal, V., Martinez Penuela, J., Udvarhely, N., El Makdissi, G., Ginestier, C., Geneix, J., Charafe-Jauffret, E., Xerri, L., Eisinger, F., Birnbaum, D., and Sobol, H. Typical medullary breast carcinomas have a basal/myoepithelial phenotype. J Pathol, *207:* 260-268, 2005.
138. Laakso, M., Loman, N., Borg, A., and Isola, J. Cytokeratin 5/14-positive breast cancer: true basal phenotype confined to BRCA1 tumors. Mod Pathol, *18:* 1321-1328, 2005.
139. Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Eystein Lonning, P., and Borresen-Dale, A. L. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A, *98:* 10869-10874, 2001.
140. Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J. S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., Demeter, J., Perou, C. M., Lonning, P. E., Brown, P. O., Borresen-Dale, A. L., and Botstein, D. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci U S A, *100:* 8418-8423, 2003.
141. Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, O., Pergamenschikov, A., Williams, C., Zhu, S. X., Lonning, P. E., Borresen-

Dale, A. L., Brown, P. O., and Botstein, D. Molecular portraits of human breast tumours. Nature, *406:* 747-752, 2000.

142. Sotiriou, C., Neo, S. Y., McShane, L. M., Korn, E. L., Long, P. M., Jazaeri, A., Martiat, P., Fox, S. B., Harris, A. L., and Liu, E. T. Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci U S A, *100:* 10393-10398, 2003.
143. Zhao, H., Langerod, A., Ji, Y., Nowels, K. W., Nesland, J. M., Tibshirani, R., Bukholm, I. K., Karesen, R., Botstein, D., Borresen-Dale, A. L., and Jeffrey, S. S. Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. Mol Biol Cell, *15:* 2523-2536, 2004.
144. Ribeiro-Silva, A., Ramalho, L. N., Garcia, S. B., Brandao, D. F., Chahud, F., and Zucoloto, S. p63 correlates with both BRCA1 and cytokeratin 5 in invasive breast carcinomas: further evidence for the pathogenesis of the basal phenotype of breast cancer. Histopathology, *47:* 458-466, 2005.
145. Rakha, E. A., Putti, T. C., Abd El-Rehim, D. M., Paish, C., Green, A. R., Powe, D. G., Lee, A. H., Robertson, J. F., and Ellis, I. O. Morphological and immunophenotypic analysis of breast carcinomas with basal and myoepithelial differentiation. J Pathol, *208:* 495-506, 2006.
146. Shien, T., Tashiro, T., Omatsu, M., Masuda, T., Furuta, K., Sato, N., Akashi-Tanaka, S., Uehara, M., Iwamoto, E., Kinoshita, T., Fukutomi, T., Tsuda, H., and Hasegawa, T. Frequent overexpression of epidermal growth factor receptor (EGFR) in mammary high grade ductal carcinomas with myoepithelial differentiation. J Clin Pathol, *58:* 1299-1304, 2005.
147. Allen, J. D., Brinkhuis, R. F., Wijnholds, J., and Schinkel, A. H. The mouse Bcrp1/Mxr/Abcp gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. Cancer Res, *59:* 4237-4241, 1999.
148. Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., Gao, Y., Rishi, A. K., and Ross, D. D. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A, *95:* 15665-15670, 1998.
149. Allikmets, R., Schriml, L. M., Hutchinson, A., Romano-Spica, V., and Dean, M. A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. Cancer Res, *58:* 5337-5339, 1998.
150. Miyake, K., Mickley, L., Litman, T., Zhan, Z., Robey, R., Cristensen, B., Brangi, M., Greenberger, L., Dean, M., Fojo, T., and Bates, S. E. Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. Cancer Res, *59:* 8-13, 1999.
151. Yano, S., Ito, Y., Fujimoto, M., Hamazaki, T. S., Tamaki, K., and Okochi, H. Characterization and localization of side population cells in mouse skin. Stem Cells, *23:* 834-841, 2005.
152. Woodward, W. A., Chen, M. S., Behbod, F., Alfarao, M. P., Buchholz, T. A., and Rosen, J. M. WNT/β-catenin mediates radiation resistance of mouse mammary progenitor cells. Proc Natl Acad Sci U S A, *104:* 618-623, 2007.

153. Bhatia, R., Holtz, M., Niu, N., Gray, R., Snyder, D. S., Sawyers, C. L., Arber, D. A., Slovak, M. L., and Forman, S. J. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. Blood, *101:* 4701-4707, 2003.
154. Hughes, T. P., Kaeda, J., Branford, S., Rudzki, Z., Hochhaus, A., Hensley, M. L., Gathmann, I., Bolton, A. E., van Hoomissen, I. C., Goldman, J. M., and Radich, J. P. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med, *349:* 1423-1432, 2003.
155. Gorre, M. E., Mohammed, M., Ellwood, K., Hsu, N., Paquette, R., Rao, P. N., and Sawyers, C. L. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science, *293:* 876-880, 2001.
156. Scharenberg, C. W., Harkey, M. A., and Torok-Storb, B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood, *99:* 507-512, 2002.
157. Iwano, M., Plieth, D., Danoff, T. M., Xue, C., Okada, H., and Neilson, E. G. Evidence that fibroblasts derive from epithelium during tissue fibrosis. J Clin Invest, *110:* 341-350, 2002.
158. Yang, J. and Liu, Y. Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. Am J Pathol, *159:* 1465-1475, 2001.
159. Grunert, S., Jechlinger, M., and Beug, H. Diverse cellular and molecular mechanisms contribute to epithelial plasticity and metastasis. Nat Rev Mol Cell Biol, *4:* 657-665, 2003.
160. Thiery, J. P. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer, *2:* 442-454, 2002.
161. Kang, Y. and Massague, J. Epithelial-mesenchymal transitions: twist in development and metastasis. Cell, *118:* 277-279, 2004.
162. Daniel, C. W., Strickland, P., and Friedmann, Y. Expression and functional role of E- and P-cadherins in mouse mammary ductal morphogenesis and growth. Dev Biol, *169:* 511-519, 1995.
163. Nanba, D., Nakanishi, Y., and Hieda, Y. Changes in adhesive properties of epithelial cells during early morphogenesis of the mammary gland. Dev Growth Differ, *43:* 535-544, 2001.
164. Foschini, M. P. and Eusebi, V. Carcinomas of the breast showing myoepithelial cell differentiation. A review of the literature. Virchows Arch, *432:* 303-310, 1998.
165. Lakhani, S. R., O'Hare, M. J., Monaghan, P., Winehouse, J., Gazet, J. C., and Sloane, J. P. Malignant myoepithelioma (myoepithelial carcinoma) of the breast: a detailed cytokeratin study. J Clin Pathol, *48:* 164-167, 1995.
166. Thorner, P. S., Kahn, H. J., Baumal, R., Lee, K., and Moffatt, W. Malignant myoepithelioma of the breast. An immunohistochemical study by light and electron microscopy. Cancer, *57:* 745-750, 1986.

167. Hungermann, D., Buerger, H., Oehlschlegel, C., Herbst, H., and Boecker, W. Adenomyoepithelial tumours and myoepithelial carcinomas of the breast--a spectrum of monophasic and biphasic tumours dominated by immature myoepithelial cells. BMC Cancer, *5:* 92, 2005.
168. Anbazhagan, R., Bartek, J., Monaghan, P., and Gusterson, B. A. Growth and development of the human infant breast. Am J Anat, *192:* 407-417, 1991.
169. Moody, S. E., Perez, D., Pan, T. C., Sarkisian, C. J., Portocarrero, C. P., Sterner, C. J., Notorfrancesco, K. L., Cardiff, R. D., and Chodosh, L. A. The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer Cell, *8:* 197-209, 2005.
170. Thomas, P. A., Kirschmann, D. A., Cerhan, J. R., Folberg, R., Seftor, E. A., Sellers, T. A., and Hendrix, M. J. Association between keratin and vimentin expression, malignant phenotype, and survival in postmenopausal breast cancer patients. Clin Cancer Res, *5:* 2698-2703, 1999.
171. Korsching, E., Packeisen, J., Liedtke, C., Hungermann, D., Wulfing, P., van Diest, P. J., Brandt, B., Boecker, W., and Buerger, H. The origin of vimentin expression in invasive breast cancer: epithelial-mesenchymal transition, myoepithelial histogenesis or histogenesis from progenitor cells with bilinear differentiation potential? J Pathol, *206:* 451-457, 2005.
172. Blume-Jensen, P. and Hunter, T. Oncogenic kinase signalling. Nature, *411:* 355-365, 2001.
173. Cohen, S., Ushiro, H., Stoscheck, C., and Chinkers, M. A native 170,000 epidermal growth factor receptor-kinase complex from shed plasma membrane vesicles. J Biol Chem, *257:* 1523-1531, 1982.
174. Frykberg, L., Palmieri, S., Beug, H., Graf, T., Hayman, M. J., and Vennstrom, B. Transforming capacities of avian erythroblastosis virus mutants deleted in the erbA or erbB oncogenes. Cell, *32:* 227-238, 1983.
175. Besmer, P., Murphy, J. E., George, P. C., Qiu, F. H., Bergold, P. J., Lederman, L., Snyder, H. W., Jr., Brodeur, D., Zuckerman, E. E., and Hardy, W. D. A new acute transforming feline retrovirus and relationship of its oncogene v-kit with the protein kinase gene family. Nature, *320:* 415-421, 1986.
176. Coussens, L., Van Beveren, C., Smith, D., Chen, E., Mitchell, R. L., Isacke, C. M., Verma, I. M., and Ullrich, A. Structural alteration of viral homologue of receptor proto-oncogene fms at carboxyl terminus. Nature, *320:* 277-280, 1986.
177. Huff, J. L., Jelinek, M. A., Borgman, C. A., Lansing, T. J., and Parsons, J. T. The protooncogene c-sea encodes a transmembrane protein-tyrosine kinase related to the Met/hepatocyte growth factor/scatter factor receptor. Proc Natl Acad Sci U S A, *90:* 6140-6144, 1993.
178. Neckameyer, W. S., Shibuya, M., Hsu, M. T., and Wang, L. H. Proto-oncogene c-ros codes for a molecule with structural features common to those of growth factor receptors and displays tissue specific and developmentally regulated expression. Mol Cell Biol, *6:* 1478-1486, 1986.
179. Cooper, C. S., Park, M., Blari, D. G., Tainsky, M. A., Huebner, K., Croce, C. M., and Vande Woude, G. F. Molecular cloning of a new transforming gene from a chemically transformed human cell line. Nature, *311:* 29-33, 1984.

180. Park, M., Dean, M., Cooper, C. S., Schmidt, M., O'Brien, S. J., Blair, D. G., and Vande Woude, G. F. Mechanism of met oncogene activation. Cell, *45:* 895-904, 1986.
181. Park, M., Testa, J. R., Blair, D. G., Parsa, N. Z., and Vande Woude, G. F. Two rearranged MET alleles in MNNG-HOS cells reveal the orientation of MET on chromosome 7 to other markers tightly linked to the cystic fibrosis locus. Proc Natl Acad Sci U S A, *85:* 2667-2671, 1988.
182. Cooper, C. S., Blair, D. G., Oskarsson, M. K., Tainsky, M. A., Eader, L. A., and Vande Woude, G. F. Characterization of human transforming genes from chemically transformed, teratocarcinoma, and pancreatic carcinoma cell lines. Cancer Res, *44:* 1-10, 1984.
183. Tempest, P. R., Reeves, B. R., Spurr, N. K., Rance, A. J., Chan, A. M., and Brookes, P. Activation of the met oncogene in the human MNNG-HOS cell line involves a chromosomal rearrangement. Carcinogenesis, *7:* 2051-2057, 1986.
184. Dean, M., Park, M., Le Beau, M. M., Robins, T. S., Diaz, M. O., Rowley, J. D., Blair, D. G., and Vande Woude, G. F. The human met oncogene is related to the tyrosine kinase oncogenes. Nature, *318:* 385-388, 1985.
185. Tempest, P. R., Cooper, C. S., and Major, G. N. The activated human met gene encodes a protein tyrosine kinase. FEBS Lett, *209:* 357-361, 1986.
186. Gonzatti-Haces, M., Seth, A., Park, M., Copeland, T., Oroszlan, S., and Vande Woude, G. F. Characterization of the TPR-MET oncogene p65 and the MET protooncogene p140 protein-tyrosine kinases. Proc Natl Acad Sci U S A, *85:* 21-25, 1988.
187. Park, M., Dean, M., Kaul, K., Braun, M. J., Gonda, M. A., and Vande Woude, G. Sequence of MET protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci U S A, *84:* 6379-6383, 1987.
188. Bottaro, D. P., Rubin, J. S., Faletto, D. L., Chan, A. M., Kmiecik, T. E., Vande Woude, G. F., and Aaronson, S. A. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. Science, *251:* 802-804, 1991.
189. Stoker, M., Gherardi, E., Perryman, M., and Gray, J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. Nature, *327:* 239-242, 1987.
190. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. Molecular cloning and expression of human hepatocyte growth factor. Nature, *342:* 440-443, 1989.
191. Zarnegar, R. and Michalopoulos, G. Purification and biological characterization of human hepatopoietin A, a polypeptide growth factor for hepatocytes. Cancer Res, *49:* 3314-3320, 1989.
192. Miyazawa, K., Tsubouchi, H., Naka, D., Takahashi, K., Okigaki, M., Arakaki, N., Nakayama, H., Hirono, S., Sakiyama, O., and et al. Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. Biochem Biophys Res Commun, *163:* 967-973, 1989.

193. Weidner, K. M., Arakaki, N., Hartmann, G., Vandekerckhove, J., Weingart, S., Rieder, H., Fonatsch, C., Tsubouchi, H., Hishida, T., Daikuhara, Y., and et al. Evidence for the identity of human scatter factor and human hepatocyte growth factor. Proc Natl Acad Sci U S A, *88:* 7001-7005, 1991.
194. Gherardi, E. and Stoker, M. Hepatocytes and scatter factor. Nature, *346:* 228, 1990.
195. Naldini, L., Weidner, K. M., Vigna, E., Gaudino, G., Bardelli, A., Ponzetto, C., Narsimhan, R. P., Hartmann, G., Zarnegar, R., Michalopoulos, G. K., and et al. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. Embo J, *10:* 2867-2878, 1991.
196. Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature, *376:* 768-771, 1995.
197. Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. Scatter factor/hepatocyte growth factor is essential for liver development. Nature, *373:* 699-702, 1995.
198. Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T., and Kitamura, N. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature, *373:* 702-705, 1995.
199. Ronsin, C., Muscatelli, F., Mattei, M. G., and Breathnach, R. A novel putative receptor protein tyrosine kinase of the met family. Oncogene, *8:* 1195-1202, 1993.
200. Iwama, A., Okano, K., Sudo, T., Matsuda, Y., and Suda, T. Molecular cloning of a novel receptor tyrosine kinase gene, STK, derived from enriched hematopoietic stem cells. Blood, *83:* 3160-3169, 1994.
201. Maestrini, E., Tamagnone, L., Longati, P., Cremona, O., Gulisano, M., Bione, S., Tamanini, F., Neel, B. G., Toniolo, D., and Comoglio, P. M. A family of transmembrane proteins with homology to the MET-hepatocyte growth factor receptor. Proc Natl Acad Sci U S A, *93:* 674-678, 1996.
202. Gaudino, G., Follenzi, A., Naldini, L., Collesi, C., Santoro, M., Gallo, K. A., Godowski, P. J., and Comoglio, P. M. RON is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. Embo J, *13:* 3524-3532, 1994.
203. Wang, M. H., Ronsin, C., Gesnel, M. C., Coupey, L., Skeel, A., Leonard, E. J., and Breathnach, R. Identification of the ron gene product as the receptor for the human macrophage stimulating protein. Science, *266:* 117-119, 1994.
204. Donate, L. E., Gherardi, E., Srinivasan, N., Sowdhamini, R., Aparicio, S., and Blundell, T. L. Molecular evolution and domain structure of plasminogen-related growth factors (HGF/SF and HGF1/MSP). Protein Sci, *3:* 2378-2394, 1994.
205. Wahl, R. C., Hsu, R. Y., Huff, J. L., Jelinek, M. A., Chen, K., Courchesne, P., Patterson, S. D., Parsons, J. T., and Welcher, A. A. Chicken macrophage stimulating protein is a ligand of the receptor protein-tyrosine kinase Sea. J Biol Chem, *274:* 26361-26368, 1999.

206. Cottage, A., Clark, M., Hawker, K., Umrania, Y., Wheller, D., Bishop, M., and Elgar, G. Three receptor genes for plasminogen related growth factors in the genome of the puffer fish Fugu rubripes. FEBS Lett, *443:* 370-374, 1999.
207. Komada, M., Hatsuzawa, K., Shibamoto, S., Ito, F., Nakayama, K., and Kitamura, N. Proteolytic processing of the hepatocyte growth factor/scatter factor receptor by furin. FEBS Lett, *328:* 25-29, 1993.
208. Mark, M. R., Lokker, N. A., Zioncheck, T. F., Luis, E. A., and Godowski, P. J. Expression and characterization of hepatocyte growth factor receptor-IgG fusion proteins. Effects of mutations in the potential proteolytic cleavage site on processing and ligand binding. J Biol Chem, *267:* 26166-26171, 1992.
209. Giordano, S., Di Renzo, M. F., Narsimhan, R. P., Cooper, C. S., Rosa, C., and Comoglio, P. M. Biosynthesis of the protein encoded by the c-met proto-oncogene. Oncogene, *4:* 1383-1388, 1989.
210. Tempest, P. R., Stratton, M. R., and Cooper, C. S. Structure of the met protein and variation of met protein kinase activity among human tumour cell lines. Br J Cancer, *58:* 3-7, 1988.
211. Gherardi, E., Youles, M. E., Miguel, R. N., Blundell, T. L., Iamele, L., Gough, J., Bandyopadhyay, A., Hartmann, G., and Butler, P. J. Functional map and domain structure of MET, the product of the c-met protooncogene and receptor for hepatocyte growth factor/scatter factor. Proc Natl Acad Sci U S A, *100:* 12039-12044, 2003.
212. Kong-Beltran, M., Stamos, J., and Wickramasinghe, D. The Sema domain of Met is necessary for receptor dimerization and activation. Cancer Cell, *6:* 75-84, 2004.
213. Stamos, J., Lazarus, R. A., Yao, X., Kirchhofer, D., and Wiesmann, C. Crystal structure of the HGF beta-chain in complex with the Sema domain of the Met receptor. Embo J, *23:* 2325-2335, 2004.
214. Chirgadze, D. Y., Hepple, J. P., Zhou, H., Byrd, R. A., Blundell, T. L., and Gherardi, E. Crystal structure of the NK1 fragment of HGF/SF suggests a novel mode for growth factor dimerization and receptor binding. Nat Struct Biol, *6:* 72-79, 1999.
215. Gherardi, E., Sandin, S., Petoukhov, M. V., Finch, J., Youles, M. E., Ofverstedt, L. G., Miguel, R. N., Blundell, T. L., Vande Woude, G. F., Skoglund, U., and Svergun, D. I. Structural basis of hepatocyte growth factor/scatter factor and MET signalling. Proc Natl Acad Sci U S A, *103:* 4046-4051, 2006.
216. Di Renzo, M. F., Narsimhan, R. P., Olivero, M., Bretti, S., Giordano, S., Medico, E., Gaglia, P., Zara, P., and Comoglio, P. M. Expression of the Met/HGF receptor in normal and neoplastic human tissues. Oncogene, *6:* 1997-2003, 1991.
217. Tajima, H., Higuchi, O., Mizuno, K., and Nakamura, T. Tissue distribution of hepatocyte growth factor receptor and its exclusive down-regulation in a regenerating organ after injury. J Biochem (Tokyo), *111:* 401-406, 1992.
218. Jeffers, M., Rao, M. S., Rulong, S., Reddy, J. K., Subbarao, V., Hudson, E., Vande Woude, G. F., and Resau, J. H. Hepatocyte growth factor/scatter

factor-Met signaling induces proliferation, migration, and morphogenesis of pancreatic oval cells. Cell Growth Differ, *7:* 1805-1813, 1996.
219. Ebert, M., Yokoyama, M., Friess, H., Buchler, M. W., and Korc, M. Coexpression of the c-met proto-oncogene and hepatocyte growth factor in human pancreatic cancer. Cancer Res, *54:* 5775-5778, 1994.
220. Takahashi, M., Ota, S., Ogura, K., Nakamura, T., and Omata, M. Hepatocyte growth factor stimulates wound repair of the rabbit esophageal epithelial cells in primary culture. Biochem Biophys Res Commun, *216:* 298-305, 1995.
221. Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., and Kamalati, T. HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. Development, *121:* 2897-2908, 1995.
222. Pepper, M. S., Soriano, J. V., Menoud, P. A., Sappino, A. P., Orci, L., and Montesano, R. Modulation of hepatocyte growth factor and c-met in the rat mammary gland during pregnancy, lactation, and involution. Exp Cell Res, *219:* 204-210, 1995.
223. Tabata, M. J., Kim, K., Liu, J. G., Yamashita, K., Matsumura, T., Kato, J., Iwamoto, M., Wakisaka, S., Matsumoto, K., Nakamura, T., Kumegawa, M., and Kurisu, K. Hepatocyte growth factor is involved in the morphogenesis of tooth germ in murine molars. Development, *122:* 1243-1251, 1996.
224. Bussolino, F., Di Renzo, M. F., Ziche, M., Bocchietto, E., Olivero, M., Naldini, L., Gaudino, G., Tamagnone, L., Coffer, A., and Comoglio, P. M. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol, *119:* 629-641, 1992.
225. Galimi, F., Bagnara, G. P., Bonsi, L., Cottone, E., Follenzi, A., Simeone, A., and Comoglio, P. M. Hepatocyte growth factor induces proliferation and differentiation of multipotent and erythroid hemopoietic progenitors. J Cell Biol, *127:* 1743-1754, 1994.
226. Galimi, F., Cottone, E., Vigna, E., Arena, N., Boccaccio, C., Giordano, S., Naldini, L., and Comoglio, P. M. Hepatocyte growth factor is a regulator of monocyte-macrophage function. J Immunol, *166:* 1241-1247, 2001.
227. Di Renzo, M. F., Bertolotto, A., Olivero, M., Putzolu, P., Crepaldi, T., Schiffer, D., Pagni, C. A., and Comoglio, P. M. Selective expression of the Met/HGF receptor in human central nervous system microglia. Oncogene, *8:* 219-222, 1993.
228. Otsuka, T., Takayama, H., Sharp, R., Celli, G., LaRochelle, W. J., Bottaro, D. P., Ellmore, N., Vieira, W., Owens, J. W., Anver, M., and Merlino, G. c-Met autocrine activation induces development of malignant melanoma and acquisition of the metastatic phenotype. Cancer Res, *58:* 5157-5167, 1998.
229. Takayama, H., La Rochelle, W. J., Anver, M., Bockman, D. E., and Merlino, G. Scatter factor/hepatocyte growth factor as a regulator of skeletal muscle and neural crest development. Proc Natl Acad Sci U S A, *93:* 5866-5871, 1996.
230. Schirmacher, P., Geerts, A., Pietrangelo, A., Dienes, H. P., and Rogler, C. E. Hepatocyte growth factor/hepatopoietin A is expressed in fat-storing cells

from rat liver but not myofibroblast-like cells derived from fat-storing cells. Hepatology, *15:* 5-11, 1992.

231. Woolf, A. S., Kolatsi-Joannou, M., Hardman, P., Andermarcher, E., Moorby, C., Fine, L. G., Jat, P. S., Noble, M. D., and Gherardi, E. Roles of hepatocyte growth factor/scatter factor and the met receptor in the early development of the metanephros. J Cell Biol, *128:* 171-184, 1995.
232. Sonnenberg, E., Weidner, K. M., and Birchmeier, C. Expression of the met-receptor and its ligand, HGF-SF during mouse embryogenesis. Exs, *65:* 381-394, 1993.
233. Kinoshita, Y., Nakata, H., Hassan, S., Asahara, M., Kawanami, C., Matsushima, Y., Naribayashi-Inomoto, Y., Ping, C. Y., Min, D., Nakamura, A., and et al. Gene expression of keratinocyte and hepatocyte growth factors during the healing of rat gastric mucosal lesions. Gastroenterology, *109:* 1068-1077, 1995.
234. Ohmichi, H., Koshimizu, U., Matsumoto, K., and Nakamura, T. Hepatocyte growth factor (HGF) acts as a mesenchyme-derived morphogenic factor during fetal lung development. Development, *125:* 1315-1324, 1998.
235. Kajihara, T., Ohnishi, T., Arakaki, N., Semba, I., and Daikuhara, Y. Expression of hepatocyte growth factor/scatter factor and c-Met in human dental papilla and fibroblasts from dental papilla. Arch Oral Biol, *44:* 135-147, 1999.
236. Patel, Y., Kim, H., and Rappolee, D. A. A role for hepatocyte growth factor during early postimplantation growth of the placental lineage in mice. Biol Reprod, *62:* 904-912, 2000.
237. Yanagita, K., Nagaike, M., Ishibashi, H., Niho, Y., Matsumoto, K., and Nakamura, T. Lung may have an endocrine function producing hepatocyte growth factor in response to injury of distal organs. Biochem Biophys Res Commun, *182:* 802-809, 1992.
238. Nakamura, T., Nawa, K., Ichihara, A., Kaise, N., and Nishino, T. Purification and subunit structure of hepatocyte growth factor from rat platelets. FEBS Lett, *224:* 311-316, 1987.
239. Mizuno, K., Tanoue, Y., Okano, I., Harano, T., Takada, K., and Nakamura, T. Purification and characterization of hepatocyte growth factor (HGF)-converting enzyme: activation of pro-HGF. Biochem Biophys Res Commun, *198:* 1161-1169, 1994.
240. Shimomura, T., Kondo, J., Ochiai, M., Naka, D., Miyazawa, K., Morimoto, Y., and Kitamura, N. Activation of the zymogen of hepatocyte growth factor activator by thrombin. J Biol Chem, *268:* 22927-22932, 1993.
241. Naldini, L., Vigna, E., Bardelli, A., Follenzi, A., Galimi, F., and Comoglio, P. M. Biological activation of pro-HGF (hepatocyte growth factor) by urokinase is controlled by a stoichiometric reaction. J Biol Chem, *270:* 603-611, 1995.
242. Mars, W. M., Zarnegar, R., and Michalopoulos, G. K. Activation of hepatocyte growth factor by the plasminogen activators uPA and tPA. Am J Pathol, *143:* 949-958, 1993.

243. Nakamura, T., Nawa, K., and Ichihara, A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. Biochem Biophys Res Commun, *122:* 1450-1459, 1984.
244. Lyon, M., Deakin, J. A., Mizuno, K., Nakamura, T., and Gallagher, J. T. Interaction of hepatocyte growth factor with heparan sulfate. Elucidation of the major heparan sulfate structural determinants. J Biol Chem, *269:* 11216-11223, 1994.
245. Lyon, M., Deakin, J. A., Rahmoune, H., Fernig, D. G., Nakamura, T., and Gallagher, J. T. Hepatocyte growth factor/scatter factor binds with high affinity to dermatan sulfate. J Biol Chem, *273:* 271-278, 1998.
246. Moscatelli, D. Basic fibroblast growth factor (bFGF) dissociates rapidly from heparan sulfates but slowly from receptors. Implications for mechanisms of bFGF release from pericellular matrix. J Biol Chem, *267:* 25803-25809, 1992.
247. Uchida, K., Inoue, M., Otake, K., Yoshiyama, S., Toiyama, Y., Hiro, J., Araki, T., Miki, C., and Kusunoki, M. The significance of serum hepatocyte growth factor levels in planning follow-up of postoperative jaundice-free patients with biliary atresia. J Pediatr Surg, *41:* 1657-1662, 2006.
248. Toi, M., Taniguchi, T., Ueno, T., Asano, M., Funata, N., Sekiguchi, K., Iwanari, H., and Tominaga, T. Significance of circulating hepatocyte growth factor level as a prognostic indicator in primary breast cancer. Clin Cancer Res, *4:* 659-664, 1998.
249. Satani, K., Konya, H., Hamaguchi, T., Umehara, A., Katsuno, T., Ishikawa, T., Kohri, K., Hasegawa, Y., Suehiro, A., Kakishita, E., and Namba, M. Clinical significance of circulating hepatocyte growth factor, a new risk marker of carotid atherosclerosis in patients with Type 2 diabetes. Diabet Med, *23:* 617-622, 2006.
250. Alexandrakis, M. G., Passam, F. H., Sfiridaki, A., Kandidaki, E., Roussou, P., and Kyriakou, D. S. Elevated serum concentration of hepatocyte growth factor in patients with multiple myeloma: correlation with markers of disease activity. Am J Hematol, *72:* 229-233, 2003.
251. Ernst, S., Langer, R., Cooney, C. L., and Sasisekharan, R. Enzymatic degradation of glycosaminoglycans. Crit Rev Biochem Mol Biol, *30:* 387-444, 1995.
252. Miyazawa, K., Shimomura, T., and Kitamura, N. Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator. J Biol Chem, *271:* 3615-3618, 1996.
253. Rubin, J. S., Bottaro, D. P., and Aaronson, S. A. Hepatocyte growth factor/scatter factor and its receptor, the c-met proto-oncogene product. Biochim Biophys Acta, *1155:* 357-371, 1993.
254. Schwall, R. H., Chang, L. Y., Godowski, P. J., Kahn, D. W., Hillan, K. J., Bauer, K. D., and Zioncheck, T. F. Heparin induces dimerization and confers proliferative activity onto the hepatocyte growth factor antagonists NK1 and NK2. J Cell Biol, *133:* 709-718, 1996.
255. Chan, A. M., Rubin, J. S., Bottaro, D. P., Hirschfield, D. W., Chedid, M., and Aaronson, S. A. Identification of a competitive HGF antagonist encoded by an alternative transcript. Science, *254:* 1382-1385, 1991.

256. Zioncheck, T. F., Richardson, L., Liu, J., Chang, L., King, K. L., Bennett, G. L., Fugedi, P., Chamow, S. M., Schwall, R. H., and Stack, R. J. Sulfated oligosaccharides promote hepatocyte growth factor association and govern its mitogenic activity. J Biol Chem, *270:* 16871-16878, 1995.
257. Cioce, V., Csaky, K. G., Chan, A. M., Bottaro, D. P., Taylor, W. G., Jensen, R., Aaronson, S. A., and Rubin, J. S. Hepatocyte growth factor (HGF)/NK1 is a naturally occurring HGF/scatter factor variant with partial agonist/antagonist activity. J Biol Chem, *271:* 13110-13115, 1996.
258. Lokker, N. A. and Godowski, P. J. Generation and characterization of a competitive antagonist of human hepatocyte growth factor, HGF/NK1. J Biol Chem, *268:* 17145-17150, 1993.
259. Date, K., Matsumoto, K., Shimura, H., Tanaka, M., and Nakamura, T. HGF/NK4 is a specific antagonist for pleiotrophic actions of hepatocyte growth factor. FEBS Lett, *420:* 1-6, 1997.
260. Uehara, Y., Mori, C., Noda, T., Shiota, K., and Kitamura, N. Rescue of embryonic lethality in hepatocyte growth factor/scatter factor knockout mice. Genesis, *27:* 99-103, 2000.
261. Kolatsi-Joannou, M., Moore, R., Winyard, P. J., and Woolf, A. S. Expression of hepatocyte growth factor/scatter factor and its receptor, MET, suggests roles in human embryonic organogenesis. Pediatr Res, *41:* 657-665, 1997.
262. Kermorgant, S., Walker, F., Hormi, K., Dessirier, V., Lewin, M. J., and Lehy, T. Developmental expression and functionality of hepatocyte growth factor and c-Met in human fetal digestive tissues. Gastroenterology, *112:* 1635-1647, 1997.
263. Sweeney, W. E., Jr. and Avner, E. D. The role of hepatocyte growth factor (HGF) at progressive stages of metanephric development. In Vitro Cell Dev Biol Anim, *34:* 189-194, 1998.
264. Matsubara, Y., Ichinose, M., Yahagi, N., Tsukada, S., Oka, M., Miki, K., Kimura, S., Omata, M., Shiokawa, K., Kitamura, N., Kaneko, Y., and Fukamachi, H. Hepatocyte growth factor activator: a possible regulator of morphogenesis during fetal development of the rat gastrointestinal tract. Biochem Biophys Res Commun, *253:* 477-484, 1998.
265. Otonkoski, T., Beattie, G. M., Rubin, J. S., Lopez, A. D., Baird, A., and Hayek, A. Hepatocyte growth factor/scatter factor has insulinotropic activity in human fetal pancreatic cells. Diabetes, *43:* 947-953, 1994.
266. Yant, J., Buluwela, L., Niranjan, B., Gusterson, B., and Kamalati, T. In vivo effects of hepatocyte growth factor/scatter factor on mouse mammary gland development. Exp Cell Res, *241:* 476-481, 1998.
267. Niemann, C., Brinkmann, V., Spitzer, E., Hartmann, G., Sachs, M., Naundorf, H., and Birchmeier, W. Reconstitution of mammary gland development in vitro: requirement of c-met and c-erbB2 signaling for branching and alveolar morphogenesis. J Cell Biol, *143:* 533-545, 1998.
268. Matsumoto, K., Date, K., Ohmichi, H., and Nakamura, T. Hepatocyte growth factor in lung morphogenesis and tumor invasion: role as a mediator in epithelium-mesenchyme and tumor-stroma interactions. Cancer Chemother Pharmacol, *38 Suppl:* S42-47, 1996.

269. Rosario, M. and Birchmeier, W. How to make tubes: signaling by the Met receptor tyrosine kinase. Trends Cell Biol, *13:* 328-335, 2003.
270. Saiki, A., Watanabe, F., Murano, T., Miyashita, Y., and Shirai, K. Hepatocyte growth factor secreted by cultured adipocytes promotes tube formation of vascular endothelial cells in vitro. Int J Obes (Lond), 2006.
271. Van Belle, E., Witzenbichler, B., Chen, D., Silver, M., Chang, L., Schwall, R., and Isner, J. M. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. Circulation, *97:* 381-390, 1998.
272. Rosen, E. M., Jaken, S., Carley, W., Luckett, P. M., Setter, E., Bhargava, M., and Goldberg, I. D. Regulation of motility in bovine brain endothelial cells. J Cell Physiol, *146:* 325-335, 1991.
273. Morimoto, A., Okamura, K., Hamanaka, R., Sato, Y., Shima, N., Higashio, K., and Kuwano, M. Hepatocyte growth factor modulates migration and proliferation of human microvascular endothelial cells in culture. Biochem Biophys Res Commun, *179:* 1042-1049, 1991.
274. Grant, D. S., Kleinman, H. K., Goldberg, I. D., Bhargava, M. M., Nickoloff, B. J., Kinsella, J. L., Polverini, P., and Rosen, E. M. Scatter factor induces blood vessel formation in vivo. Proc Natl Acad Sci U S A, *90:* 1937-1941, 1993.
275. Rosen, E. M., Grant, D. S., Kleinman, H. K., Goldberg, I. D., Bhargava, M. M., Nickoloff, B. J., Kinsella, J. L., and Polverini, P. Scatter factor (hepatocyte growth factor) is a potent angiogenesis factor in vivo. Symp Soc Exp Biol, *47:* 227-234, 1993.
276. Wojta, J., Kaun, C., Breuss, J. M., Koshelnick, Y., Beckmann, R., Hattey, E., Mildner, M., Weninger, W., Nakamura, T., Tschachler, E., and Binder, B. R. Hepatocyte growth factor increases expression of vascular endothelial growth factor and plasminogen activator inhibitor-1 in human keratinocytes and the vascular endothelial growth factor receptor flk-1 in human endothelial cells. Lab Invest, *79:* 427-438, 1999.
277. Nakabayashi, M., Morishita, R., Nakagami, H., Kuba, K., Matsumoto, K., Nakamura, T., Tano, Y., and Kaneda, Y. HGF/NK4 inhibited VEGF-induced angiogenesis in in vitro cultured endothelial cells and in vivo rabbit model. Diabetologia, *46:* 115-123, 2003.
278. Shiota, G., Wang, T. C., Nakamura, T., and Schmidt, E. V. Hepatocyte growth factor in transgenic mice: effects on hepatocyte growth, liver regeneration and gene expression. Hepatology, *19:* 962-972, 1994.
279. Huh, C. G., Factor, V. M., Sanchez, A., Uchida, K., Conner, E. A., and Thorgeirsson, S. S. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci U S A, *101:* 4477-4482, 2004.
280. Matsumoto, K. and Nakamura, T. Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases. Kidney Int, *59:* 2023-2038, 2001.
281. Yanagita, K., Matsumoto, K., Sekiguchi, K., Ishibashi, H., Niho, Y., and Nakamura, T. Hepatocyte growth factor may act as a pulmotrophic factor on

lung regeneration after acute lung injury. J Biol Chem, *268:* 21212-21217, 1993.

282. Nakamura, T., Mizuno, S., Matsumoto, K., Sawa, Y., and Matsuda, H. Myocardial protection from ischemia/reperfusion injury by endogenous and exogenous HGF. J Clin Invest, *106:* 1511-1519, 2000.
283. Mizuno, S., Matsumoto, K., Kurosawa, T., Mizuno-Horikawa, Y., and Nakamura, T. Reciprocal balance of hepatocyte growth factor and transforming growth factor-beta 1 in renal fibrosis in mice. Kidney Int, *57:* 937-948, 2000.
284. Mizuno, S., Kurosawa, T., Matsumoto, K., Mizuno-Horikawa, Y., Okamoto, M., and Nakamura, T. Hepatocyte growth factor prevents renal fibrosis and dysfunction in a mouse model of chronic renal disease. J Clin Invest, *101:* 1827-1834, 1998.
285. Michalopoulos, G., Houck, K. A., Dolan, M. L., and Leutteke, N. C. Control of hepatocyte replication by two serum factors. Cancer Res, *44:* 4414-4419, 1984.
286. Igawa, T., Kanda, S., Kanetake, H., Saitoh, Y., Ichihara, A., Tomita, Y., and Nakamura, T. Hepatocyte growth factor is a potent mitogen for cultured rabbit renal tubular epithelial cells. Biochem Biophys Res Commun, *174:* 831-838, 1991.
287. Kan, M., Zhang, G. H., Zarnegar, R., Michalopoulos, G., Myoken, Y., McKeehan, W. L., and Stevens, J. I. Hepatocyte growth factor/hepatopoietin A stimulates the growth of rat kidney proximal tubule epithelial cells (RPTE), rat nonparenchymal liver cells, human melanoma cells, mouse keratinocytes and stimulates anchorage-independent growth of SV-40 transformed RPTE. Biochem Biophys Res Commun, *174:* 331-337, 1991.
288. Matsumoto, K., Hashimoto, K., Yoshikawa, K., and Nakamura, T. Marked stimulation of growth and motility of human keratinocytes by hepatocyte growth factor. Exp Cell Res, *196:* 114-120, 1991.
289. Rubin, J. S., Chan, A. M., Bottaro, D. P., Burgess, W. H., Taylor, W. G., Cech, A. C., Hirschfield, D. W., Wong, J., Miki, T., Finch, P. W., and et al. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. Proc Natl Acad Sci U S A, *88:* 415-419, 1991.
290. Gherardi, E., Gray, J., Stoker, M., Perryman, M., and Furlong, R. Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interactions and movement. Proc Natl Acad Sci U S A, *86:* 5844-5848, 1989.
291. Rosen, E. M., Meromsky, L., Setter, E., Vinter, D. W., and Goldberg, I. D. Purified scatter factor stimulates epithelial and vascular endothelial cell migration. Proc Soc Exp Biol Med, *195:* 34-43, 1990.
292. Royal, I. and Park, M. Hepatocyte growth factor-induced scatter of Madin-Darby canine kidney cells requires phosphatidylinositol 3-kinase. J Biol Chem, *270:* 27780-27787, 1995.
293. Royal, I., Lamarche-Vane, N., Lamorte, L., Kaibuchi, K., and Park, M. Activation of cdc42, rac, PAK, and rho-kinase in response to hepatocyte growth factor differentially regulates epithelial cell colony spreading and dissociation. Mol Biol Cell, *11:* 1709-1725, 2000.

294. Dowrick, P. G., Prescott, A. R., and Warn, R. M. Scatter factor affects major changes in the cytoskeletal organization of epithelial cells. Cytokine, *3:* 299-310, 1991.
295. Dunsmore, S. E., Rubin, J. S., Kovacs, S. O., Chedid, M., Parks, W. C., and Welgus, H. G. Mechanisms of hepatocyte growth factor stimulation of keratinocyte metalloproteinase production. J Biol Chem, *271:* 24576-24582, 1996.
296. McCawley, L. J., O'Brien, P., and Hudson, L. G. Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)- mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. J Cell Physiol, *176:* 255-265, 1998.
297. Weidner, K. M., Behrens, J., Vandekerckhove, J., and Birchmeier, W. Scatter factor: molecular characteristics and effect on the invasiveness of epithelial cells. J Cell Biol, *111:* 2097-2108, 1990.
298. Sunitha, I., Meighen, D. L., Hartman, D. P., Thompson, E. W., Byers, S. W., and Avigan, M. I. Hepatocyte growth factor stimulates invasion across reconstituted basement membranes by a new human small intestinal cell line. Clin Exp Metastasis, *12:* 143-154, 1994.
299. Khoury, H., Naujokas, M. A., Zuo, D., Sangwan, V., Frigault, M. M., Petkiewicz, S., Dankort, D. L., Muller, W. J., and Park, M. HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion. Mol Biol Cell, *16:* 550-561, 2005.
300. Rodrigues, S. P., Fathers, K. E., Chan, G., Zuo, D., Halwani, F., Meterissian, S., and Park, M. CrkI and CrkII function as key signaling integrators for migration and invasion of cancer cells. Mol Cancer Res, *3:* 183-194, 2005.
301. Brinkmann, V., Foroutan, H., Sachs, M., Weidner, K. M., and Birchmeier, W. Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J Cell Biol, *131:* 1573-1586, 1995.
302. Montesano, R., Schaller, G., and Orci, L. Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. Cell, *66:* 697-711, 1991.
303. Montesano, R., Soriano, J. V., Malinda, K. M., Ponce, M. L., Bafico, A., Kleinman, H. K., Bottaro, D. P., and Aaronson, S. A. Differential effects of hepatocyte growth factor isoforms on epithelial and endothelial tubulogenesis. Cell Growth Differ, *9:* 355-365, 1998.
304. Soriano, J. V., Pepper, M. S., Nakamura, T., Orci, L., and Montesano, R. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. J Cell Sci, *108 (Pt 2):* 413-430, 1995.
305. Tsarfaty, I., Resau, J. H., Rulong, S., Keydar, I., Faletto, D. L., and Vande Woude, G. F. The met proto-oncogene receptor and lumen formation. Science, *257:* 1258-1261, 1992.
306. Montesano, R., Matsumoto, K., Nakamura, T., and Orci, L. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. Cell, *67:* 901-908, 1991.

307. Pollack, A. L., Runyan, R. B., and Mostov, K. E. Morphogenetic mechanisms of epithelial tubulogenesis: MDCK cell polarity is transiently rearranged without loss of cell-cell contact during scatter factor/hepatocyte growth factor-induced tubulogenesis. Dev Biol, *204:* 64-79, 1998.
308. Naldini, L., Vigna, E., Narsimhan, R. P., Gaudino, G., Zarnegar, R., Michalopoulos, G. K., and Comoglio, P. M. Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET. Oncogene, *6:* 501-504, 1991.
309. Zhu, H., Naujokas, M. A., Fixman, E. D., Torossian, K., and Park, M. Tyrosine 1356 in the carboxyl-terminal tail of the HGF/SF receptor is essential for the transduction of signals for cell motility and morphogenesis. J Biol Chem, *269:* 29943-29948, 1994.
310. Rodrigues, G. A. and Park, M. Autophosphorylation modulates the kinase activity and oncogenic potential of the Met receptor tyrosine kinase. Oncogene, *9:* 2019-2027, 1994.
311. Weidner, K. M., Di Cesare, S., Sachs, M., Brinkmann, V., Behrens, J., and Birchmeier, W. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. Nature, *384:* 173-176, 1996.
312. Nguyen, L., Holgado-Madruga, M., Maroun, C., Fixman, E. D., Kamikura, D., Fournier, T., Charest, A., Tremblay, M. L., Wong, A. J., and Park, M. Association of the multisubstrate docking protein Gab1 with the hepatocyte growth factor receptor requires a functional Grb2 binding site involving tyrosine 1356. J Biol Chem, *272:* 20811-20819, 1997.
313. Maroun, C. R., Holgado-Madruga, M., Royal, I., Naujokas, M. A., Fournier, T. M., Wong, A. J., and Park, M. The Gab1 PH domain is required for localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase. Mol Cell Biol, *19:* 1784-1799, 1999.
314. Fournier, T. M., Kamikura, D., Teng, K., and Park, M. Branching tubulogenesis but not scatter of madin-darby canine kidney cells requires a functional Grb2 binding site in the Met receptor tyrosine kinase. J Biol Chem, *271:* 22211-22217, 1996.
315. Sachs, M., Brohmann, H., Zechner, D., Muller, T., Hulsken, J., Walther, I., Schaeper, U., Birchmeier, C., and Birchmeier, W. Essential role of Gab1 for signaling by the c-Met receptor in vivo. J Cell Biol, *150:* 1375-1384, 2000.
316. Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P. M. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. Cell, *77:* 261-271, 1994.
317. Fixman, E. D., Fournier, T. M., Kamikura, D. M., Naujokas, M. A., and Park, M. Pathways downstream of Shc and Grb2 are required for cell transformation by the tpr-Met oncoprotein. J Biol Chem, *271:* 13116-13122, 1996.
318. Ponzetto, C., Bardelli, A., Maina, F., Longati, P., Panayotou, G., Dhand, R., Waterfield, M. D., and Comoglio, P. M. A novel recognition motif for phosphatidylinositol 3-kinase binding mediates its association with the

hepatocyte growth factor/scatter factor receptor. Mol Cell Biol, *13:* 4600-4608, 1993.

319. Stefan, M., Koch, A., Mancini, A., Mohr, A., Weidner, K. M., Niemann, H., and Tamura, T. Src homology 2-containing inositol 5-phosphatase 1 binds to the multifunctional docking site of c-Met and potentiates hepatocyte growth factor-induced branching tubulogenesis. J Biol Chem, *276:* 3017-3023, 2001.
320. Park, C. Y. and Hayman, M. J. The tyrosines in the bidentate motif of the env-sea oncoprotein are essential for cell transformation and are binding sites for Grb2 and the tyrosine phosphatase SHP-2. J Biol Chem, *274:* 7583-7590, 1999.
321. Boccaccio, C., Ando, M., Tamagnone, L., Bardelli, A., Michieli, P., Battistini, C., and Comoglio, P. M. Induction of epithelial tubules by growth factor HGF depends on the STAT pathway. Nature, *391:* 285-288, 1998.
322. Porter, A. C. and Vaillancourt, R. R. Tyrosine kinase receptor-activated signal transduction pathways which lead to oncogenesis. Oncogene, *17:* 1343-1352, 1998.
323. Bowers, D. C., Fan, S., Walter, K. A., Abounader, R., Williams, J. A., Rosen, E. M., and Laterra, J. Scatter factor/hepatocyte growth factor protects against cytotoxic death in human glioblastoma via phosphatidylinositol 3-kinase- and AKT-dependent pathways. Cancer Res, *60:* 4277-4283, 2000.
324. Fan, S., Ma, Y. X., Wang, J. A., Yuan, R. Q., Meng, Q., Cao, Y., Laterra, J. J., Goldberg, I. D., and Rosen, E. M. The cytokine hepatocyte growth factor/scatter factor inhibits apoptosis and enhances DNA repair by a common mechanism involving signaling through phosphatidyl inositol 3' kinase. Oncogene, *19:* 2212-2223, 2000.
325. Potempa, S. and Ridley, A. J. Activation of both MAP kinase and phosphatidylinositide 3-kinase by Ras is required for hepatocyte growth factor/scatter factor-induced adherens junction disassembly. Mol Biol Cell, *9:* 2185-2200, 1998.
326. Karihaloo, A., O'Rourke, D. A., Nickel, C., Spokes, K., and Cantley, L. G. Differential MAPK pathways utilized for HGF- and EGF-dependent renal epithelial morphogenesis. J Biol Chem, *276:* 9166-9173, 2001.
327. Ridley, A. J., Comoglio, P. M., and Hall, A. Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac, and Rho in MDCK cells. Mol Cell Biol, *15:* 1110-1122, 1995.
328. Lamorte, L., Kamikura, D. M., and Park, M. A switch from p130Cas/Crk to Gab1/Crk signaling correlates with anchorage independent growth and JNK activation in cells transformed by the Met receptor oncoprotein. Oncogene, *19:* 5973-5981, 2000.
329. Sakkab, D., Lewitzky, M., Posern, G., Schaeper, U., Sachs, M., Birchmeier, W., and Feller, S. M. Signaling of hepatocyte growth factor/scatter factor (HGF) to the small GTPase Rap1 via the large docking protein Gab1 and the adapter protein CRKL. J Biol Chem, *275:* 10772-10778, 2000.
330. Kuniyasu, H., Yasui, W., Kitadai, Y., Yokozaki, H., Ito, H., and Tahara, E. Frequent amplification of the c-met gene in scirrhous type stomach cancer. Biochem Biophys Res Commun, *189:* 227-232, 1992.

331. Miller, C. T., Lin, L., Casper, A. M., Lim, J., Thomas, D. G., Orringer, M. B., Chang, A. C., Chambers, A. F., Giordano, T. J., Glover, T. W., and Beer, D. G. Genomic amplification of MET with boundaries within fragile site FRA7G and upregulation of MET pathways in esophageal adenocarcinoma. Oncogene, *25:* 409-418, 2006.
332. Faletto, D. L., Tsarfaty, I., Kmiecik, T. E., Gonzatti, M., Suzuki, T., and Vande Woude, G. F. Evidence for non-covalent clusters of the c-met proto-oncogene product. Oncogene, *7:* 1149-1157, 1992.
333. Rodrigues, G. A. and Park, M. Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. Mol Cell Biol, *13:* 6711-6722, 1993.
334. Soman, N. R., Correa, P., Ruiz, B. A., and Wogan, G. N. The TPR-MET oncogenic rearrangement is present and expressed in human gastric carcinoma and precursor lesions. Proc Natl Acad Sci U S A, *88:* 4892-4896, 1991.
335. Soman, N. R., Wogan, G. N., and Rhim, J. S. TPR-MET oncogenic rearrangement: detection by polymerase chain reaction amplification of the transcript and expression in human tumor cell lines. Proc Natl Acad Sci U S A, *87:* 738-742, 1990.
336. Yu, J., Miehlke, S., Ebert, M. P., Hoffmann, J., Breidert, M., Alpen, B., Starzynska, T., Stolte Prof, M., Malfertheiner, P., and Bayerdorffer, E. Frequency of TPR-MET rearrangement in patients with gastric carcinoma and in first-degree relatives. Cancer, *88:* 1801-1806, 2000.
337. Osaki, M., Miyata, H., Hayashi, A., Gomyo, Y., Tatebe, S., and Ito, H. Lack of rearranged Tpr-met mRNA expression in human gastric cancer cell lines and gastric mucosa and carcinoma. Anticancer Res, *16:* 2881-2884, 1996.
338. Heideman, D. A., Snijders, P. J., Bloemena, E., Meijer, C. J., Offerhaus, G. J., Meuwissen, S. G., Gerritsen, W. R., and Craanen, M. E. Absence of tpr-met and expression of c-met in human gastric mucosa and carcinoma. J Pathol, *194:* 428-435, 2001.
339. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. Met, metastasis, motility and more. Nat Rev Mol Cell Biol, *4:* 915-925, 2003.
340. Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell, *8:* 995-1004, 2001.
341. Vigna, E., Gramaglia, D., Longati, P., Bardelli, A., and Comoglio, P. M. Loss of the exon encoding the juxtamembrane domain is essential for the oncogenic activation of TPR-MET. Oncogene, *18:* 4275-4281, 1999.
342. Weidner, K. M., Sachs, M., Riethmacher, D., and Birchmeier, W. Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. Proc Natl Acad Sci U S A, *92:* 2597-2601, 1995.
343. Bardelli, A., Longati, P., Gramaglia, D., Stella, M. C., and Comoglio, P. M. Gab1 coupling to the HGF/Met receptor multifunctional docking site requires binding of Grb2 and correlates with the transforming potential. Oncogene, *15:* 3103-3111, 1997.

344. Ma, P. C., Kijima, T., Maulik, G., Fox, E. A., Sattler, M., Griffin, J. D., Johnson, B. E., and Salgia, R. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res, *63:* 6272-6281, 2003.
345. Kong-Beltran, M., Seshagiri, S., Zha, J., Zhu, W., Bhawe, K., Mendoza, N., Holcomb, T., Pujara, K., Stinson, J., Fu, L., Severin, C., Rangell, L., Schwall, R., Amler, L., Wickramasinghe, D., and Yauch, R. Somatic mutations lead to an oncogenic deletion of met in lung cancer. Cancer Res, *66:* 283-289, 2006.
346. Schmidt, L., Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J., Walther, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., Zbar, B., and et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet, *16:* 68-73, 1997.
347. Bardelli, A., Longati, P., Gramaglia, D., Basilico, C., Tamagnone, L., Giordano, S., Ballinari, D., Michieli, P., and Comoglio, P. M. Uncoupling signal transducers from oncogenic MET mutants abrogates cell transformation and inhibits invasive growth. Proc Natl Acad Sci U S A, *95:* 14379-14383, 1998.
348. Giordano, S., Maffe, A., Williams, T. A., Artigiani, S., Gual, P., Bardelli, A., Basilico, C., Michieli, P., and Comoglio, P. M. Different point mutations in the met oncogene elicit distinct biological properties. Faseb J, *14:* 399-406, 2000.
349. Maritano, D., Accornero, P., Bonifaci, N., and Ponzetto, C. Two mutations affecting conserved residues in the Met receptor operate via different mechanisms. Oncogene, *19:* 1354-1361, 2000.
350. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci U S A, *94:* 11445-11450, 1997.
351. Jeffers, M., Fiscella, M., Webb, C. P., Anver, M., Koochekpour, S., and Vande Woude, G. F. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci U S A, *95:* 14417-14422, 1998.
352. Jhiang, S. M. The RET proto-oncogene in human cancers. Oncogene, *19:* 5590-5597, 2000.
353. Miller, M., Ginalski, K., Lesyng, B., Nakaigawa, N., Schmidt, L., and Zbar, B. Structural basis of oncogenic activation caused by point mutations in the kinase domain of the MET proto-oncogene: modeling studies. Proteins, *44:* 32-43, 2001.
354. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. Central role of the threonine residue within the p+1 loop of receptor tyrosine kinase in STAT3 constitutive phosphorylation in metastatic cancer cells. Mol Cell Biol, *24:* 9390-9400, 2004.
355. Kamikura, D. M., Khoury, H., Maroun, C., Naujokas, M. A., and Park, M. Enhanced transformation by a plasma membrane-associated met oncoprotein:

activation of a phosphoinositide 3'-kinase-dependent autocrine loop involving hyaluronic acid and CD44. Mol Cell Biol, *20:* 3482-3496, 2000.

356. Fixman, E. D., Naujokas, M. A., Rodrigues, G. A., Moran, M. F., and Park, M. Efficient cell transformation by the Tpr-Met oncoprotein is dependent upon tyrosine 489 in the carboxy-terminus. Oncogene, *10:* 237-249, 1995.
357. Saucier, C., Papavasiliou, V., Palazzo, A., Naujokas, M. A., Kremer, R., and Park, M. Use of signal specific receptor tyrosine kinase oncoproteins reveals that pathways downstream from Grb2 or Shc are sufficient for cell transformation and metastasis. Oncogene, *21:* 1800-1811, 2002.
358. Fischer, J., Palmedo, G., von Knobloch, R., Bugert, P., Prayer-Galetti, T., Pagano, F., and Kovacs, G. Duplication and overexpression of the mutant allele of the MET proto-oncogene in multiple hereditary papillary renal cell tumours. Oncogene, *17:* 733-739, 1998.
359. Olivero, M., Valente, G., Bardelli, A., Longati, P., Ferrero, N., Cracco, C., Terrone, C., Rocca-Rossetti, S., Comoglio, P. M., and Di Renzo, M. F. Novel mutation in the ATP-binding site of the MET oncogene tyrosine kinase in a HPRCC family. Int J Cancer, *82:* 640-643, 1999.
360. Schmidt, L., Junker, K., Nakaigawa, N., Kinjerski, T., Weirich, G., Miller, M., Lubensky, I., Neumann, H. P., Brauch, H., Decker, J., Vocke, C., Brown, J. A., Jenkins, R., Richard, S., Bergerheim, U., Gerrard, B., Dean, M., Linehan, W. M., and Zbar, B. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene, *18:* 2343-2350, 1999.
361. Park, W. S., Dong, S. M., Kim, S. Y., Na, E. Y., Shin, M. S., Pi, J. H., Kim, B. J., Bae, J. H., Hong, Y. K., Lee, K. S., Lee, S. H., Yoo, N. J., Jang, J. J., Pack, S., Zhuang, Z., Schmidt, L., Zbar, B., and Lee, J. Y. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. Cancer Res, *59:* 307-310, 1999.
362. Jagadeeswaran, R., Ma, P. C., Seiwert, T. Y., Jagadeeswaran, S., Zumba, O., Nallasura, V., Ahmed, S., Filiberti, R., Paganuzzi, M., Puntoni, R., Kratzke, R. A., Gordon, G. J., Sugarbaker, D. J., Bueno, R., Janamanchi, V., Bindokas, V. P., Kindler, H. L., and Salgia, R. Functional analysis of c-Met/hepatocyte growth factor pathway in malignant pleural mesothelioma. Cancer Res, *66:* 352-361, 2006.
363. Lee, J. H., Han, S. U., Cho, H., Jennings, B., Gerrard, B., Dean, M., Schmidt, L., Zbar, B., and Vande Woude, G. F. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene, *19:* 4947-4953, 2000.
364. Tanyi, J., Tory, K., Rigo, J., Jr., Nagy, B., and Papp, Z. Evaluation of the tyrosine kinase domain of the Met proto-oncogene in sporadic ovarian carcinomas*. Pathol Oncol Res, *5:* 187-191, 1999.
365. Aebersold, D. M., Landt, O., Berthou, S., Gruber, G., Beer, K. T., Greiner, R. H., and Zimmer, Y. Prevalence and clinical impact of Met Y1253D-activating point mutation in radiotherapy-treated squamous cell cancer of the oropharynx. Oncogene, *22:* 8519-8523, 2003.
366. Liu, C., Park, M., and Tsao, M. S. Overexpression of c-met proto-oncogene but not epidermal growth factor receptor or c-erbB-2 in primary human colorectal carcinomas. Oncogene, *7:* 181-185, 1992.

367. Umeki, K., Shiota, G., and Kawasaki, H. Clinical significance of c-met oncogene alterations in human colorectal cancer. Oncology, *56:* 314-321, 1999.
368. Kuniyasu, H., Yasui, W., Yokozaki, H., Kitadai, Y., and Tahara, E. Aberrant expression of c-met mRNA in human gastric carcinomas. Int J Cancer, *55:* 72-75, 1993.
369. Tolnay, E., Kuhnen, C., Wiethege, T., Konig, J. E., Voss, B., and Muller, K. M. Hepatocyte growth factor/scatter factor and its receptor c-Met are overexpressed and associated with an increased microvessel density in malignant pleural mesothelioma. J Cancer Res Clin Oncol, *124:* 291-296, 1998.
370. Murai, M., Shen, X., Huang, L., Carpenter, W. M., Lin, C. S., Silverman, S., Regezi, J., and Kramer, R. H. Overexpression of c-met in oral SCC promotes hepatocyte growth factor-induced disruption of cadherin junctions and invasion. Int J Oncol, *25:* 831-840, 2004.
371. Yucel, O. T., Sungur, A., and Kaya, S. c-met overexpression in supraglottic laryngeal squamous cell carcinoma and its relation to lymph node metastases. Otolaryngol Head Neck Surg, *130:* 698-703, 2004.
372. Morello, S., Olivero, M., Aimetti, M., Bernardi, M., Berrone, S., Di Renzo, M. F., and Giordano, S. MET receptor is overexpressed but not mutated in oral squamous cell carcinomas. J Cell Physiol, *189:* 285-290, 2001.
373. Marshall, D. D. and Kornberg, L. J. Overexpression of scatter factor and its receptor (c-met) in oral squamous cell carcinoma. Laryngoscope, *108:* 1413-1417, 1998.
374. Porte, H., Triboulet, J. P., Kotelevets, L., Carrat, F., Prevot, S., Nordlinger, B., DiGioia, Y., Wurtz, A., Comoglio, P., Gespach, C., and Chastre, E. Overexpression of stromelysin-3, BM-40/SPARC, and MET genes in human esophageal carcinoma: implications for prognosis. Clin Cancer Res, *4:* 1375-1382, 1998.
375. Sawatsubashi, M., Sasatomi, E., Mizokami, H., Tokunaga, O., and Shin, T. Expression of c-Met in laryngeal carcinoma. Virchows Arch, *432:* 331-335, 1998.
376. Di Renzo, M. F., Olivero, M., Ferro, S., Prat, M., Bongarzone, I., Pilotti, S., Belfiore, A., Costantino, A., Vigneri, R., Pierotti, M. A., and et al. Overexpression of the c-MET/HGF receptor gene in human thyroid carcinomas. Oncogene, *7:* 2549-2553, 1992.
377. Fluge, O., Haugen, D. R., Lillehaug, J. R., and Varhaug, J. E. Difference in patterns of Met expression in papillary thyroid carcinomas and nonneoplastic thyroid tissue. World J Surg, *25:* 623-631, 2001.
378. Trovato, M., Villari, D., Bartolone, L., Spinella, S., Simone, A., Violi, M. A., Trimarchi, F., Batolo, D., and Benvenga, S. Expression of the hepatocyte growth factor and c-met in normal thyroid, non-neoplastic, and neoplastic nodules. Thyroid, *8:* 125-131, 1998.
379. Olivero, M., Rizzo, M., Madeddu, R., Casadio, C., Pennacchietti, S., Nicotra, M. R., Prat, M., Maggi, G., Arena, N., Natali, P. G., Comoglio, P. M., and Di Renzo, M. F. Overexpression and activation of hepatocyte growth

factor/scatter factor in human non-small-cell lung carcinomas. Br J Cancer, *74:* 1862-1868, 1996.

380. Cheng, H. L., Trink, B., Tzai, T. S., Liu, H. S., Chan, S. H., Ho, C. L., Sidransky, D., and Chow, N. H. Overexpression of c-met as a prognostic indicator for transitional cell carcinoma of the urinary bladder: a comparison with p53 nuclear accumulation. J Clin Oncol, *20:* 1544-1550, 2002.
381. Ghoussoub, R. A., Dillon, D. A., D'Aquila, T., Rimm, E. B., Fearon, E. R., and Rimm, D. L. Expression of c-met is a strong independent prognostic factor in breast carcinoma. Cancer, *82:* 1513-1520, 1998.
382. Camp, R. L., Rimm, E. B., and Rimm, D. L. Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. Cancer, *86:* 2259-2265, 1999.
383. Beviglia, L., Matsumoto, K., Lin, C. S., Ziober, B. L., and Kramer, R. H. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int J Cancer, *74:* 301-309, 1997.
384. Tsarfaty, I., Alvord, W. G., Resau, J. H., Altstock, R. T., Lidereau, R., Bieche, I., Bertrand, F., Horev, J., Klabansky, R. L., Keydar, I., and Vande Woude, G. F. Alteration of Met protooncogene product expression and prognosis in breast carcinomas. Anal Quant Cytol Histol, *21:* 397-408, 1999.
385. Jin, L., Fuchs, A., Schnitt, S. J., Yao, Y., Joseph, A., Lamszus, K., Park, M., Goldberg, I. D., and Rosen, E. M. Expression of scatter factor and c-met receptor in benign and malignant breast tissue. Cancer, *79:* 749-760, 1997.
386. Kuhnen, C., Muehlberger, T., Honsel, M., Tolnay, E., Steinau, H. U., and Muller, K. M. Impact of c-Met expression on angiogenesis in soft tissue sarcomas: correlation to microvessel-density. J Cancer Res Clin Oncol, *129:* 415-422, 2003.
387. Kajiya, K., Hirakawa, S., Ma, B., Drinnenberg, I., and Detmar, M. Hepatocyte growth factor promotes lymphatic vessel formation and function. Embo J, *24:* 2885-2895, 2005.
388. Jiang, W. G., Davies, G., Martin, T. A., Parr, C., Watkins, G., Mansel, R. E., and Mason, M. D. The potential lymphangiogenic effects of hepatocyte growth factor/scatter factor in vitro and in vivo. Int J Mol Med, *16:* 723-728, 2005.
389. Rong, S., Segal, S., Anver, M., Resau, J. H., and Vande Woude, G. F. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc Natl Acad Sci U S A, *91:* 4731-4735, 1994.
390. Jeffers, M., Rong, S., and Vande Woude, G. F. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol, *16:* 1115-1125, 1996.
391. Sheen-Chen, S. M., Liu, Y. W., Eng, H. L., and Chou, F. F. Serum levels of hepatocyte growth factor in patients with breast cancer. Cancer Epidemiol Biomarkers Prev, *14:* 715-717, 2005.

392. Taniguchi, T., Toi, M., Inada, K., Imazawa, T., Yamamoto, Y., and Tominaga, T. Serum concentrations of hepatocyte growth factor in breast cancer patients. Clin Cancer Res, *1:* 1031-1034, 1995.
393. Maemura, M., Iino, Y., Yokoe, T., Horiguchi, J., Takei, H., Koibuchi, Y., Horii, Y., Takeyoshi, I., Ohwada, S., and Morishita, Y. Serum concentration of hepatocyte growth factor in patients with metastatic breast cancer. Cancer Lett, *126:* 215-220, 1998.
394. Bharti, A., Ma, P. C., Maulik, G., Singh, R., Khan, E., Skarin, A. T., and Salgia, R. Haptoglobin alpha-subunit and hepatocyte growth factor can potentially serve as serum tumor biomarkers in small cell lung cancer. Anticancer Res, *24:* 1031-1038, 2004.
395. Vejchapipat, P., Tangkijvanich, P., Theamboonlers, A., Chongsrisawat, V., Chittmittrapap, S., and Poovorawan, Y. Association between serum hepatocyte growth factor and survival in untreated hepatocellular carcinoma. J Gastroenterol, *39:* 1182-1188, 2004.
396. Yamagamim, H., Moriyama, M., Matsumura, H., Aoki, H., Shimizu, T., Saito, T., Kaneko, M., Shioda, A., Tanaka, N., and Arakawa, Y. Serum concentrations of human hepatocyte growth factor is a useful indicator for predicting the occurrence of hepatocellular carcinomas in C-viral chronic liver diseases. Cancer, *95:* 824-834, 2002.
397. Yu, Y. and Merlino, G. Constitutive c-Met signaling through a nonautocrine mechanism promotes metastasis in a transgenic transplantation model. Cancer Res, *62:* 2951-2956, 2002.
398. Elliott, B. E., Hung, W. L., Boag, A. H., and Tuck, A. B. The role of hepatocyte growth factor (scatter factor) in epithelial-mesenchymal transition and breast cancer. Can J Physiol Pharmacol, *80:* 91-102, 2002.
399. Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K. M., Birchmeier, C., and Birchmeier, W. Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J Cell Biol, *131:* 215-226, 1995.
400. Gallego, M. I., Bierie, B., and Hennighausen, L. Targeted expression of HGF/SF in mouse mammary epithelium leads to metastatic adenosquamous carcinomas through the activation of multiple signal transduction pathways. Oncogene, *22:* 8498-8508, 2003.
401. Lengyel, E., Prechtel, D., Resau, J. H., Gauger, K., Welk, A., Lindemann, K., Salanti, G., Richter, T., Knudsen, B., Vande Woude, G. F., and Harbeck, N. C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. Int J Cancer, *113:* 678-682, 2005.
402. Lee, W. Y., Su, W. C., Lin, P. W., Guo, H. R., Chang, T. W., and Chen, H. H. Expression of S100A4 and Met: potential predictors for metastasis and survival in early-stage breast cancer. Oncology, *66:* 429-438, 2004.
403. Charafe-Jauffret, E., Ginestier, C., Monville, F., Finetti, P., Adelaide, J., Cervera, N., Fekairi, S., Xerri, L., Jacquemier, J., Birnbaum, D., and Bertucci, F. Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene, *25:* 2273-2284, 2006.

404. Yamashita, J., Ogawa, M., Yamashita, S., Nomura, K., Kuramoto, M., Saishoji, T., and Shin, S. Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. Cancer Res, *54:* 1630-1633, 1994.
405. Liang, T. J., Reid, A. E., Xavier, R., Cardiff, R. D., and Wang, T. C. Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. J Clin Invest, *97:* 2872-2877, 1996.
406. Takayama, H., LaRochelle, W. J., Sharp, R., Otsuka, T., Kriebel, P., Anver, M., Aaronson, S. A., and Merlino, G. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc Natl Acad Sci U S A, *94:* 701-706, 1997.
407. Jakubczak, J. L., LaRochelle, W. J., and Merlino, G. NK1, a natural splice variant of hepatocyte growth factor/scatter factor, is a partial agonist in vivo. Mol Cell Biol, *18:* 1275-1283, 1998.
408. Graveel, C., Su, Y., Koeman, J., Wang, L. M., Tessarollo, L., Fiscella, M., Birchmeier, C., Swiatek, P., Bronson, R., and Vande Woude, G. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci U S A, *101:* 17198-17203, 2004.
409. Welm, A. L., Kim, S., Welm, B. E., and Bishop, J. M. MET and MYC cooperate in mammary tumorigenesis. Proc Natl Acad Sci U S A, *102:* 4324-4329, 2005.
410. Cardiff, R. D., Sinn, E., Muller, W., and Leder, P. Transgenic oncogene mice. Tumor phenotype predicts genotype. Am J Pathol, *139:* 495-501, 1991.
411. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell, *54:* 105-115, 1988.
412. Brandt, R., Eisenbrandt, R., Leenders, F., Zschiesche, W., Binas, B., Juergensen, C., and Theuring, F. Mammary gland specific hEGF receptor transgene expression induces neoplasia and inhibits differentiation. Oncogene, *19:* 2129-2137, 2000.
413. Kirma, N., Luthra, R., Jones, J., Liu, Y. G., Nair, H. B., Mandava, U., and Tekmal, R. R. Overexpression of the colony-stimulating factor (CSF-1) and/or its receptor c-fms in mammary glands of transgenic mice results in hyperplasia and tumor formation. Cancer Res, *64:* 4162-4170, 2004.
414. Lin, E. Y., Gouon-Evans, V., Nguyen, A. V., and Pollard, J. W. The macrophage growth factor CSF-1 in mammary gland development and tumor progression. J Mammary Gland Biol Neoplasia, *7:* 147-162, 2002.
415. Chen, Y. Q., Zhou, Y. Q., Fu, L. H., Wang, D., and Wang, M. H. Multiple pulmonary adenomas in the lung of transgenic mice overexpressing the RON receptor tyrosine kinase. Recepteur d'origine nantais. Carcinogenesis, *23:* 1811-1819, 2002.
416. Chen, Y. Q., Zhou, Y. Q., Fisher, J. H., and Wang, M. H. Targeted expression of the receptor tyrosine kinase RON in distal lung epithelial cells results in multiple tumor formation: oncogenic potential of RON in vivo. Oncogene, *21:* 6382-6386, 2002.

417. Waltz, S. E., Eaton, L., Toney-Earley, K., Hess, K. A., Peace, B. E., Ihlendorf, J. R., Wang, M. H., Kaestner, K. H., and Degen, S. J. Ron-mediated cytoplasmic signaling is dispensable for viability but is required to limit inflammatory responses. J Clin Invest, *108:* 567-576, 2001.
418. Peace, B. E., Toney-Earley, K., Collins, M. H., and Waltz, S. E. Ron receptor signaling augments mammary tumor formation and metastasis in a murine model of breast cancer. Cancer Res, *65:* 1285-1293, 2005.
419. Zinser, G. M., Leonis, M. A., Toney, K., Pathrose, P., Thobe, M., Kader, S. A., Peace, B. E., Beauman, S. R., Collins, M. H., and Waltz, S. E. Mammary-specific Ron receptor overexpression induces highly metastatic mammary tumors associated with β-catenin activation. Cancer Res, *66:* 11967-11974, 2006.
420. Taipale, J. and Keski-Oja, J. Growth factors in the extracellular matrix. Faseb J, *11:* 51-59, 1997.
421. Kalluri, R. and Zeisberg, M. Fibroblasts in cancer. Nat Rev Cancer, *6:* 392-401, 2006.
422. Krtolica, A. and Campisi, J. Cancer and aging: a model for the cancer promoting effects of the aging stroma. Int J Biochem Cell Biol, *34:* 1401-1414, 2002.
423. Coppe, J. P., Kauser, K., Campisi, J., and Beausejour, C. M. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. J Biol Chem, *281:* 29568-29574, 2006.
424. Barcellos-Hoff, M. H. and Ravani, S. A. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. Cancer Res, *60:* 1254-1260, 2000.
425. Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y., and Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci U S A, *98:* 12072-12077, 2001.
426. Olumi, A. F., Grossfeld, G. D., Hayward, S. W., Carroll, P. R., Tlsty, T. D., and Cunha, G. R. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. Cancer Res, *59:* 5002-5011, 1999.
427. De Wever, O. and Mareel, M. Role of tissue stroma in cancer cell invasion. J Pathol, *200:* 429-447, 2003.
428. Desmouliere, A., Guyot, C., and Gabbiani, G. The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int J Dev Biol, *48:* 509-517, 2004.
429. Powell, D. W., Mifflin, R. C., Valentich, J. D., Crowe, S. E., Saada, J. I., and West, A. B. Myofibroblasts. I. Paracrine cells important in health and disease. Am J Physiol, *277:* C1-9, 1999.
430. Monvoisin, A., Neaud, V., De Ledinghen, V., Dubuisson, L., Balabaud, C., Bioulac-Sage, P., Desmouliere, A., and Rosenbaum, J. Direct evidence that hepatocyte growth factor-induced invasion of hepatocellular carcinoma cells is mediated by urokinase. J Hepatol, *30:* 511-518, 1999.
431. Monvoisin, A., Bisson, C., Si-Tayeb, K., Balabaud, C., Desmouliere, A., and Rosenbaum, J. Involvement of matrix metalloproteinase type-3 in hepatocyte

growth factor-induced invasion of human hepatocellular carcinoma cells. Int J Cancer, *97:* 157-162, 2002.

432. Dubuisson, L., Monvoisin, A., Nielsen, B. S., Le Bail, B., Bioulac-Sage, P., and Rosenbaum, J. Expression and cellular localization of the urokinase-type plasminogen activator and its receptor in human hepatocellular carcinoma. J Pathol, *190:* 190-195, 2000.
433. Yang, L., Scott, P. G., Giuffre, J., Shankowsky, H. A., Ghahary, A., and Tredget, E. E. Peripheral blood fibrocytes from burn patients: identification and quantification of fibrocytes in adherent cells cultured from peripheral blood mononuclear cells. Lab Invest, *82:* 1183-1192, 2002.
434. Kalka, C., Tehrani, H., Laudenberg, B., Vale, P. R., Isner, J. M., Asahara, T., and Symes, J. F. VEGF gene transfer mobilizes endothelial progenitor cells in patients with inoperable coronary disease. Ann Thorac Surg, *70:* 829-834, 2000.
435. Ishii, G., Sangai, T., Oda, T., Aoyagi, Y., Hasebe, T., Kanomata, N., Endoh, Y., Okumura, C., Okuhara, Y., Magae, J., Emura, M., Ochiya, T., and Ochiai, A. Bone-marrow-derived myofibroblasts contribute to the cancer-induced stromal reaction. Biochem Biophys Res Commun, *309:* 232-240, 2003.
436. Ronnov-Jessen, L., Petersen, O. W., Koteliansky, V. E., and Bissell, M. J. The origin of the myofibroblasts in breast cancer. Recapitulation of tumor environment in culture unravels diversity and implicates converted fibroblasts and recruited smooth muscle cells. J Clin Invest, *95:* 859-873, 1995.
437. Petersen, O. W., Nielsen, H. L., Gudjonsson, T., Villadsen, R., Rank, F., Niebuhr, E., Bissell, M. J., and Ronnov-Jessen, L. Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. Am J Pathol, *162:* 391-402, 2003.
438. Nowicki, A., Szenajch, J., Ostrowska, G., Wojtowicz, A., Wojtowicz, K., Kruszewski, A. A., Maruszynski, M., Aukerman, S. L., and Wiktor-Jedrzejczak, W. Impaired tumor growth in colony-stimulating factor 1 (CSF-1)-deficient, macrophage-deficient op/op mouse: evidence for a role of CSF-1-dependent macrophages in formation of tumor stroma. Int J Cancer, *65:* 112-119, 1996.
439. de Visser, K. E., Korets, L. V., and Coussens, L. M. De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. Cancer Cell, *7:* 411-423, 2005.
440. Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C., and Evans, R. M. Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature, *300:* 611-615, 1982.
441. Andres, A. C., Schonenberger, C. A., Groner, B., Hennighausen, L., LeMeur, M., and Gerlinger, P. Ha-ras oncogene expression directed by a milk protein gene promoter: tissue specificity, hormonal regulation, and tumor induction in transgenic mice. Proc Natl Acad Sci U S A, *84:* 1299-1303, 1987.
442. Stewart, T. A., Pattengale, P. K., and Leder, P. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell, *38:* 627-637, 1984.

443. Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet, *29:* 418-425, 2001.
444. Donehower, L. A., Godley, L. A., Aldaz, C. M., Pyle, R., Shi, Y. P., Pinkel, D., Gray, J., Bradley, A., Medina, D., and Varmus, H. E. Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. Genes Dev, *9:* 882-895, 1995.
445. Furth, P. A., St Onge, L., Boger, H., Gruss, P., Gossen, M., Kistner, A., Bujard, H., and Hennighausen, L. Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. Proc Natl Acad Sci U S A, *91:* 9302-9306, 1994.
446. Moody, S. E., Sarkisian, C. J., Hahn, K. T., Gunther, E. J., Pickup, S., Dugan, K. D., Innocent, N., Cardiff, R. D., Schnall, M. D., and Chodosh, L. A. Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. Cancer Cell, *2:* 451-461, 2002.
447. Strachan, T. and Read, A. P. Human Molecular Genetics 2, 2nd edition. Oxford: BIOS Scientific Publishers, 1999.
448. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet, *21:* 70-71, 1999.
449. Friedrich, G. and Soriano, P. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. Genes Dev, *5:* 1513-1523, 1991.
450. Wagner, K. U., Krempler, A., Triplett, A. A., Qi, Y., George, N. M., Zhu, J., and Rui, H. Impaired alveologenesis and maintenance of secretory mammary epithelial cells in Jak2 conditional knockout mice. Mol Cell Biol, *24:* 5510-5520, 2004.
451. Taketo, M., Schroeder, A. C., Mobraaten, L. E., Gunning, K. B., Hanten, G., Fox, R. R., Roderick, T. H., Stewart, C. L., Lilly, F., Hansen, C. T., and et al. FVB/N: an inbred mouse strain preferable for transgenic analyses. Proc Natl Acad Sci U S A, *88:* 2065-2069, 1991.
452. Zhou, H., Chen, W. D., Qin, X., Lee, K., Liu, L., Markowitz, S. D., and Gerson, S. L. MMTV promoter hypomethylation is linked to spontaneous and MNU associated c-neu expression and mammary carcinogenesis in MMTV c-neu transgenic mice. Oncogene, *20:* 6009-6017, 2001.
453. Chaillet, J. R., Bader, D. S., and Leder, P. Regulation of genomic imprinting by gametic and embryonic processes. Genes Dev, *9:* 1177-1187, 1995.
454. Nieto, A. I., Shyamala, G., Galvez, J. J., Thordarson, G., Wakefield, L. M., and Cardiff, R. D. Persistent mammary hyperplasia in FVB/N mice. Comp Med, *53:* 433-438, 2003.
455. Wakefield, L. M., Thordarson, G., Nieto, A. I., Shyamala, G., Galvez, J. J., Anver, M. R., and Cardiff, R. D. Spontaneous pituitary abnormalities and mammary hyperplasia in FVB/NCr mice: implications for mouse modeling. Comp Med, *53:* 424-432, 2003.
456. Cardiff, R. D. Are the TDLU of the human the same as the LA of mice? J Mammary Gland Biol Neoplasia, *3:* 3-5, 1998.

457. Deome, K. B., Faulkin, L. J., Jr., Bern, H. A., and Blair, P. B. Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res, *19:* 515-520, 1959.
458. Wellings, S. R. A hypothesis of the origin of human breast cancer from the terminal ductal lobular unit. Pathol Res Pract, *166:* 515-535, 1980.
459. Wellings, S. R. Development of human breast cancer. Adv Cancer Res, *31:* 287-314, 1980.
460. Cardiff, R. D. The biology of mammary transgenes: five rules. J Mammary Gland Biol Neoplasia, *1:* 61-73, 1996.
461. Andrechek, E. R., Laing, M. A., Girgis-Gabardo, A. A., Siegel, P. M., Cardiff, R. D., and Muller, W. J. Gene expression profiling of neu-induced mammary tumors from transgenic mice reveals genetic and morphological similarities to ErbB2-expressing human breast cancers. Cancer Res, *63:* 4920-4926, 2003.
462. Lin, E. Y., Jones, J. G., Li, P., Zhu, L., Whitney, K. D., Muller, W. J., and Pollard, J. W. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. Am J Pathol, *163:* 2113-2126, 2003.
463. Brennan, K. R. and Brown, A. M. Wnt proteins in mammary development and cancer. J Mammary Gland Biol Neoplasia, *9:* 119-131, 2004.
464. Rosner, A., Miyoshi, K., Landesman-Bollag, E., Xu, X., Seldin, D. C., Moser, A. R., MacLeod, C. L., Shyamala, G., Gillgrass, A. E., and Cardiff, R. D. Pathway pathology: histological differences between ErbB/Ras and Wnt pathway transgenic mammary tumors. Am J Pathol, *161:* 1087-1097, 2002.
465. Hobbs, A. A., Richards, D. A., Kessler, D. J., and Rosen, J. M. Complex hormonal regulation of rat casein gene expression. J Biol Chem, *257:* 3598-3605, 1982.
466. Campbell, S. M., Rosen, J. M., Hennighausen, L. G., Strech-Jurk, U., and Sippel, A. E. Comparison of the whey acidic protein genes of the rat and mouse. Nucleic Acids Res, *12:* 8685-8697, 1984.
467. Huang, A. L., Ostrowski, M. C., Berard, D., and Hager, G. L. Glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from mouse mammary tumor virus. Cell, *27:* 245-255, 1981.
468. Cato, A. C., Miksicek, R., Schutz, G., Arnemann, J., and Beato, M. The hormone regulatory element of mouse mammary tumour virus mediates progesterone induction. Embo J, *5:* 2237-2240, 1986.
469. Darbre, P., Page, M., and King, R. J. Androgen regulation by the long terminal repeat of mouse mammary tumor virus. Mol Cell Biol, *6:* 2847-2854, 1986.
470. Wagner, K. U., McAllister, K., Ward, T., Davis, B., Wiseman, R., and Hennighausen, L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res, *10:* 545-553, 2001.
471. Cardiff, R. D. Mouse models of human breast cancer. Comp Med, *53:* 250-253, 2003.

472. Beatson, G. On the treatmen of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. Lancet 104-107, 162-105, 1896.
473. Gruvberger, S. K., Ringner, M., Eden, P., Borg, A., Ferno, M., Peterson, C., and Meltzer, P. S. Expression profiling to predict outcome in breast cancer: the influence of sample selection. Breast Cancer Res, *5:* 23-26, 2003.
474. Gruvberger, S., Ringner, M., Chen, Y., Panavally, S., Saal, L. H., Borg, A., Ferno, M., Peterson, C., and Meltzer, P. S. Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res, *61:* 5979-5984, 2001.
475. West, M., Blanchette, C., Dressman, H., Huang, E., Ishida, S., Spang, R., Zuzan, H., Olson, J. A., Jr., Marks, J. R., and Nevins, J. R. Predicting the clinical status of human breast cancer by using gene expression profiles. Proc Natl Acad Sci U S A, *98:* 11462-11467, 2001.
476. van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R., and Friend, S. H. Gene expression profiling predicts clinical outcome of breast cancer. Nature, *415:* 530-536, 2002.
477. Mirza, A. N., Mirza, N. Q., Vlastos, G., and Singletary, S. E. Prognostic factors in node-negative breast cancer: a review of studies with sample size more than 200 and follow-up more than 5 years. Ann Surg, *235:* 10-26, 2002.
478. Bundred, N. J. Prognostic and predictive factors in breast cancer. Cancer Treat Rev, *27:* 137-142, 2001.
479. Guy, C. T., Webster, M. A., Schaller, M., Parsons, T. J., Cardiff, R. D., and Muller, W. J. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci U S A, *89:* 10578-10582, 1992.
480. Bouchard, L., Lamarre, L., Tremblay, P. J., and Jolicoeur, P. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. Cell, *57:* 931-936, 1989.
481. Michaelson, J. S. and Leder, P. beta-catenin is a downstream effector of Wnt-mediated tumorigenesis in the mammary gland. Oncogene, *20:* 5093-5099, 2001.
482. Lane, T. F. and Leder, P. Wnt-10b directs hypermorphic development and transformation in mammary glands of male and female mice. Oncogene, *15:* 2133-2144, 1997.
483. Miyoshi, K., Shillingford, J. M., Le Provost, F., Gounari, F., Bronson, R., von Boehmer, H., Taketo, M. M., Cardiff, R. D., Hennighausen, L., and Khazaie, K. Activation of beta -catenin signaling in differentiated mammary secretory cells induces transdifferentiation into epidermis and squamous metaplasias. Proc Natl Acad Sci U S A, *99:* 219-224, 2002.
484. Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., Tan, L. K., Rosen, J. M., and Varmus, H. E. Evidence that transgenes encoding components of the Wnt

signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci U S A, *100:* 15853-15858, 2003.

Chapter 2

Ubiquitylation Suppresses the Transforming Activity of an Oncogenic Met Receptor Tyrosine Kinase *In Vivo*

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Preface

Receptor tyrosine kinases (RTKs) have long been known to be involved in cellular transformation. Many RTKs, including the Met receptor family member, c-Sea, were originally identified as the cellular homologues of retroviral oncogenes. Dysregulated RTK signaling can arise through a variety of means including chromosomal translocations, amplification, point mutations, deletions, and generation of an autocrine signaling loop.

Dysregulation of Met RTK signaling has been observed in human papillary renal carcinomas expressing a Met receptor with activating point mutations in the kinase domain. In addition, point mutations and deletions in the juxtamembrane (JM) domain of Met have recently been identified in gastric and lung cancers. Both the activating and JM mutations have been shown to be transforming by *in vitro* assays. Y1003 in the JM domain of the Met receptor, a site that has been lost in the Met deletion mutant found in lung cancer, has been shown to serve as a binding site for the tyrosine kinase binding domain of the ubiquitin ligase Cbl. A Y1003F substitution results in decreased receptor ubiquitination and prolonged activation of downstream pathways following stimulation with ligand.

Met-Y1003F demonstrated transforming ability by *in vitro* assays and a tumorigenesis assay, but it was unknown if the uncoupling of the Met RTK from ubiquitination could contribute to tumorigenicity *in vivo*. In the following manuscript the transforming ability of the Y1003F mutation is contrasted with that of an activating mutation (M1250T) and we provide the first evidence that loss of ubiquitination contributes to tumorigenesis.

Abstract

Ubiquitylation of receptor tyrosine kinases promotes ligand-dependent receptor degradation in cell culture. However, the importance of receptor ubiquitylation *in vivo* has not been examined. We have previously described the role of Y1003 in the juxtamembrane domain of the Met receptor as providing a direct binding site for the ubiquitin ligase, Cbl. A Met receptor containing Y1003F displays decreased receptor ubiquitylation and delayed degradation. Here we demonstrate that decreased receptor ubiquitylation synergises with an activating mutation (M1250T) originally isolated from papillary renal carcinomas to induce tumorigenesis. Transgenic mice expressing a Met receptor containing both Y1003F and M1250T mutations under the control of the mouse mammary tumor virus promoter/enhancer develop tumors with greater penetrance and a shorter latency than do mice expressing Met-wt or either of the single mutants. Met-M1250T, expressed in T47D cells, responds to HGF stimulation in a similar fashion as Met-wt with lessening of receptor phosphorylation over time, however, addition of the Y1003F substitution (Met-Y1003F/M1250T) results in prolonged receptor phosphorylation and MAPK signaling. Met-Y1003F/M1250T cells grow better than any of the single mutant lines in soft agar, as well as in mammary fat pad injection tumorigenesis assays. This is the first demonstration that ubiquitylation is required to suppress the transforming activity of a receptor tyrosine kinase *in vivo*.

Abbreviations

ALAS1	d-Aminolevulinate Synthase
Akt	acutely transforming retrovirus AKT8 in rodent T cell lymphoma
Cbl	Casitas B-lineage lymphoma
CSF	Colony Stimulating Factor-1
EGFR	Epidermal Growth Factor Receptor
ErbB2	Erythroblastic leukemia viral oncogene2
FVB/N	Friend's leukemia Virus B strain ($Fv1^+$)-NIH-mouse strain
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
JM	Juxtamembrane
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen activated protein Kinase Kinase
MMTV	Mouse Mammary Tumor Virus
PI3K	Phosphoinositide-3 Kinase
RTK	Receptor Tyrosine Kinase
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
TKB	Tyrosine Kinase Binding domain

Introduction

The Met receptor tyrosine kinase (RTK) is expressed by epithelial cells in a variety of tissues whereas its ligand, hepatocyte growth factor/scatter factor (HGF/SF), is expressed by cells in the stroma. In culture, HGF/Met receptor signaling can induce epithelial cell proliferation as well as a breakdown of intercellular junctions, promoting migration and invasion of epithelial cells in two-dimensional scatter assays or three-dimensional tubular morphogenesis assays (1, 2). *In vivo* Met plays a role in tissue maintenance, having an important function in liver regeneration, wound healing, and angiogenesis (3).

Dysregulated Met receptor signaling is observed in tumors from a variety of organs. Mutations or deletions in Met have been detected in mesotheliomas (4), papillary renal carcinoma (5-8), gastric (9), liver (10), ovarian (11), oropharyngeal (12), and lung cancers (13, 14). Additionally, Met is overexpressed in tumors, as compared with normal tissue, in breast (15), stomach (16), colorectal (17), and head and neck cancers (18, 19). The first Met mutations studied were in the kinase domain (5, 8, 10, 11) and it was believed that these mutations could lead to enhanced kinase activity as demonstrated by increased receptor phosphorylation and enhanced ability to phosphorylated an exogenous substrate (20, 21). Shortly thereafter, several mutations were found in the juxtamembrane domain (JM) domain including deletion of the entirety of one JM-coding exon (MetΔEx14) (9, 13). Both the JM mutant and MetΔEx14 displayed prolonged receptor phosphorylation following treatment with HGF indicating a loss of receptor negative regulation (9, 13).

Our lab has established that the specific uncoupling of the Met RTK from ubiquitylation results in oncogenic activation of Met *in vitro* and in xenograft models (22). A Y1003F substitution in the JM domain prevents direct interaction of the tyrosine kinase binding (TKB) domain of the ubiquitin ligase Cbl with the Met receptor and inhibits receptor ubiquitylation (23, 24). This residue is lost in the MetΔEx14 found in lung cancer as well as in the oncogenic fusion protein Tpr-Met that displays constitutive activation (25).

Loss of ubiquitylation of the Met receptor tyrosine kinase has been shown to result in prolonged phosphorylation of the receptor and downstream signaling

proteins of the MAPK pathway (22, 23). Although the ubiquitin-deficient Met receptor internalizes at the same rate as a wild type Met receptor, degradation is delayed and expression of the ubiquitin-deficient receptor in fibroblasts induces cellular transformation *in vitro* (22, 23).

Loss of ubiquitylation of other RTKs has also been shown to result in cellular transformation by *in vitro* assays. The binding site for the ubiquitin ligase Cbl in the Colony Stimulating Factor 1 receptor (c-Fms) is absent from the oncogenic variant, v-Fms; adding back the Cbl-binding site results in decreased transforming ability of v-Fms by soft agar assay (26). Consistent with this, a tyrosine to phenylalanine substitution in the Cbl binding motif of c-Fms results in a weakly transforming receptor as determined by focus-forming and soft agar assays (27). Signaling from the Epidermal Growth Factor Receptor (EGFR) is also regulated by Cbl-mediated downregulation. Mutation of the Cbl binding site in EGFR drives a stronger mitogenic signal than a wt receptor upon stimulation with ligand (28). Additionally, it has been shown that Cdc42, activated following EGFR ligand binding, serves to bind to Cbl and sequester it away from the EGFR. Expression of a constitutively active Cdc42 results in prolonged signaling downstream of EGFR and cellular transformation *in vitro*, as a result of Cdc42 inhibiting Cbl-mediated EGFR degradation (29). Hence, by a variety of mechanisms, escape from Cbl-mediated ubiquitylation and receptor downregulation contributes to the generation of a receptor that can be transforming *in vitro*.

Transformation of epithelial cells through expression of a Met receptor containing an activating mutation has been demonstrated by both *in vitro* and *in vivo* assays; and *in vitro* assays have demonstrated the ability of Y1003F to transform, however, it was unknown if loss of ubiquitylation would play a role in RTK oncogenic activation *in vivo*. In order to determine the comparative transforming abilities of the activating mutation and the loss of negative regulation mutation, we generated Met receptor variants containing the loss of ubiquitylation substitution, Y1003F, or the kinase domain activating mutation from papillary renal carcinoma (M1250T) (5), or a receptor that contained both mutations (Met-YF/MT). Transgenic mice expressing Met-YF/MT in the mammary epithelium under the control of the

mouse mammary tumor virus promoter/enhancer developed tumors with a shorter latency and greater penetrance than mice expressing any of the single mutant Met receptor variants.

Through these studies we have demonstrated synergy between loss of negative regulation mutations and activating mutations in RTKs in inducing transformation. This highlights the importance of ubiquitylation *in vivo* in suppressing the transforming ability of an activated receptor.

Materials and Methods

Generation of Constructs

Chimeric CSF-Met variants were generated by removing the *BsrGI-NotI* fragment from the pSL301 vector containing the Met receptor chimera that was used to generate the transgenic mice and ligated into pXM-CSF-Met wt or pXM-CSF-Met Y1003F generated as previously described (23). The intracellular domain containing either M1250T or Y1003F/M1250T was then cleaved from the pXM-CSF-Met variants using *SwaI* and ligated into pLXSN-CSF-Met wt or pLXSN-CSF-Met Y1003F.

Cell Culture and Transformation Assays

T47D mammary epithelial cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum. T47D cell populations were generated by infection with retrovirus expressing Met-wt, -Y1003F, -M1250T, and -YF/MT Met constructs as previously described (22).

Rat1 fibroblast populations were maintained under selection in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 4 μg/ml G418. Stable populations were generated by infecting with retrovirus expressing chimeric CSF-Met receptor variants of Met-wt, -Y1003F, -M1250T, -YF/MT or vector alone. Following 2 weeks of selection, soft agar assays were performed as previously described (30).

Populations of EpH4 normal murine mammary epithelial cells were generated by infection with retrovirus expressing CSF-Met chimeric Met receptors generated as previously described (31) and as described above. Cells were selected and maintained under selection in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 4 μg/ml G418. Following at least 2 weeks of selection, 1 x 10^6 cells were injected into the mammary fat pads of nude (CD1 *nu/nu*; Charles River Breeding Laboratories) mice. The mice were examined regularly for tumor development and sacrificed when tumor volume reached 1 cm^3.

Immunoprecipitation and Immunoblotting

Following stimulation with HGF, T47D cell stable populations expressing the Met receptor variants were harvested in TGH lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 µg/ml aprotinin, and 10 µg/ml leupeptin). For the ubiquitination assay T47D cells were stimulated for 5 minutes at RT with 3nM HGF and lysed 100 µl hot buffer (2% SDS, 1mM EDTA). Lysates were boiled for 10 minutes followed by the addition of buffer containing 2.5% Triton X-100, 12.5 mM Tris, pH 7.5, 187.5 mM NaCl, and proteasomal inhibitors.

For immunoprecipitations, lysates were incubated with specific antibodies for 2 hours at 4°C with gentle mixing. Antibody-bound proteins were collected on protein A-Sepharose beads and washed three times in lysis buffer.

Proteins, whether whole cell lysate or immunoprecipitations were boiled in laemmeli buffer with 10% DTT, resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose membrane. Membranes were blocked in 3% bovine serum albumin in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 0.1% Tween 20) for 1 h and incubated with primary and secondary antibodies in TBST for 2 h and 1 h, respectively. Antibody-bound proteins were visualized using an ECL detection kit (Amersham Biosciences, Buckinghamshire, UK).

Antibodies and Reagents

Antibodies for immunoblotting were as follows: Antibody Met 144 was raised against a carboxy-terminal peptide of the human Met and does not cross react with murine Met (32). Antibodies against Met DO-24 were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-c-Cbl (SC-170) and anti-ubiquitin (P4D1) are from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phosphorylated Met (Y1349) as well as total and phosphorylated protein-specific Erk1/2 (pThr202/pTyr204), MEK1/2 (pSer217/221), and Akt1 (pSer473) are from Cell Signaling Technology (Mississauga, Ontario, Canada). Secondary antibodies for Western blotting were

HRP-Protein A, HRP-anti-mouse, and HRP-anti-goat, all from Amersham Biosciences.

Transgenic Mice

The chimeric Met receptor construct was generated in pXM by ligating a 2.6 kb cDNA coding for the extracellular domain of murine Met to 2 kb cDNA coding for the transmembrane domain and intracellular domain of human Met receptor. The two mutations were introduced by site directed mutagenesis followed by ligation of the sequences into the intracellular domain of human Met. The MMTV long terminal repeat and polyadenylation sequence of the SV40 early region were excised from a modified p206 vector (a kind gift of Dr. William Muller) (33) by *SalI* and *SpeI* cleavage and ligated into pSL301 (gift from Dr. William Muller), following destruction of the *HindIII* site in the multiple cloning site of pSL301. The chimeric Met cDNA was then ligated into the *HindIII* site between the MMTV and SV40 in pSL301. The Y1003F mutation was introduced as previously described (23). The M1250T mutation was introduced by site-directed mutagenesis using the QuikChange kit (Stratagene-La Jolla, CA) according to the manufacturers instructions. Methionine 1250 was converted to Thr using the 5'-GCCAGTGAAGTGGACGG CTCTAGAAAGTC -3' primer and its complementary primer. The silent mutations of the second C and A were added in order to supply a new restriction site for easy identification of clones. The 8.7 kb fragment containing the Met cDNA and the MMTV promoter and SV40 polyA was excised by *SfiI-SpeI* cleavage and injected into the pronuclei of FVB/N zygotes and implanted into FVB/N hosts by the McIntyre Transgenic Core Facility in Montreal, Canada. Mice were housed in the Royal Victoria Hospital (Montreal, Canada) transgenic mouse facility in accordance with McGill University Animal Ethics Committee guidelines.

DNA extraction and PCR

Founder animals were selected and all progeny genotyped by polymerase chain reaction using DNA extracted from tail clippings and primers binding in the C-

terminus of human Met and in the SV40 poly A tail of the construct (Forward=5'GATGAGGTGGACACACGACCAGCC, Reverse= 5'GCATTCC ACCACTGCTCCCATTC) yielding a band of 492 bp [95°C denaturation-5 minutes, 94°C-30s, 60°C-30s, 72°C-45s]. DNA was obtained from tail clippings following overnight digestion with proteinase K (100μg/mL) at 56°C (2M Tris (pH8.5), 0.05M EDTA (pH 7.0), 20% SDS, 5 M NaCl), phenol-chloroform extraction, and ethanol precipitation.

qRT-PCR:

To identify founder lines with highest levels of expression of transgene transcript, RNA was extracted from mammary tissue following the Trizol protocol (Invitrogen, Frederick, MA). Following resuspension in diethyl pyrocarbonate treated water, ~30 μg of RNA was treated with DNaseI (Roche, Mannheim, Germany) (0.5U RNase inhibitor/μg RNA, 2U DNaseI/μg RNA in 10x buffer-100mM Tris pH8.4, 500mM KCl, 15mM $MgCl_2$, 0.01% gelatin) and isolated using phenol-chloroform and isopropanol precipitation. The DNase-treated RNA was then amplified to cDNA using oligo dT primers (Invitrogen) and SuperScript II (Invitrogen). The Qiagen (Mississauga, Canada) SYBR green kit was used for qRT-PCR. The primers as used for the qRT-PCR are specific to the SV40 polyadenylation sequence (Forward-5'GGAACCTTACTTCTGTGGTGT Reverse-5' GGAAAGTC CTTGGGGTCTTCT, product =300 bp [anneal 60°C]) were utilized to detect transgene cDNA. GAPDH was used as an internal control (GAPDH Forward-5'-ACCACAGTCCATGCCATCAC Reverse-5'-TCCACCACCCTGTTGCTGTA, [anneal 55°C] product=300 bp). For quantification, the cycle threshold number (Ct) exhibiting the maximum growth curve rate was determined by the Rotor-Gene software (Corbett Research, Sydney, Australia).

Whole Mount Preparation

Whole mounts were prepared by mounting the left abdominal mammary fat pad on a glass slide and processed as previously described (34).

Results

Loss of Cbl TKB Binding Site Inhibits Ubiquitylation of an Activated Met RTK

Previously we have shown that the Y1003 residue in the juxtamembrane domain of the Met receptor provides a direct binding site for the Cbl ubiquitin ligase and is required for efficient receptor ubiquitylation. Substitution of the tyrosine residue with a phenylalanine reduces receptor ubiquitylation and promotes oncogenic activation of the receptor in tissue culture and nude mouse tumorigenesis assays (22, 23). In order to determine if the presence of an activating mutation (M1250T) affects the ubiquitylation status of the receptor with or without a Y1003F substitution we utilized the mammary epithelial cell line T47D to generate stable cell lines that expressed each of the Met receptor variants. T47D cells are one of the few epithelial cell lines that has undetectable levels of expression of endogenous Met receptor (35), making these cells ideal for the study of mutant Met receptor ubiquitination. Following 5 minutes of stimulation with 3 nM HGF the cells were lysed and Met receptor immunoprecipitated and blotted for ubiquitin. Both the wt and M1250T receptors underwent ubiquitylation following stimulation whereas both the Y1003F and the YF/MT variants exhibited decreased receptor ubiquitylation consistent with loss of binding of the Cbl TKB domain to the Met receptor (fig. 1B).

Presence of Y1003F in the Met Receptor Prolongs Receptor Signaling

Previous reports from our lab have demonstrated that the loss of receptor ubiquitylation through the Met Y1003F substitution results in sustained receptor phosphorylation and activation of downstream signaling pathways following treatment with HGF (22, 23). To determine how each Met receptor variant behaved following treatment with ligand-whether one mutation was dominant over the other in dictating the response-we stimulated stable populations of the mammary epithelial cell line T47D with HGF and examined the response of the receptor and downstream signaling proteins over time. Through 4 hours of stimulation with 1.5 nM HGF, cells expressing the Y1003F and YF/MT mutatants show strong receptor phosphorylation whereas the Met-wt and Met-M1250T show decreased levels of receptor

phosphorylation at 4 hours (fig. 2A). Both Met-wt and Met-M1250T display an initial rise in phosphorylation but by 240 minutes the levels of phospho-Met have returned to near baseline whereas the receptors containing the Y1003F mutation retain high levels of phosphorylation with respect to the amount of total Met through 240 mintues (fig. 2C). The Met receptor mediates downstream signaling through the adaptor proteins Grb2 and Shc which couple the receptor to the Ras-MAPK pathway and the scaffold protein Gab1. Gab1 functions to also couple Met with the Ras-MAPK pathway, but additionally to the phosphoinositol-3-kinase (PI3K)-AKT pathway (36, 37). Consistent with sustained receptor phosphorylation, in receptors containing the Y1003F substitution, MAPK pathway proteins Erk1/2, MEK1/2 as well as PI3K pathway protein Akt remain highly phosphorylated through 4 hours following receptor stimulation whereas Met-wt and Met-M1250T display a decline in phospho-protein levels after 60 minutes post-stimulation.

Met Receptor Variants Are Transforming *in vitro*

Expression of Met-M1250T in fibroblasts has previously been shown to be transforming *in vitro* by focus forming assay and *in vivo* by subcutaneous injection of NIH3T3 cells expressing the receptor variant (38). As determined by HGF stimulation of the T47D populations, the addition of the Y1003F substitution to Met-M1250T resulted in prolonged receptor phosphorylation and a pattern of phosphorylated downstream proteins similar to that of the Met-Y1003F. Although Met-YF/MT did not appear to signal differently from Met-Y1003F, it was not clear if the Y1003F substitution synergised with M1250T resulting in greater transforming efficiency than either single mutant Met variant.

Expression of the M1250T Met receptor variants in Rat1 fibroblasts resulted in morphological changes in the cells with acquisition of a more stellate shape and decreased cell spreading (fig. 3C). The phenotype of the Met-Y1003F Rat1 cell populations was not significantly different from the Met-wt and vector control cells. The Met-M1250T and Met-YF/MT variants were poorly adherent, and frequently detached from the dish as the cells became confluent.

In order to examine the transforming abilities of the Met receptor variants in an assay in which adhesion is not a factor, we performed soft agar assays using Rat1 populations stably expressing similar levels of the chimeric Met receptor variants (fig. 3D). Met-Y1003F formed a higher number of colonies per plate (~186) than the Met-M1250T or Met-YF/MT (~98 and ~161, respectively) however they were much smaller than those formed by Met-M1250T or Met-YF/MT. Interestingly, the Met-YF/MT displayed a phenotype intermediate between the Met-Y1003F and Met-M1250T and generated a high number of small colonies but approximately the same fraction of large colonies as the Met-M1250T (~10%) whereas only ~5% of the Met-Y1003F colonies were large. Nonetheless, all three variants formed at least 4 times more colonies than did the wt Met or the vector control demonstrating that all three variants do have some degree of transforming ability *in vitro*.

Loss of Receptor Ubiquitination Results in Transformation *in vivo*

The effect of loss of receptor ubiquitination alone or in combination with an activating mutation was assayed *in vivo* using the normal murine mammary epithelial cell line, EpH4. Stable populations of cells expressing equal levels of the CSF-Met chimeric receptor variants were established following infection with retrovirus and antibiotic selection (fig. 4B). As a positive control we also generated stable lines expressing the oncogenic Met receptor fusion protein, Tpr-Met. There were no obvious phenotypic differences between the Met variant epithelial cell lines (data not shown). Following injection of $1x10^6$ cells into the mammary fat pads of nude mice, tumors developed with varying latencies. With 100% penetrance, EpH4 cells expressing Met-YF/MT developed tumors with the same latency as the Tpr-Met expressing cells, at approximately 70 days. By 100 days, less than 50% of the Met-Y1003F and Met-M1250T injected mice had developed tumors. Met-wt and vector control did not form tumors through 200 days (fig. 4A). All tumors that developed in the nude mice strongly expressed the Met receptor variant proteins (fig. 4C). Hence, the activating and the loss of negative regulation mutations acted synergistically to consistently induce tumor development with the shortest latency.

Y1003F and M1250T Act Synergistically to Induce the Formation of Mammary Tumors in Transgenic Mice

Fifteen to twenty percent of human breast tumors exhibit elevated levels of Met expression compared with normal mammary epithelium (15, 39-41). The sensitivity of the mammary epithelium to transformation by dysregulated Met signaling has been demonstrated in transgenic mice expressing Met-M1250T under the control of the ubiquitously expressed metallothionein promoter that developed mammary adenocarcinomas at 10 months of age (38). In order to determine if loss of negative regulation alone was sufficient to induce mammary tumors or if it could enhance the tumorigenicity of Met-M1250T in the mammary epithelium, we generated transgenic mice expressing wt Met, Met-Y1003F, Met-M1250T and Met-YF/MT under the control of the mouse mammary tumor virus promoter/enhancer (MMTV) (Supp. fig. 1A). A chimeric receptor was utilized in order to distinguish, by immunoblotting, between the endogenous mouse Met receptor and the exogenous trangenic receptor using an antibody specific to an epitope in the C-terminus of the human Met receptor (32). The chimeric receptor (extracellular mouse/intracellular human) has been shown, in NIH3T3 cells injected subcutaneously into nude mice, to have equal transforming ability as a wholly mouse receptor (42).

Between eight and twelve founder animals were generated for each construct. Two lines for each Met receptor variant were selected for further study on the basis of higher transgene transcript in females at 10 weeks of age and at lactational day 18 as determined by RT-PCR (Supp. fig. 1B).

For each transgenic line two cohorts of mice were established-multiparous and nulliparous animals. Of the two lines, a primary line was selected to establish a cohort of at least 13 female mice that were continuously bred from 10 weeks of age in order to enhance transgene expression in the mammary epithelium. A cohort of nulliparous mice was established from both founder lines of each transgene. In addition to the transgenic animals, two cohorts of non-transgenic FVB mice were maintained-multiparous and nulliparous-in order to control for spontaneous lesions that arise in the FVB population.

None of the transgenic lines displayed any abnormal breeding or lactating phenotypes and litter sizes were similar to those from the FVB parental line. Whole mount analysis of the mammary fat pad 4 from 3 and 9 week nulliparous mice revealed no significant difference from the FVB controls (Supp. fig. 1C).

Multiparous mice from all lines, and the FVB controls, developed mammary tumors however, multiparous Met-YF/MT mice developed tumors with a decreased latency and increased penetrance compared with the other lines. From two lines of Met-YF/MT mice, multiparous mice developed tumors at approximately 419 days with 44% penetrance (Table 1). The M1250T mice developed tumors at approximately 547 days, but only 21% penetrance. Compared with FVB tumor latency, the Met-YF/MT tumors arose statistically significantly earlier ($p=6x10^{-5}$, student's t-test) whereas none of the other lines arose significantly earlier than FVB. Both Met-YF/MT and Met-M1250T tumors exhibited expression of the exogenous protein by immunoblotting, however none of the Met Y1003F nor the Met wt tumors expressed exogenous Met protein (data not shown). Additionally, the tumors that developed in the Met-YF/MT nulliparous mice exhibited expression of exogenous Met, whereas none of the other nulliparous mouse tumors did, again indicating the potency of Met-YF/MT to induce cellular transformation. The failure of the Met-wt, and Met-Y1003F tumors to express exogenous protein, coupled with the latencies suggests that these tumors are spontaneous tumors seen in aged mice. Thus, expression of Met-wt or Met-Y1003F alone is not sufficient to transform mammary epithelium in transgenic animals. Met-M1250T is not highly transforming either, however, together, the Y1003F contributes to the transforming ability of Met-M1250T.

Discussion

Growth factor receptor tyrosine kinases control growth, proliferation, differentiation, survival and migration. Since deregulation of more than 30 RTKs has been observed in human cancers (43), it is essential to understand how RTKs are regulated. Increased RTK signaling occurs through multiple mechanisms in human cancers including amplification of the gene locus, increased transcription, or point mutations that cause structural alterations resulting in increased kinase activity, altered substrate specificity or ligand-independent activation of the receptor. Recent data also indicate that failure of RTKs to be appropriately downmodulated following activation may contribute to cancer development or progression.

A major pathway involved in the downmodulation of RTKs involves their ligand-induced internalization through endocytosis followed by their degradation in the lysosome. The accelerated ligand-dependent degradation of RTKs is also a means of switching off initiated signaling pathways through the sequestration of the receptor cytosolic domain in the late endosome/multivesicular body. Receptor targeting for internalization into the multivesicular body is dependent on ubiquitylation (44-46). Although loss of ubiquitylation of the Met RTK has been shown to result in enhanced transforming activity of the receptor *in vitro*, the consequence of this oncogenic activation of the receptor *in vivo* was unknown.

Here we show that the loss of Met receptor ubiquitylation acts synergistically with receptor activation to induce tumorigenesis in a transgenic model of mammary tumorigenesis. The dramatic differences in tumor development in the MMTV-Met transgenic mice highlights the cooperation between the Y1003F and M1250T mutations. The failure of the Met-Y1003F mice to develop mammary tumors that express exogenous protein indicates that in non-immortalized cells Met-Y1003F is poorly transforming. Similarly, few MMTV-Met-M1250T mice developed mammary tumors, however, there were three tumors that did express exogenous Met protein indicating that Met-M1250T can be weakly transforming *in vivo*. The decreased latency and increased penetrance of tumors in the MMTV-Met-YF/MT demonstrates that loss of receptor ubiquitylation greatly enhances the transforming potential of the M1250T activating mutation. Fifty percent of MMTV-Met-YF/MT tumor bearing

mice carried multiple tumors, again indicating the potency of the Met-YF/MT in transforming cells. Graveel et al. generated knock-in mice that expressed a Met receptor containing one or two activating mutations (47). Fifty-nine percent of their Met-M1250T mice developed non-mammary tumors but when M1250T was combined with a second kinase domain activating mutation, the incidence of tumors was only 50% (47), thus there appears to be no synergy in tumorigenesis between the two activating mutations. We have created a unique model that demonstrates how two classes of mutations can have an additive effect on transformation.

When stably expressed in the mammary epithelial cell line T47D, the Met-Y1003F substitution results in decreased receptor ubiquitylation even in the presence of the M1250T activating mutation (fig. 1B) and the reduced ubiquitylation led to prolonged receptor phosphorylation following stimulation with HGF. Met-M1250T in T47D cells displayed signaling kinetics similar to that of Met-wt with near extinction of phospho-Erk1/2 and phospho-MEK four hours after addition of HGF to the medium whereas the variants containing the Y1003F substitution displayed sustained MAPK pathway activation (fig. 2). Phospho-Akt was induced and sustained in all Met variants except for Met-M1250T, thus the Y1003F appears dominant in maintaining phospho-Akt in Met-YF/MT. Importantly, as was previously observed in our laboratory, the receptor variants that contain the Y1003F mutation are expressed at higher levels in the stable T47D populations (22). The increased levels of protein are consistent with a study that demonstrated that Met receptor ubiquitylation correlates with ligand-induced degradation (48).

Similarly, when expressed in the normal murine mammary epithelial cell line EpH4, the additive effects of the Y1003F and M1250T mutations were observed. Singly, Met-Y1003F and Met-M1250T are only weakly transforming, however when combined the transforming ability was equivalent to that of the potent oncogenic form of Met, Tpr-Met. It is possible that because the Y1003F substitution causes the receptor to be inefficiently degraded (22) that Met-YF/MT may continue to signal intracellularly as does the cytoplasmic protein Tpr-Met (49), whereas Met-wt or Met-M1250T does not signal from within in the cell, but only at the membrane. Alternatively, perhaps the duration of the signal, as a result of impaired receptor

degradation, is a causal factor in cellular transformation. Tpr-Met is constitutively dimerized by the Tpr leucine zipper (25) but it is also lacking residue Y1003 and may not be properly degraded, thus the prolonged signals may be similar between Met-YF/MT and Tpr-Met.

Impaired receptor ubiquitylation of an RTK may play a role in breast cancers that overexpress the EGFR family member, ErbB2. Twenty to thirty percent of human breast cancers overexpress ErbB2 and this finding indicates poor prognosis and decreased survival (50, 51). There is no known ligand for ErbB2 and overexpression of ErbB2 drives the formation of heterodimers with other EGFR family members that propagate an EGFR signal intracellularly (52). ErbB2 is resistant to Cbl-mediated degradation and confers this resistance to active heterodimers possibly by failing to phosphorylate the Cbl-binding site on the other member of the receptor pair (53). EGFR-ErbB2 heterodimers are slowly internalized and quickly recycle back to the plasma membrane (53, 54), allowing for potentiation of the EGFR-dependent signal. Thus, by dimerizing with ErbB2, EGFR escapes Cbl-mediated downregulation, however, whether this occurs *in vivo* is unknown.

In human lung cancer, a Met receptor splice variant was identified that is lacking exon 14 (MetΔEx14), coding for a large portion of the juxtamembrane domain and including Y1003 (13). Similar to the Met-Y1003F, MetΔEx14 displayed reduced ubiquitylation and prolonged phosphorylation of both receptor and downstream signaling proteins upon treatment with ligand (13). Additionally, expression of MetΔEx14 in fibroblasts was shown to induce tumor formation in a xenograft model (13, 55). Interestingly, in neither tumorigenesis assay was Met-Y1003F used as a control, thus it is still unknown if Y1003F alone transforms to the same extent as MetΔEx14. The discovery of MetΔEx14 in human lung cancer, but not in nonneoplastic tissue from the same patient suggests that the dysregulated receptor plays a role in the initiation or maintenance of the tumor. As the juxtamembrane domain of RTKs frequently plays a role in receptor negative regulation (56), it is possible that MetΔEx14 is more transforming than Met-Y1003F as a result of disruption of other negative regulatory functions that the JM domain

may serve. Comparison of the *in vivo* transforming abilities of the two Met JM mutants may yield insight into other negative regulatory functions that the Met JM domain may have. If the JM domain serves only to permit receptor ubiquitylation, our studies suggest that it is unlikely that expression of MetΔEx14 was the initiating event in tumorigenesis and may be an incidental finding.

Through these studies we have demonstrated that loss of negative regulation of an activated receptor tyrosine kinase can contribute to cellular transformation. Although, Met-Y1003F alone was not a strongly transforming signal, when combined with the activating Met-M1250T, there was an additive effect on transformation observed in the *in vivo* tumorigenesis assays. As point mutations and deletions have already been observed in tumors in the JM domain of the Met receptor (13, 14), perhaps loss of negative regulation of RTKs is a more common event than previously believed and should not be overlooked.

References

1. Royal, I. and Park, M. Hepatocyte growth factor-induced scatter of Madin-Darby canine kidney cells requires phosphatidylinositol 3-kinase. J Biol Chem, *270:* 27780-27787, 1995.
2. Brinkmann, V., Foroutan, H., Sachs, M., Weidner, K. M., and Birchmeier, W. Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J Cell Biol, *131:* 1573-1586, 1995.
3. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. Met, metastasis, motility and more. Nat Rev Mol Cell Biol, *4:* 915-925, 2003.
4. Jagadeeswaran, R., Ma, P. C., Seiwert, T. Y., Jagadeeswaran, S., Zumba, O., Nallasura, V., Ahmed, S., Filiberti, R., Paganuzzi, M., Puntoni, R., Kratzke, R. A., Gordon, G. J., Sugarbaker, D. J., Bueno, R., Janamanchi, V., Bindokas, V. P., Kindler, H. L., and Salgia, R. Functional analysis of c-Met/hepatocyte growth factor pathway in malignant pleural mesothelioma. Cancer Res, *66:* 352-361, 2006.
5. Schmidt, L., Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J., Walther, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., Zbar, B., and et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet, *16:* 68-73, 1997.
6. Fischer, J., Palmedo, G., von Knobloch, R., Bugert, P., Prayer-Galetti, T., Pagano, F., and Kovacs, G. Duplication and overexpression of the mutant allele of the MET proto-oncogene in multiple hereditary papillary renal cell tumours. Oncogene, *17:* 733-739, 1998.
7. Schmidt, L., Junker, K., Nakaigawa, N., Kinjerski, T., Weirich, G., Miller, M., Lubensky, I., Neumann, H. P., Brauch, H., Decker, J., Vocke, C., Brown, J. A., Jenkins, R., Richard, S., Bergerheim, U., Gerrard, B., Dean, M., Linehan, W. M., and Zbar, B. Novel mutations of the MET proto-oncogene in papillary renal carcinomas. Oncogene, *18:* 2343-2350, 1999.
8. Olivero, M., Valente, G., Bardelli, A., Longati, P., Ferrero, N., Cracco, C., Terrone, C., Rocca-Rossetti, S., Comoglio, P. M., and Di Renzo, M. F. Novel mutation in the ATP-binding site of the MET oncogene tyrosine kinase in a HPRCC family. Int J Cancer, *82:* 640-643, 1999.
9. Lee, J. H., Han, S. U., Cho, H., Jennings, B., Gerrard, B., Dean, M., Schmidt, L., Zbar, B., and Vande Woude, G. F. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene, *19:* 4947-4953, 2000.
10. Park, W. S., Dong, S. M., Kim, S. Y., Na, E. Y., Shin, M. S., Pi, J. H., Kim, B. J., Bae, J. H., Hong, Y. K., Lee, K. S., Lee, S. H., Yoo, N. J., Jang, J. J., Pack, S., Zhuang, Z., Schmidt, L., Zbar, B., and Lee, J. Y. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. Cancer Res, *59:* 307-310, 1999.

11. Tanyi, J., Tory, K., Rigo, J., Jr., Nagy, B., and Papp, Z. Evaluation of the tyrosine kinase domain of the Met proto-oncogene in sporadic ovarian carcinomas*. Pathol Oncol Res, *5:* 187-191, 1999.
12. Aebersold, D. M., Landt, O., Berthou, S., Gruber, G., Beer, K. T., Greiner, R. H., and Zimmer, Y. Prevalence and clinical impact of Met Y1253D-activating point mutation in radiotherapy-treated squamous cell cancer of the oropharynx. Oncogene, *22:* 8519-8523, 2003.
13. Kong-Beltran, M., Seshagiri, S., Zha, J., Zhu, W., Bhawe, K., Mendoza, N., Holcomb, T., Pujara, K., Stinson, J., Fu, L., Severin, C., Rangell, L., Schwall, R., Amler, L., Wickramasinghe, D., and Yauch, R. Somatic mutations lead to an oncogenic deletion of met in lung cancer. Cancer Res, *66:* 283-289, 2006.
14. Ma, P. C., Kijima, T., Maulik, G., Fox, E. A., Sattler, M., Griffin, J. D., Johnson, B. E., and Salgia, R. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res, *63:* 6272-6281, 2003.
15. Tsarfaty, I., Alvord, W. G., Resau, J. H., Altstock, R. T., Lidereau, R., Bieche, I., Bertrand, F., Horev, J., Klabansky, R. L., Keydar, I., and Vande Woude, G. F. Alteration of Met protooncogene product expression and prognosis in breast carcinomas. Anal Quant Cytol Histol, *21:* 397-408, 1999.
16. Kuniyasu, H., Yasui, W., Kitadai, Y., Yokozaki, H., Ito, H., and Tahara, E. Frequent amplification of the c-met gene in scirrhous type stomach cancer. Biochem Biophys Res Commun, *189:* 227-232, 1992.
17. Liu, C., Park, M., and Tsao, M. S. Overexpression of c-met proto-oncogene but not epidermal growth factor receptor or c-erbB-2 in primary human colorectal carcinomas. Oncogene, *7:* 181-185, 1992.
18. Murai, M., Shen, X., Huang, L., Carpenter, W. M., Lin, C. S., Silverman, S., Regezi, J., and Kramer, R. H. Overexpression of c-met in oral SCC promotes hepatocyte growth factor-induced disruption of cadherin junctions and invasion. Int J Oncol, *25:* 831-840, 2004.
19. Yucel, O. T., Sungur, A., and Kaya, S. c-met overexpression in supraglottic laryngeal squamous cell carcinoma and its relation to lymph node metastases. Otolaryngol Head Neck Surg, *130:* 698-703, 2004.
20. Maritano, D., Accornero, P., Bonifaci, N., and Ponzetto, C. Two mutations affecting conserved residues in the Met receptor operate via different mechanisms. Oncogene, *19:* 1354-1361, 2000.
21. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci U S A, *94:* 11445-11450, 1997.
22. Abella, J. V., Peschard, P., Naujokas, M. A., Lin, T., Saucier, C., Urbe, S., and Park, M. Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol Cell Biol, *25:* 9632-9645, 2005.
23. Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. Mutation of the c-Cbl TKB domain binding

site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell, *8:* 995-1004, 2001.

24. Peschard, P., Ishiyama, N., Lin, T., Lipkowitz, S., and Park, M. A conserved DpYR motif in the juxtamembrane domain of the met receptor family forms an atypical c-Cbl/Cbl-b tyrosine kinase binding domain binding site required for suppression of oncogenic activation. J Biol Chem, *279:* 29565-29571, 2004.
25. Rodrigues, G. A. and Park, M. Dimerization mediated through a leucine zipper activates the oncogenic potential of the met receptor tyrosine kinase. Mol Cell Biol, *13:* 6711-6722, 1993.
26. Mancini, A., Koch, A., Wilms, R., and Tamura, T. c-Cbl associates directly with the C-terminal tail of the receptor for the macrophage colony-stimulating factor, c-Fms, and down-modulates this receptor but not the viral oncogene v-Fms. J Biol Chem, *277:* 14635-14640, 2002.
27. Roussel, M. F., Downing, J. R., Rettenmier, C. W., and Sherr, C. J. A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogene product) activates its transforming potential. Cell, *55:* 979-988, 1988.
28. Waterman, H., Katz, M., Rubin, C., Shtiegman, K., Lavi, S., Elson, A., Jovin, T., and Yarden, Y. A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. Embo J, *21:* 303-313, 2002.
29. Wu, W. J., Tu, S., and Cerione, R. A. Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation. Cell, *114:* 715-725, 2003.
30. Rodrigues, G. A., Park, M., and Schlessinger, J. Activation of the JNK pathway is essential for transformation by the Met oncogene. Embo J, *16:* 2634-2645, 1997.
31. Zhu, H., Naujokas, M. A., and Park, M. Receptor chimeras indicate that the met tyrosine kinase mediates the motility and morphogenic responses of hepatocyte growth/scatter factor. Cell Growth Differ, *5:* 359-366, 1994.
32. Rodrigues, G. A., Naujokas, M. A., and Park, M. Alternative splicing generates isoforms of the met receptor tyrosine kinase which undergo differential processing. Mol Cell Biol, *11:* 2962-2970, 1991.
33. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell, *49:* 465-475, 1987.
34. Webster, M. A., Hutchinson, J. N., Rauh, M. J., Muthuswamy, S. K., Anton, M., Tortorice, C. G., Cardiff, R. D., Graham, F. L., Hassell, J. A., and Muller, W. J. Requirement for both Shc and phosphatidylinositol 3' kinase signaling pathways in polyomavirus middle T-mediated mammary tumorigenesis. Mol Cell Biol, *18:* 2344-2359, 1998.
35. Shen, Y., Naujokas, M., Park, M., and Ireton, K. InlB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. Cell, *103:* 501-510, 2000.
36. Maroun, C. R., Holgado-Madruga, M., Royal, I., Naujokas, M. A., Fournier, T. M., Wong, A. J., and Park, M. The Gab1 PH domain is required for

localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase. Mol Cell Biol, *19:* 1784-1799, 1999.

37. Fixman, E. D., Holgado-Madruga, M., Nguyen, L., Kamikura, D. M., Fournier, T. M., Wong, A. J., and Park, M. Efficient cellular transformation by the Met oncoprotein requires a functional Grb2 binding site and correlates with phosphorylation of the Grb2-associated proteins, Cbl and Gab1. J Biol Chem, *272:* 20167-20172, 1997.
38. Jeffers, M., Fiscella, M., Webb, C. P., Anver, M., Koochekpour, S., and Vande Woude, G. F. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci U S A, *95:* 14417-14422, 1998.
39. Ghoussoub, R. A., Dillon, D. A., D'Aquila, T., Rimm, E. B., Fearon, E. R., and Rimm, D. L. Expression of c-met is a strong independent prognostic factor in breast carcinoma. Cancer, *82:* 1513-1520, 1998.
40. Camp, R. L., Rimm, E. B., and Rimm, D. L. Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. Cancer, *86:* 2259-2265, 1999.
41. Beviglia, L., Matsumoto, K., Lin, C. S., Ziober, B. L., and Kramer, R. H. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int J Cancer, *74:* 301-309, 1997.
42. Rong, S., Bodescot, M., Blair, D., Dunn, J., Nakamura, T., Mizuno, K., Park, M., Chan, A., Aaronson, S., and Vande Woude, G. F. Tumorigenicity of the met proto-oncogene and the gene for hepatocyte growth factor. Mol Cell Biol, *12:* 5152-5158, 1992.
43. Blume-Jensen, P. and Hunter, T. Oncogenic kinase signalling. Nature, *411:* 355-365, 2001.
44. Grovdal, L. M., Stang, E., Sorkin, A., and Madshus, I. H. Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation. Exp Cell Res, *300:* 388-395, 2004.
45. Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P. P., and Dikic, I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol, *5:* 461-466, 2003.
46. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor. Genes Dev, *12:* 3663-3674, 1998.
47. Graveel, C., Su, Y., Koeman, J., Wang, L. M., Tessarollo, L., Fiscella, M., Birchmeier, C., Swiatek, P., Bronson, R., and Vande Woude, G. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci U S A, *101:* 17198-17203, 2004.
48. Jeffers, M., Taylor, G. A., Weidner, K. M., Omura, S., and Vande Woude, G. F. Degradation of the Met tyrosine kinase receptor by the ubiquitin-proteasome pathway. Mol Cell Biol, *17:* 799-808, 1997.

49. Rodrigues, G. A. and Park, M. Autophosphorylation modulates the kinase activity and oncogenic potential of the Met receptor tyrosine kinase. Oncogene, *9:* 2019-2027, 1994.
50. Andrulis, I. L., Bull, S. B., Blackstein, M. E., Sutherland, D., Mak, C., Sidlofsky, S., Pritzker, K. P., Hartwick, R. W., Hanna, W., Lickley, L., Wilkinson, R., Qizilbash, A., Ambus, U., Lipa, M., Weizel, H., Katz, A., Baida, M., Mariz, S., Stoik, G., Dacamara, P., Strongitharm, D., Geddie, W., and McCready, D. neu/erbB-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer. Toronto Breast Cancer Study Group. J Clin Oncol, *16:* 1340-1349, 1998.
51. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science, *235:* 177-182, 1987.
52. Kokai, Y., Myers, J. N., Wada, T., Brown, V. I., LeVea, C. M., Davis, J. G., Dobashi, K., and Greene, M. I. Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts. Cell, *58:* 287-292, 1989.
53. Muthuswamy, S. K., Gilman, M., and Brugge, J. S. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol Cell Biol, *19:* 6845-6857, 1999.
54. Lenferink, A. E., Pinkas-Kramarski, R., van de Poll, M. L., van Vugt, M. J., Klapper, L. N., Tzahar, E., Waterman, H., Sela, M., van Zoelen, E. J., and Yarden, Y. Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers. Embo J, *17:* 3385-3397, 1998.
55. Lee, J. H., Gao, C. F., Lee, C. C., Kim, M. D., and Vande Woude, G. F. An alternatively spliced form of Met receptor is tumorigenic. Exp Mol Med, *38:* 565-573, 2006.
56. Hubbard, S. R. Juxtamembrane autoinhibition in receptor tyrosine kinases. Nat Rev Mol Cell Biol, *5:* 464-471, 2004.

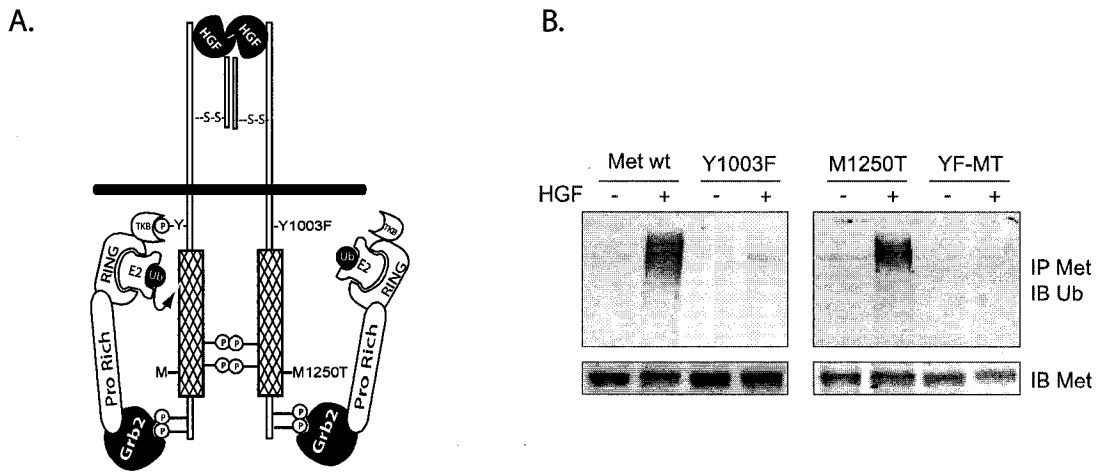


Figure 1. The Y1003F juxtamembrane substitution results in decreased receptor ubiquitylation. A. Schematic of the Met receptor variants. Receptor contains either no mutations, the Y1003F mutation alone, M1250T alone, or a combination of both YF/MT. B. Following 2 minutes of stimulation with 3nM HGF, T47D cells stably expressing Met receptor variants were lysed in 2% SDS. Lysates were boiled for 10 minutes, diluted to 0.4% SDS, 2% Triton and Met receptor protein was immunoprecipitated and blotted with anti-ubiquitin antibodies; the membrane was stripped and reblotted with antibodies against Met.

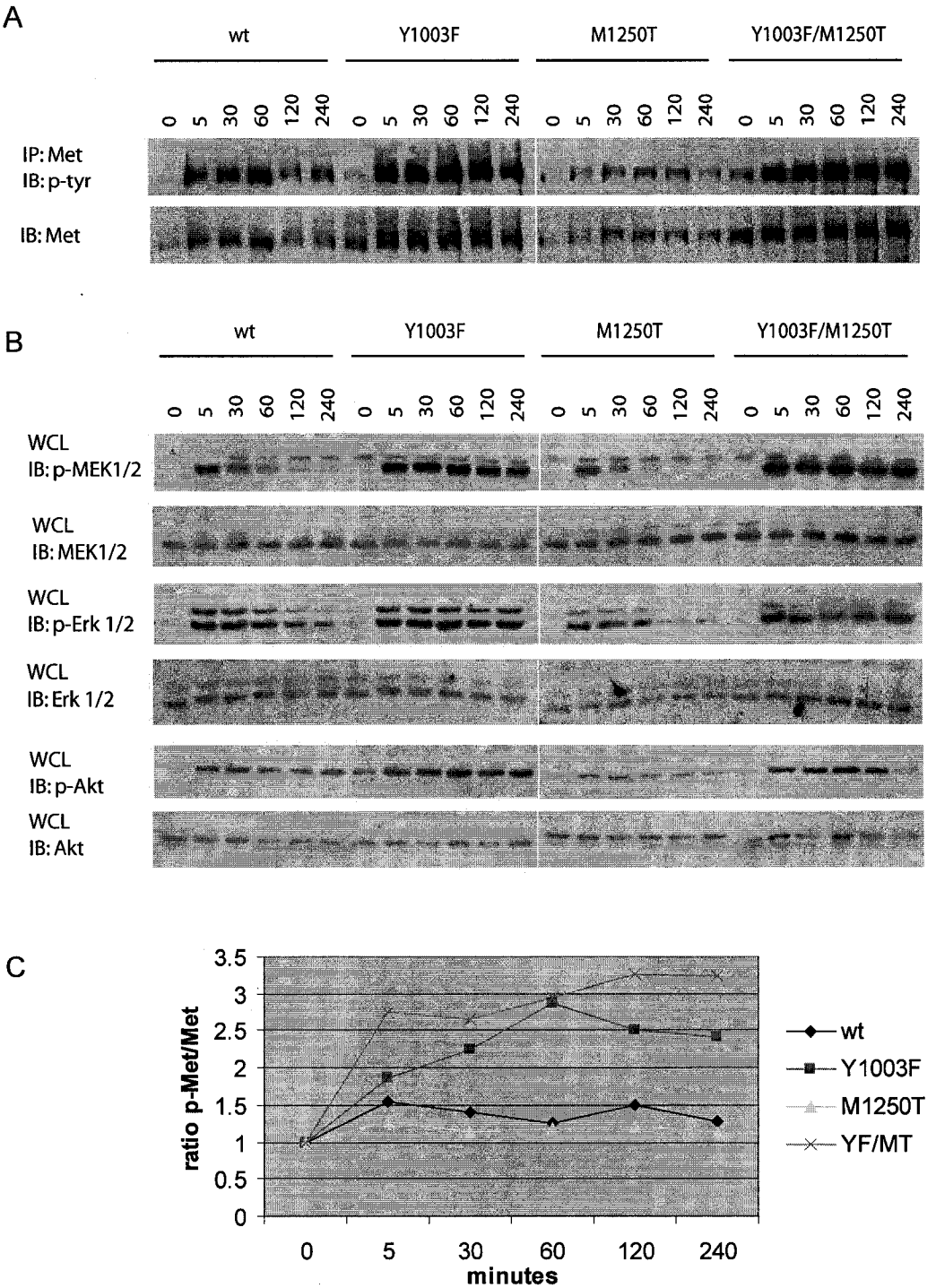


Figure 2. Activation of the Met-Y1003F receptor and downstream signaling proteins is sustained following stimulation with HGF. A. T47D cells were stimulated with 1.5nM HGF for the indicated times and lysed in TGH buffer. Met protein was immunoprecipitated and immunoblotted with phosphotyrosine antibodies (top). The membrane was stripped and re-blotted for total Met protein. B. T47D cells were stimulated with 1.5nM HGF and lysed as a above. Whole cell lysate was blotted with antibodies to phospho-Ser217/221 MEK1/2, total MEK, phospho-Thr202/Tyr204 Erk1/2, total Erk1/2, phospho-Ser473 Akt, and total Akt. C. Quantitation of bands indicating trend of p-Met over time.

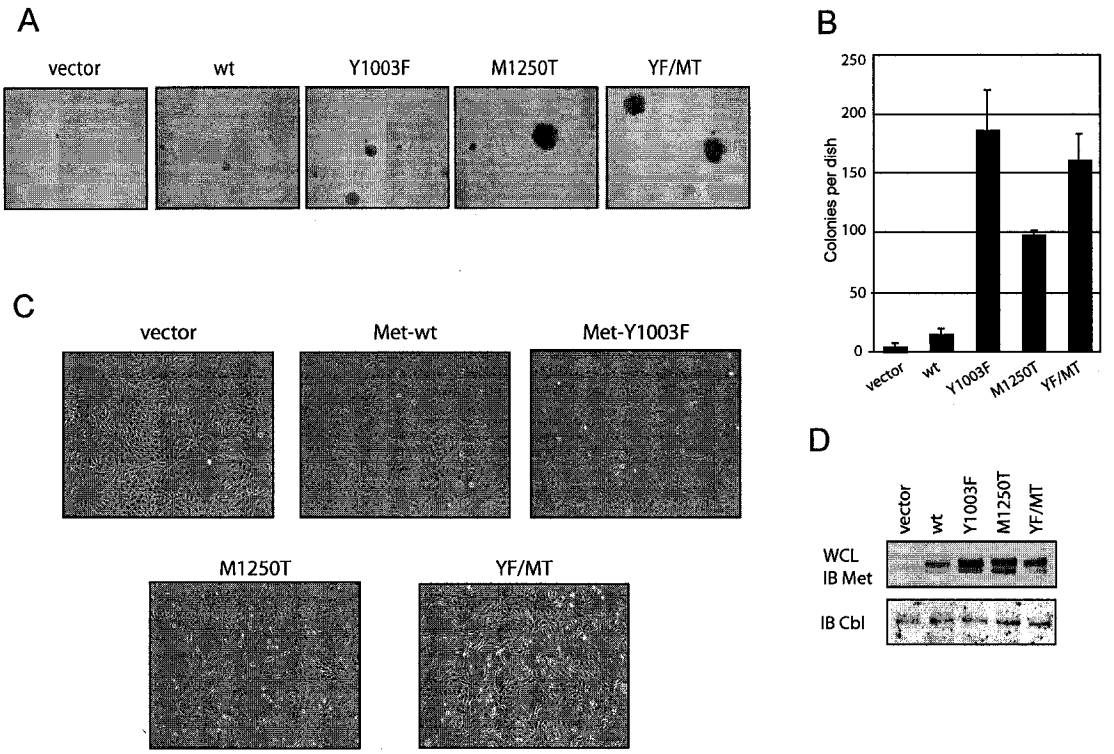


Figure 3. Transformation assays utilizing Rat1 cells expressing chimeric CSF-Met receptor variants. A. Soft agar assay-1x10^6 cells were plated in agar and grew for 3 weeks in the absence of ligand. B. Quantification of colonies in soft agar. C. Rat1 fibroblast populations stably expressing Met receptor variants. Cells were maintained in DMEM/10% FCS under G418 selection. Phase contrast images taken 2 days after plating D. Protein expression levels of Met receptor variants in the Rat-1 populations used in the soft agar assay.

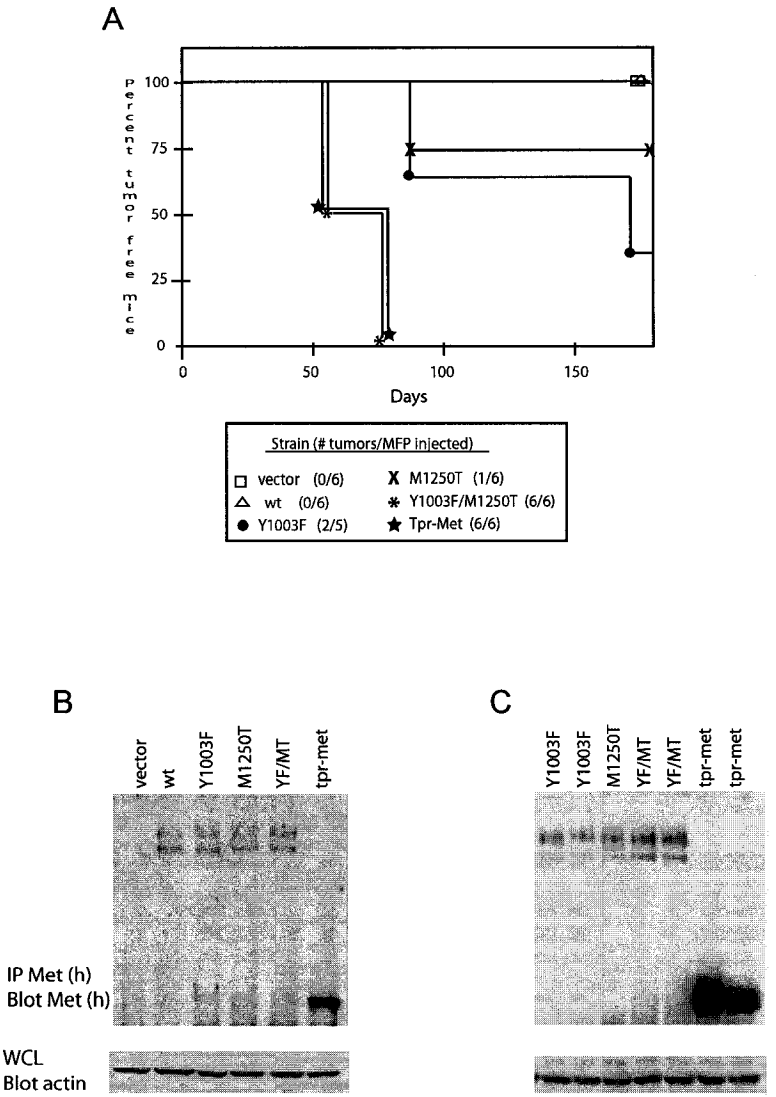
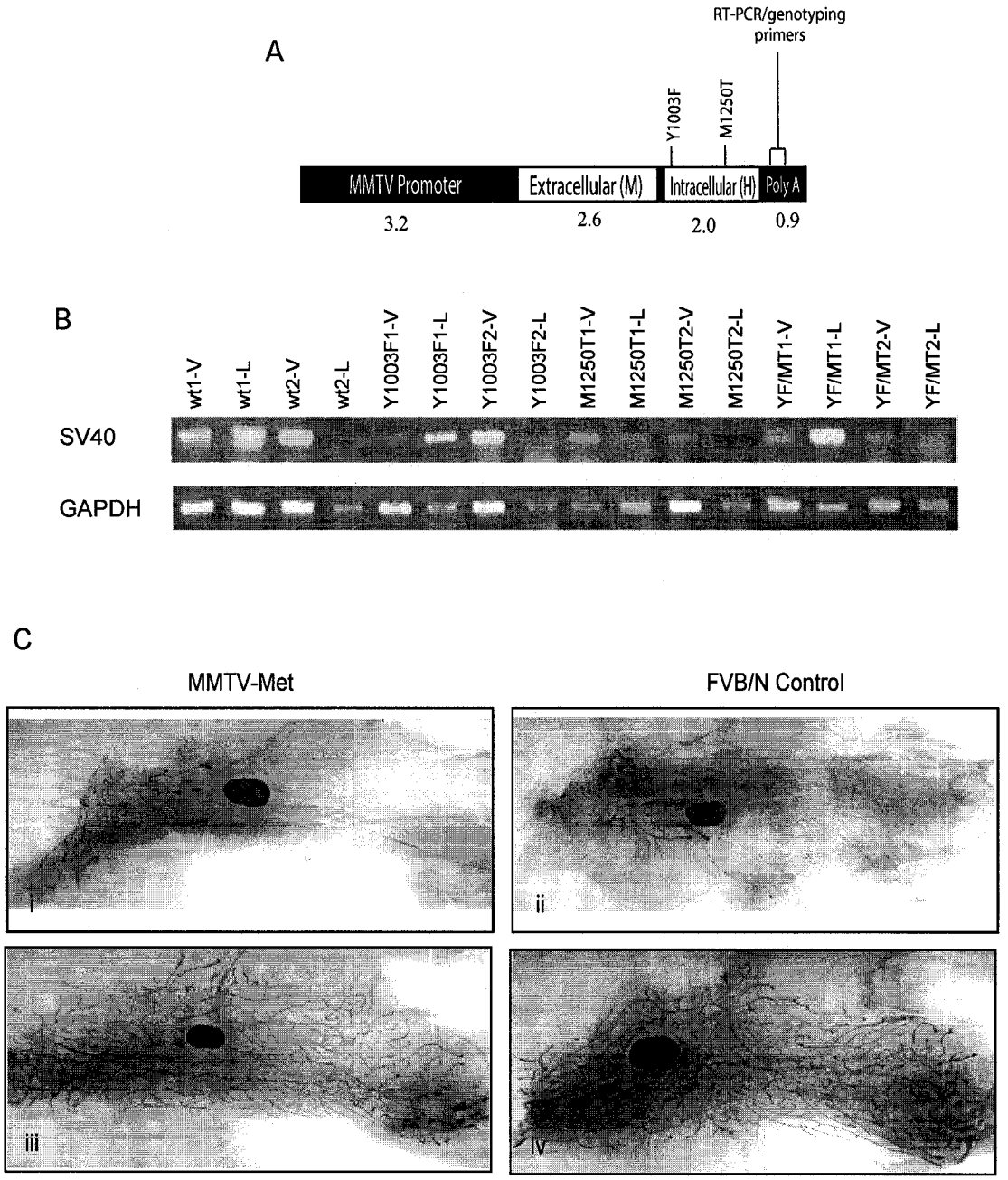


Figure 4. EpH4 cells expressing Met receptor variants form tumors following mammary fat pad injection into nude mice. A. Time course of tumor development in the mammary fat pad of nude mice injected with stable EpH4 populations expressing Met receptor variants. B. Immunoblot of levels of protein expression of Met receptor variants in EpH4 populations prior to injection. C. Immunoblot of protein levels of Met receptor variants in lysates from tumors resulting from the mammary fat pad injections.

Tumor Latency and Penetrance in MMTV-Met Transgenic Mice

	Nulliparous			Multiparous			
Strain	mice with tumors/total mice	average latency	multifocal tumors	mice with tumors/total mice	average latency	multifocal tumors	expressing Met (h)
FVB	0/15	N/A	0	4/21 (19%)	610	2/4 (50%)	0
MMTV-Met wt	0/25	N/A	0	3/13 (23%)	513	1/3 (33%)	0
MMTV-Met Y1003F	1/26 (4%)	821	0	3/17 (18%)	642	0	0
MMTV-Met M1250T	1/24 (4%)	614	0	3/14 (21%)	547	1/3 (33%)	50%
MMTV-Met-YF/MT	2/19 (11%)	441	1/2 (50%)	15/34 (44%)	419*	9/18 (50%)	82%

Table 1. Tumor incidence and latencies in MMTV-Met transgenic lines.
* $p=6x10^{-5}$. No statistically significant ($p<0.05$) differences amongst other mean values.



Supplemental Figure 1. Basic characterization of MMTV-Met transgenic lines. A. Schematic of construct used to generate the trangenic mice. B. RT-PCR of RNA extracted from the mammary glands of virgin (V) and lactating d18 (L) females from each of the transgenic lines selected. C. Whole mounts of mammary fat pad 4 from 3 (i, ii) and 9 (iii, iv) week old transgenic and wild type mice. i, iii-MMTV-Met; ii, iv-FVB control mice. All images taken at 0.6x magnification.

Chapter 3

Activating Mutations in the Met Receptor Tyrosine Kinase Induce Tumors with a Basal Phenotype in a Transgenic Mouse Model

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Manuscript in preparation

Preface

Expression of the Met receptor tyrosine kinase is elevated when compared with normal tissue in 15-20% of human breast cancers. Overexpression of Met in breast tumors predicts a shorter disease-free interval and shortened survival time independent of patient age, tumor size and ErbB2 status. In order to examine Met-dependent mammary tumorigenesis we have generated transgenic mice that, through the use of a mammary-specific promoter, express dysregulated Met receptors in the mammary epithelium. The Met receptor variants used contained one or two mutations in the intracellular domain. The first mutation (Y1003F) is a loss of negative regulation mutation in the juxtamembrane domain that prohibits binding of the tyrosine kinase binding domain of the ubiquitin ligase Cbl, resulting in prolonged signaling downstream of the receptor due to decreased receptor ubiquitination. The second mutation (M1250T) is an activating mutation in the kinase domain that has been observed in the Met receptor in patients with papillary renal carcinoma. Both mutations have been shown to be transforming independently *in vitro* and have an additive effect on transformation when both mutations are present in the receptor (Met-YF/MT).

Previously, other Met-related transgenic models had been generated and did yield mammary tumors, however, none has been characterized in detail nor has any expressed the Met receptor under a mammary-specific promoter. In this manuscript the Met-expressing tumors are characterized, yielding insight into how the Met receptor contributes to mammary tumorigenesis by cooperating with other oncogenic events and possibly affecting the progenitor cell population.

Abstract

Elevated expression of the Met receptor tyrosine kinase when compared with normal epithelium is observed in 15-20% of human breast cancers and, independent of patient age, tumor size and ErbB2 expression status, predicts a poor prognosis and a shorter disease-free interval. In order to examine the role of dysregulated Met signals in mammary tumorigenesis we have generated transgenic mice using the mouse mammary tumor virus (MMTV) promoter/enhancer to drive expression of either a wild type or a dysregulated Met receptor in the mammary epithelium. The dysregulated receptor contains either one or both an activating mutation originally isolated from patients with hereditary and sporadic papillary renal carcinoma (M1250T) or a loss of negative regulation mutation (Y1003F) that results in enhanced receptor stabilization.

Mammary development and lactation in the transgenic mice are normal, however, multiparous mice from both the Met-M1250T and Met-YF/MT lines, but not Met-wt nor Met-Y1003F, develop mammary adenocarcinomas with long latencies and low penetrance. Multiparous Met-YF/MT mice develop tumors in approximately 435 days whereas multiparous Met-M1250T mice develop tumors at approximately 547 days. Tumors from the Met-YF/MT mice are comprised mainly of luminal cells and, unlike many transgenic models, display a range of histological phenotypes. The Met-M1250T tumors display a basal phenotype as determined by staining for basal cytokeratins and markers of progenitor cells. Additionally, the Met-M1250T tumors display nuclear p53 staining. Microarray data obtained from the Met-YF/MT and Met-M1250T tumors clusters according to Met variant expressed, supporting the observed differences in phenotypes. Exogenous protein is expressed in the tumors and established signaling pathways downstream from the Met receptor are activated as evidenced by phosphorylation of Gab1 and proteins of the MAPK pathway. The difference in phenotypes between Met-M1250T and Met-YF/MT tumors highlights the impact of the cooperation of the activating and loss of negative regulation mutations in inducing transformation. Through mammary expression of dysregulated Met receptors we have generated a murine model for human tumors that recapitulates the diversity of breast cancers seen in the human population.

Abbreviations

ALAS1	d-Aminolevulinate Synthase
Cbl	Casitas B-lineage Lymphoma
CD31	Cluster of differentiation 31-endothelial cell marker
E-cadherin	Epithelial cadherin
ErbB2	Erythroblastic leukemia viral oncogene2
Gab1	Grb2 Associated Binding protein 1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
Grb2	Growth factor Receptor-Bound protein2
HER2	Human Epidermal growth factor Receptor2
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
K6	Cytokeratin6-putative progenitor cell marker
K8/18	Cytokeratin8/18-luminal cell marker
K14	Cytokeratin14-myoepithelial cell marker
MAPK	Mitogen Activated Protein Kinase
MEK	Mitogen activated protein kinase kinase
MMTV	Mouse Mammary Tumor Virus
P-cadherin	Placental cadherin
PCNA	Proliferating Cell Nuclear Antigen
RTK	Receptor Tyrosine Kinase
TNM	Tumor/Lymph Node/Metastases
TUNEL	Terminal deoxynucleotidyl Transferase mediated-dUTP Nick End Labeling
VEGF	Vascular Endothelial Growth Factor
WAP	Whey Acidic Protein

Introduction

Breast cancer is the second most common cancer in women, affecting one in eight women in the USA (1). Receptor tyrosine kinases (RTK), a family of transmembrane signaling proteins, have documented roles in the development of breast cancer as well as other cancers (review-(2)). Amplification of the HER2 RTK is observed in 20-30% of human breast cancers and is inversely correlated with patient survival (3). Additionally, the hepatocyte growth factor/Met receptor tyrosine kinase is expressed at elevated levels compared with normal tissue in ~20% of breast cancers (4-6) and the overexpression predicts poor prognosis in lymph node-positive patients independent of HER2 expression status (7). Additionally, high serum levels of hepatocyte growth factor/scatter factor (HGF/SF), the ligand of the Met receptor, have been correlated with a shorter disease-free interval following surgery (8) and a higher tumor/lymph node/metastasis (TNM) score in women with invasive breast cancers (9-12).

Met receptor signaling, following binding of HGF, propagates signals for a number of cellular processes that may have implications in the development or progression of cancer such as induction of cell motility, mitogenesis and angiogenesis (review-(13)). Met receptor signaling is consistent with a role in normal mammary development. Expression of Met and HGF has been shown in mice (14) and rats (15) to be upregulated during puberty, early pregnancy and involution, but downregulated during lactation. Elevated levels of HGF in the mammary fat pad (MFP) during glandular development results in increased ductal branching (16, 17). Moreover, HGF treatment of three-dimensional cultures of mammary epithelial cells induces branching morphogenesis recapitulating the formation of a normal ductal system *in vitro* (18-20)

Several transgenic mouse models of Met receptor signaling have been generated, however none has specifically targeted Met expression to the mammary epithelium. When either HGF or an activated Met receptor is expressed under the ubiquitously expressed metallothionein promoter, the mice develop mammary tumors, as well as other malignancies following breeding (21-23). Additionally, 89% of multiparous transgenic mice expressing HGF under the mammary-specific whey

acidic protein (WAP) promoter developed tumors with a latency of less than one year (16). These transgenic mice highlight the susceptibility of the mammary epithelium to transformation by an enhanced Met/HGF signal.

In order to explore the role of a dysregulated Met receptor specifically in the mammary epithelium we have generated transgenic mice expressing either the wild type Met receptor or a mutant Met receptor containing either a loss of negative regulation mutation (Met-Y1003F), an activating mutation (Met-M1250T) or a combination of both mutations (Met-YF/MT) under the MMTV promoter/enhancer. In contrast to the WAP-HGF transgenics in which the promoter is activated in pregnancy and lactation as cells differentiate to become milk-producing cells (16), the MMTV promoter is a hormonally-responsive promoter that drives low levels of expression in all stages of mammary gland development but expression is enhanced during pregnancy and lactation.

The M1250T mutation in the Met receptor was originally isolated from patients with either hereditary or sporadic papillary renal carcinomas (24) whereas the Y1003F mutation leads to enhanced stability of the Met receptor (25, 26). Loss of Y1003 has been observed in human non-small cell lung carcinomas through the deletion of the entirety of exon 14 (27). Other juxtamembrane residues in the Met receptor have been found to be altered in cancers such as lung and gastric (27-29). Independently, both the M1250T and the Y1003F mutations have been shown to be transforming by both *in vitro* (26, 30) and *in vivo* subcutaneous tumor assays (23, 25), however, the Met-YF/MT receptor is significantly more transforming than either mutation alone as determined by *in vitro* assays and in transgenic mice (S. Petkiewicz, manuscript in preparation).

In this manuscript we show that whereas many RTK mammary tumor models display one primary phenotype, the Met-YF/MT tumors displayed a range of histological phenotypes. The Met-M1250T tumors, did not resemble the Met-YF/MT tumors and whereas the Met-YF/MT tumors were comprised mainly of luminal epithelial cells, the tumors expressing the Met-M1250T mutant contained both luminal and basal cytokeratin-expressing cells indicating the possibility of progenitor cell transformation. Through these experiments we have demonstrated that

expression of a dysregulated Met receptor in the mammary epithelium can result in the development of tumors with a range of histological phenotypes similar to those observed in human breast cancers.

Materials and Methods

Transgenic Animals

The MMTV-Met transgenic animals were generated as described previously (S. Petkiewicz, manuscript in preparation). Mice were housed in the Royal Victoria Hospital (Montreal, Canada) transgenic mouse facility and all experiments were carried out in accordance with McGill University Animal Ethics Committee guidelines. Genotyping and selection of founder lines was performed as previously described (S. Petkiewicz, manuscript in preparation).

Histology and Immunohistochemistry

Tissues harvested from the MMTV-Met or nontransgenic littermates were fixed overnight in 10% buffered formalin (Surgipath, Richmond, IL), dehydrated, embedded in paraffin using standard techniques and sectioned at 5 μm. Paraffin sections were stained with haematoxylin and eosin for histology. Immunohistochemical assays on paraffin sections were carried out by deparaffinization, rehydration to water, heat-induced antigen retrieval in 10 mM sodium citrate buffer pH 6.0, blocking of endogenous peroxidases using 3% (v/v) hydrogen peroxide for 15 minutes, and blocking using either BSA or serum specific to the secondary antibody used. The primary antibodies used and their dilutions were PCNA (1:1400) from DAKO Cytomation (Glostrup, Denmark), E-cadherin (1:100) from Transduction Labs (Franklin Lakes, NJ), Met 905 (1:200) from Cedarlane (Hornby, ON, Canada), p53 (1:250) from Santa Cruz Biotechnologies (Santa Cruz, CA), cytokeratin 8/18 from Fitzgerald Industries International (Concord, MA), cytokeratin 14, cytokeratin 6 from Covance (Berkley, CA). Bound antibody was detected using the Vectastain Elite ABC Kit from Vector labs (Burlingame, CA, USA). The secondary antibodies used were biotin-conjugated anti-rabbit, anti-mouse, and anti-guinea pig (Vector Labs). All were visualized using DAB + (DAKO) and counterstained with 25% haematoxylin.

Determination of Microvessel Density

Microvessel density was determined by counting the number of CD31 positive ducts present per high power field (20x objective, 10x objective-200x final) in areas of highest vascular density. Single, staining cells were counted as were clusters of endothelial cells even if lumens were not apparent. Branching vessels within a field were counted as only one vessel. Areas of necrosis and peritumoral vasculature was not counted. Five fields were counted per tumor section and an average taken.

Immunoblotting

Mammary tissue lysates were generated by crushing frozen tissue with a mortar and pestle and lysing in 1% Triton lysis buffer (TGH) (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Forty μg of whole cell lysate was resolved by SDS-PAGE and separated proteins were transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham-Buckinghamshire, UK). Membranes were blocked with 3% bovine serum albumin and blotted with antibodies binding the C-terminus of human Met (Met 144) (31), murine Met (B2), HGF (H-145), (Santa Cruz-Santa Cruz, CA) phosphotyrosine Met (pY1234/35), phospho-ERK1/2 (pThr202/pTyr204), ERK1/2, phospho-MEK1/2 (pSer217/221), MEK 1/2, phospho-Akt1 (pSer473), Akt1, phospho-Stat3 (pTyr705), Stat3 (Cell Signaling-Danvers, MA, USA); phospho-Gab1 (Biosource-Camarillo, CA); Gab1 (Upstate-Lake Placid, NY); alpha-tubulin (Sigma-St. Louis, MO); cytokeratin 8 (Novocastra, Norwell, MA).

qPCR

DNA was extracted from the Triton-insoluble pellet following tissue lysis. Pellets were incubated overnight at 56°C in tissue lysis buffer [(2M Tris (pH8.5), 0.05M EDTA (pH 7.0), 20% SDS, 5 M NaCl)], with proteinase K (100μg/mL). DNA was then phenol-chloroform extracted, and precipitated with ethanol. Primers used for determining transgene copy number bound within the SV40 polyadenylation sequence. SV40 Forward-5'GGAACCTTACTTCTGTGGTGT Reverse-5'

GGAAAGTCCTTGGGGTCTTCT, annealing 60°C, product of 300 bp. Copy number was normalized to GAPDH copy number. GAPDH Forward-5'-ACCACAGTCCATGCCATCAC Reverse-5'-TCCACCACCCTGTTGCTGTA, annealing 55°C, product of 300 bp.

Laser Capture Microdissection and Microarray Preparation

Tumor and matched normal tissue was embedded in OCT (TissueTek) and fixed, H&E stained, frozen sections were laser capture microdissected with a PixCell IIe LCM system (Arcturus). Total RNA from laser captured cells was isolated using phenol-chloroform extraction and subjected to DNase treatment, as previously described (32). Total RNA was quantified using RiboGreen reagen (Molecular Probes) and RNA quality was assessed using Agilent's Bioanalyzer. 4 ng of total RNA from each tissue underwent two rounds of linear T7-based RNA amplification (Amino Allyl MessageAMP kit, Ambion-Austin, Texas), and the resulting amino allyl RNA was conjugated to Cy3 and Cy5 dyes (Amersham) according to manufacturer's protocol. Universal mouse reference RNA (Stratagene-La Jolla, CA) was subjected to amplification and labeling using the same method. Duplicate hybridizations were performed for all samples using reverse-dye labeling. RNA concentration and dye incorporation was determined by UV-vis spectorphotometer (Nanodrop ND-1000), and RNA quality was assessed using the Agilent 2100 Bioanalyzer. 0.75μg of Cy3 and Cy5-labeled RNA was hybridized to 44k Whole Mouse Genome Oligo microarrays (G4122A, Agilient). Arrays were washed, dried, and scanned using Agilent's dual laser scanner (Model G2505B) according to the manufacturer's procedure.

Statistical Analysis

Scanned images were feature extracted using Feature Extraction 7.11 software (Agilent). Class discovery under Pearson correlation distance metrics was performed. To assess the significance of the clusters, 1000 bootstrap iterations were done using the pvclust package for R (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=16595560&dopt=Abstract&holding=f1000,f1000m,isrctn)

Class distinction between pooled tumor samples and pooled normal samples was performed. The top 2000 differentially expressed genes were used to generate the heat map.

Results

Dysregulated Met Receptor Induces Mammary Tumors

To examine the tumor-inducing capacity of an activated Met RTK when expressed in mammary epithelia we generated transgenic mice that expressed either the wild type Met, Met-Y1003F, Met-M1250T or Met-YF/MT under the control of the MMTV promoter/enhancer (fig. 1). For each transgenic strain, two founder lines were established. From the primary founder line, at least 13 females were selected for continuous breeding from 10 weeks of age and from both the primary and secondary lines, at least 8 females were aged as nulliparous mice. All original founder animals were kept after they ceased to breed and three separate Met-YF/MT founder animals developed tumors at approximately 1 year of age indicating that tumors developing in the Met-YF/MT cohorts are not dependent on integration site. Additionally, tumor development did not appear to be dependent on the number of copies of transgene as was determined by qPCR (Supp. fig. 1B). Tumors that developed in Met-wt and Met-Y1003F animals failed to express exogenous protein, however, both Met-M1250T and Met-YF/MT mice grew tumors that expressed exogenous protein (fig. 2). Tumors, once discovered by palpation, grew rapidly and typically the mice were sacrificed within 3 weeks. In the Met-YF/MT lines, tumors appeared in two founder lines before selection of primary and secondary lines was complete, thus multiparous mice from two Met-YF/MT lines were maintained (table 1). 57% of multiparous animals from the Met-YF/MT1 line developed mammary tumors at a mean of 406 days, but none of the nulliparous animals developed tumors. In contrast, in the Met-YF/MT2 line, three out of thirteen (23%) multiparous animal developed mammary tumors at approximately 460 days, but significantly, two nulliparous animals developed mammary tumors at an average latency of 441 days; in one of the nulliparous mice five mammary fat pads were affected. Four tumors developed in three multiparous Met-M1250T mice, but much later than the Met-YF/MT tumors, at an average latency of 547 days (table 1).

Exogenous Met Protein is Expressed in Mammary Adenocarcinomas

In order to determine the activation state of the exogenous Met protein expressed in the mammary tumors, lysates from the mammary tumors were immunoblotted using an antibody specific to the intracellular domain of the human Met receptor (31). Strong expression of exogenous Met is visible in the tumors whereas the protein is weak or undetectable in an unaffected mammary fat pad from the same animal (fig. 2A). Importantly, none of the Met-wt nor Met-Y1003F tumors, despite carrying the transgene, demonstrated exogenous Met receptor expression by either immunoblotting or immunohistochemistry (data not shown), thus the tumors from these mice may simply be spontaneous lesions as would be suggested by their long latency.

In addition to being highly expressed in the tumors, the exogenous Met receptor is phosphorylated on residues Y1234/1235 as determined by blotting with a phospho-specific antibody. Phosphorylation of Y1234/35 in the activation loop is required for Met kinase activation (33, 34). Notably, the level of phosphorylation observed was proportional to the level of exogenous Met expressed in the tumors (fig. 2A).

In humans, coexpression of ligand and receptor has been documented (35, 36) implicating an autocrine loop in increasing signaling from the Met receptor. To establish if HGF was elevated in tumor tissue and thus was responsible for the activation of the exogenous Met receptor, membranes were probed for endogenous HGF protein. Tumors showed no increase in HGF expression compared with normal tissue, thus, upregulation of ligand was most likely not responsible for the strong receptor phosphorylation (fig. 2B). The elevated levels of phospho-Met could also be due to either upregulation of or heterodimerization with endogenous, murine Met. In order to rule out these possibilities, levels of endogenous murine Met were assayed using an antibody specific for murine Met. Endogenous mouse Met receptor was expressed at similar levels amongst all samples tested and between normal and tumor tissue, demonstrating no upregulation of endogenous Met receptor in the mammary tumors. The Met receptor has been shown to heterodimerize with its closely related family member, Ron (37), however tumor lysates did not show upregulation of Ron

compared with normal tissue (data not shown), thus, Ron-Met heterodimers are most likely not responsible for the strong phospho-Met. Therefore, the elevated levels of phospho-Met are either due to stimulation by endogenous HGF or receptor homodimerization in the absence of ligand as a result of high concentrations at the plasma membrane, as has been observed in transiently transfected cells (38).

In several studies of human tumors (39-42) and in one Met receptor knock-in model (43), the Met receptor locus has been shown to be amplified in tumor tissue. In order to determine if amplification of the transgene had occurred, we performed quantitative PCR on DNA extracted from the tumors and contrasted it with DNA obtained from unaffected tissues from the same animal and normalized all DNA levels to GAPDH. The analysis of this data revealed that the inserted Met construct DNA had not been amplified in the tumors (Supp. fig. 1). Additionally, the transgene present in the tumors was sequenced and no additional mutations were identified (data not shown).

Downstream Signaling in the Tumors

As the exogenous Met receptor was phosphorylated and active, the activation state of various proteins known to be downstream from Met were assayed by immunoblotting. The primary binding partner of the Met receptor, the scaffold protein Gab1, was found to be highly phosphorylated in tumors but not phosphorylated in the normal tissue, and the level of phosphorylation was proportional to the amount of exogenous Met expressed in the tumors (fig. 3). Through the adapter protein Grb2 and the scaffold protein Gab1, Met is coupled to the MAPK pathway (44, 45). Phosphorylated MEK1/2 was detected in tumor lysates but not in normal gland, however, the levels of phospho-MEK1/2 did not always correlate with the levels of phospho-Met indicating there may be cooperating activated oncogenic pathways present in the tumors (fig. 3). The phosphorylation of Erk1/2 in tumors versus normal was more consistent, however, the total levels of Erk are upregulated in tumor compared with normal tissue. The Met receptor is also coupled to the PI3K/Akt pathway through Gab1 (44) and by binding directly to the p85 subunit of PI3K (46) . Tumor samples had slightly higher levels of

phosphorylated Akt compared with normal tissue, and importantly, AKT levels were stable between tumor and normal (fig. 3). The M1250T mutation has been shown induce binding of Stat3 to the Met receptor (47), thus, as all the Met-expressing tumors expressed a Met receptor that contained the M1250T mutation, we blotted for phosphorylated Stat3. Tumors contained higher levels of p-Stat3, but levels were not proportional to the amount of exogenous Met expressed. Notably, levels of Stat3 were increased in the tumor compared to normal tissue (fig. 3). As Met and Myc were shown to cooperate in mammary tumorigenesis in a mouse model (48), we blotted for Myc protein but did not find any upregulation of Myc in tumor versus normal lysates (data not shown).

Histological Analysis of MMTV-Met mutant Mammary Tumors

Of the other Met-related transgenic animals, only the WAP-HGF mice provide a thorough description of histology of the resulting mammary tumors (16). All Met-related transgenic mice were described as having adenocarcinomas (16, 22, 23), however, little other detail is provided making it difficult to determine if there is a consistent Met mammary tumor phenotype. Histological examination of the Met-YF/MT mammary tumors revealed that all tumors were adenocarcinomas, but several histological subtypes and degrees of differentiation were noted. The most common phenotype was the poorly-differentiated, solid, nodular, tumor similar to tumors observed in ErbB2 transgenic mice (fig. 4A, table 2). More differentiated papillary tumors were observed in several animals (fig. 4D). Additionally, a secretory phenotype was seen in four tumors (fig. 4B) showing the ductal structures containing a proteinaceous secretion. Several tumors had invasive margins and fig. 5C shows a well-differentiated invasive tubular adenocarcinoma invading through the muscle tissue adjacent to the mammary fat pad. The Met-M1250T tumors contained areas of squamous metaplasia (fig. 4F) but were otherwise poorly differentiatated aside from a few obvious ductal structures (fig.5D, 4E-arrow) .

MMTV-Met-YF/MT Tumors Rarely Metastasize

Met receptor signaling *in vitro* stimulates cellular migration and invasion in a variety of epithelial cell lines, including mammary cells (49). To determine if expression of a dysregulated Met receptor in the tumor tissue could result in invasive behavior leading to metastases we examined the lungs of all tumor-bearing mice both at the gross and the histological levels. Extravascular lung metastases were found only in one animal from the Met-YF/MT1 line and the third Met-YF/MT founder that had developed a mammary tumor. Histological examination of these metastases revealed recapitulation of the glandular structure of the mammary tumor and demonstrated expression of the exogenous Met receptor protein as determined by immunohistochemistry (fig. 5E). The expression of exogenous protein and morphology distinguished these lung nodules from the common lung carcinomas that FVB mice are present in ~26% of females by 14 months (50). None of the other tumor-bearing mice was found to have lung metastases.

Immunohistochemical Analysis of MMTV-Met Mammary Tumors

To characterize the tumors for the degree of differentiation and determine if all cells in the tumors were expressing exogenous Met protein, tumors were stained with an antibody specific to the C-terminus of human Met as well as for markers of proliferation and expression of E-cadherin. Strong Met staining was seen in most luminal cells of the Met-YF/MT tumors (fig. 5). The Met-M1250T tumor samples also displayed Met staining, but not all cells stained; in particular there was exclusion from small ductal structures scattered throughout the tumor (fig. 5D). As a control, a section of tumor transplanted into a non-transgenic mouse was stained revealing Met receptor staining of the transplanted tumor but not of the adjacent normal, non-transgenic host mammary epithelium (fig. 5F).

Met signaling has been shown to induce an epithelial-mesenchymal-like (EM-like) transition with loss of adherens junctions and internalization of E-cadherin (51). In order to determine if the MMTV-Met tumors were undergoing an EM-like transition we stained tumor sections for E-cadherin. All Met-YF/MT tumors stained for E-cadherin but not all areas of the tumor were strongly positive (fig. 6A,B). The

regions that stained poorly for E-cadherin were found to contain a higher percentage of proliferating cells, as determined by staining serial sections for E-cadherin and PCNA (fig. 6D,E). In contrast, the Met-M1250T tumors were mostly E-cadherin negative (fig. 6C). Small ductal structures retained strong E-cadherin staining, and when contrasted with PCNA staining, the majority of the tumor mass was proliferating, whereas the ductal structures do not stain positively for PCNA (fig. 6F).

In addition to the range of histological phenotypes, there was variability in the number and size of blood vessels within the tumor. Met receptor signaling cooperates with vascular endothelial growth factor (VEGF) to induce the formation of new blood vessels from existing vessels (52-54), hence, we stained sections for the endothelial marker CD31 and determined microvessel density in the tumor samples. Samples were divided into high and low microvessel densities as determined by taking the top 1/3 of the tumors as high microvessel density. The majority of the non-solid tumors were within the high microvessel density class, potentially as a result of the non-solid tumors containing more stroma within the tumor mass (table 2, Supp. fig. 3).

MMTV-Met M1250T Tumors Contain Basal Cells

The finding that Met-M1250T tumors did not stain positively for E-cadherin argued in favor of the tumors undergoing an EMT (EMT tumor). Alternatively, as myoepithelial cells express P-cadherin rather than E-cadherin (55) the tumors could be comprised of myoepithelial cells. In order to better verify the identity of the cells in the Met-M1250T tumors, we stained tumor sections for markers of cell lineages. Although the Met-YF/MT tumors did not overtly appear to be undergoing EMT or contain cells of mixed lineages, they did display a range of histologies and were examined alongside the Met-M1250T tumors.

All tumors contained cells that were cytokeratin 8/18 (K8/18) positive, with all cells in the solid adenocarcinomas displaying K8/18 positivity. Approximately 50% of cells in the Met-M1250T tumors stained for K8/18 (fig. 7D). Staining for K14, a myoepithelial cell marker (56, 57) revealed that the majority (76%) of Met-YF/MT tumors were K14 negative, but all of the Met-M1250T tumors contained clusters of K14 positive cells (fig. 7E).

Mammary adenocarcinomas generated by Welm et al. (2005) by infecting primary mammary epithelial cells from inducible Met transgenics with a modified stem cell retrovirus that expressed Myc resulted in mammary tumors that displayed not only K14 positive cells but expansion of the progenitor population, as determined by cytokeratin 6 staining (48). The WAP-HGF transgenic mouse tumors also contained K6 positive cells but they were restricted to near areas of squamous metaplasia (16). Staining of the Met-YF/MT tumors for cytokeratin 6 was negative in the majority of the tumors, however, all of the Met-M1250T tumors displayed K6 positive cells (fig. 7F).

There were several exceptions to Met-YF/MT tumors being negative for K14 and K6. The invasive tubular adenocarcinomas contained K14 and K6 positive cells and these populations were expanded when the tumor was transplanted (Supp. fig. 4). Additionally, one papillary adenocarcinoma and two solid, but highly vascular, solid, adenocarcinomas contained numerous K14 positive cells, but no K6 positive cells. Thus, the mixed lineage/basal phenotype was not entirely restricted to the Met-M1250T tumors.

Tumors Containing Mixed Lineage Cells Display p53 Nuclear Positivity

Amplification of the *met* locus is frequently observed in the tumors of $BRCA1^{-/-}p53^{-/-}$ (58). Additionally tumors with a basal phenotype often have mutant p53 (59-62). In order to determine if there is cooperation between Met and p53 in the MMTV-Met tumors, we performed immunohistochemistry for p53 nuclear localization. Those tumors that contained mixed population cells displayed p53 nuclear localization, especially the Met-M1250T tumors (fig. 8A), whereas the luminal-only tumors were generally devoid of p53 nuclear staining (fig. 8C). Thus, there appears to be an association between Met receptor signaling and p53 dysregulation in the MMTV-Met tumors that contain mixed lineage cells.

Met-M1250T Tumors Cluster Separately from Met-YF/MT Tumors

In order to better characterize and examine differences between the Met-M1250T and Met-YF/MT tumors, we performed microarray analysis on epithelial

tissue isolated by laser capture microdissection from frozen tumor and normal samples. Following normalization, hierarchical clustering was applied to nine normal epithelial samples and nine tumor samples, and the complete panel of genes (44K genome features). All normal samples clustered together, indicating few differences in the normal epithelium between mice of different ages and genotypes. The tumor samples clustered together, with complete separation from normal tissue. Two of the tumor samples included in the microarray analysis were from MMTV-Met-M250T mice and these two tumors clustered away from the Met-YF/MT tumors, indicating differences in expression profiles between the two tumor types. This again highlights the differences in signaling between Met-M1250T alone and Met-YF/MT.

Discussion

Evidence for a role of Met receptor signaling in human mammary tumorigenesis has been mostly circumstantial. Several studies have demonstrated that high levels of expression of the Met receptor and/or HGF in mammary tumors or high serum levels of HGF correlate with a poor prognosis and a shorter disease-free interval (4-8). To determine if dysregulated Met receptor signaling could play a more causal role in mammary tumorigenesis, we have expressed a mutant Met receptor in the mammary epithelium and found that a receptor containing both an activating mutation and a loss of negative regulation mutation is capable of inducing mammary tumors, albeit with a long latency. The shortest latency to tumor development was in the MMTV-Met-YF/MT1 line in which 57% of multiparous animals developed tumors with an average latency of 409 days whereas only 21% of multiparous Met-M1250T mice developed tumors at 547 days.

There have been a number of other Met-related transgenic models that developed mammary adenocarcinomas. Multiparous WAP-HGF mice developed tumors within 10 months and with 89% penetrance (16). This has been, by far, the most tumorigenic Met-related model of mammary carcinoma. Expression of the oncogenic fusion protein, Tpr-Met, under the control of the ubiquitously expressed metallothionein promoter (MT-Tpr-Met) resulted in the development of a variety of tumors, but the most common was the mammary adenocarcinoma, indicating the sensitivity of the mammary epithelium to dysregulated Met signaling. Forty-two percent of MT-Tpr-Met mice developed mammary tumors with an average latency of 381 days (22). The M1250T mutation was used in a knock-in model of tumorigenesis, but the Met-M1250T knock-ins did not develop mammary tumors, perhaps as a result of being on a mixed background, rather than on the FVB/N background (43). The transgenic animal most similar to ours, expressing Met-M1250T under the control of the MT promoter, developed a mammary tumor, however, the founder animals had difficulty breeding, thus the lines were not able to be expanded (23). The development of tumors under the ubiquitously expressed promoter, again, demonstrates the sensitivity of the mammary epithelium to transformation by dysregulated Met receptor signaling.

Selecting founders lines was challenging due to low levels of expression of the transgene in the epithelium. The expression level was not consistently enhanced by lactation (Supp. fig. 1) and protein was undetectable even in older, multiparous animals, as evidenced by immunoblotting normal glands from tumor-bearing mice (fig. 2). The failure to induce strong expression under this MMTV promoter is specific to Met receptor as the same MMTV-SV40 construct has been used to generate several other transgenic mice in which the expression of the transgene was easily detected (63).

Failure to detect expression of exogenous Met protein in normal tissue was unexpected, however, as the protein was easily detected in most of the tumors from the Met-YF/MT and Met-M1250T lines, we believe that high levels of Met expression are only tolerated by cells that have undergone genetic alterations that partially transform the cells. Support for this hypothesis is provided by the long latency to tumor formation. Mice with the earliest onset tumors required sacrifice at an average of 409 days. In a mice, whose lifespan is generally two years, this makes them middle age, approximately the time when tumors begin to arise in the human population. Throughout this time there has been ample opportunity for cells to accumulate genetic mutations.

The MMTV promoter is active throughout mammary ductal development, thus it was possible that the mammary epithelial cells that expressed exogenous Met underwent apoptosis. Terminal deoxynucleotidyl Transferase mediated-dUTP Nick End Labeling (TUNEL) staining of mammary ducts and terminal end buds of 3 and 6 week old mice did not reveal enhanced apoptosis at early stages of development nor was there any delay in ductal outgrowth (Supp. fig. 2). Additionally, examination of these developing mammary glands for exogenous Met expression by immunofluorescence did not reveal any pockets of expression (data not shown). Alternatively, it was possible that exogenous Met expression, as it is known to stimulate an EM-like transition (51), was driving cells through an EMT, thereby shutting off expression from MMTV, as observed in the MMTV-ILK. The MMTV-ILK mice developed spindle cell carcinomas that failed to express exogenous ILK (63). We did not observe an increase in the number of spindle cells nor any break

down of glandular structures in any of the transgenic lines nor did the mice develop spindle cell tumors. Thus, by exclusion, it appears that exogenous Met expression is repressed in normal mammary epithelium of the mice.

The tumors that arise in the Met-YF/MT mice display a range of histological phenotypes supporting the idea that through the long latency the mammary epithelial cells acquire genetic mutations that cooperate with dysregulated Met, and it is the combination of dysregulated pathways that drives the tumor phenotype. It is uncommon for a mammary-directed RTK transgenic animal to generate tumors with a range of phenotypes. Transgenic models such as MMTV-ErbB2 or MMTV-Ras develop tumors that display consistent phenotypes but appear with a shorter tumor latency (64, 65). Crossing transgenic models has demonstrated that between two models there is always a dominant oncogene that determines the histological phenotype of the tumor (66). Thus, it may be that the histological phenotype observed in the tumors may be the result of the dominant transforming event in the tumor cells.

Neither MMTV-Met-wt nor MMTV-Met-Y1003F induced the development of mammary tumors, as determined by the lack of exogenous wt- or Y1003F-Met expression in the tumors that arose in these cohorts, thus, only the activated receptor was able to promote tumorigenesis. Expression of Met-M1250T or Met-YF/MT must be selected for during tumor progression. Either the few cells that may express Met-M1250T or Met-YF/MT are predisposed to transformation or transformed cells have a growth advantage if they express one of the dysregulated Met proteins. In either case, dysregulated Met does not aggressively drive tumorigenesis but may provide an excellent model to identify other transforming events that contribute to the development of tumors.

The general failure of the MMTV-Met tumors to metastasize was surprising. Met receptor activation is known to induce cell dispersal and invasion *in vitro* (49, 67, 68). Additionally, experimental metastasis assays have demonstrated that co-expression of Met and HGF in either NIH3T3 (69) or leiomyosarcoma (70) cells can encourage the development of lung metastases. However, the development of metastases requires more than invasive behavior; it requires the ability to survive and

proliferate in a foreign environment, and perhaps the majority of the tumors did not have the capacity to thrive outside of the mammary fat pad.

Expression of exogenous Met was visualized by IHC but was not localized to the plasma membrane, possibly as a result of a high rate of receptor turnover in cells that are continuously binding the endogenously produced HGF. All Met-YF/MT tumors retained expression of E-cadherin, however, areas with lower E-cadherin expression had higher rates of proliferation as seen by PCNA staining of serial sections (fig. 4). The decreased expression of E-cadherin may indicate a less differentiated state as seen in the E-cadherin negative cap cells of the terminal end bud (55). Consistent with this, *in vitro* experiments have shown that inducing expression of E-cadherin in cell lines decreases cellular proliferation (71-73). The decreased proliferation may be mediated through regulation of β-catenin-mediated transcription (72), or induction of expression of the cyclin-dependent kinase inhibitor p27 (73). The loss of E-cadherin expression was most pronounced in the Met-M1250T tumors in which nearly all cells were E-cadherin negative by IHC. The cells that did retain E-cadherin expression were well-differentiated cells that organized into tubular structures (fig. 6C, F). As with the Met-YF/MT tumors, these cells did not stain for PCNA.

The Met-M1250T tumors had a histological phenotype distinct from the Met-YF/MT tumors. The differences between the two tumor types was highlighted by the differences in cell types being expanded in the tumors. The majority of Met-YF/MT tumors were comprised solely of luminal epithelial cells, as defined by positive staining for K8/18, whereas Met-M1250T tumors contained luminal and myoepithelial cells as well as potential progenitor cells (K6 positive) (fig. 7). This is consistent with expansion of the progenitor population in tumors that developed from cells that co-expressed both wt Met and Myc (48). WAP-HGF mouse tumors also contained K6 positive cells (16), however they were restricted to areas of squamous metaplasia and not scattered throughout the tumor as in the Met-M1250T tumors (fig. 7F). Finding mixed cell lineages in the tumors is consistent with transformation of a progenitor or stem cell as is observed in the Wnt pathway transgenics (74).

Met receptor tyrosine kinase signaling is apparent in the mammary tumors by immunoblotting. Exogenous Met is phosphorylated (fig. 2A) as is the primary downstream signaling protein, Gab1 (fig. 3). Despite differences in the tumor phenotypes and in signaling observed following HGF stimulation of stable cell lines (S. Petkiewicz, manuscript in preparation), Met-YF/MT and Met-M1250T tumors show similar activation of downstream pathways. The MAPK pathway is activated in the tumors as seen by a significantly stronger phospho-Erk1/2 and phospho-MEK1/2 in the tumor than in the normal tissue. Additionally, phospho-Akt is higher in tumors than normal, as would be expected downstream from an activated Met receptor (75). Increased levels of phosphorylated Akt were also noted in the MT-HGF mice at the invasive front of the tumor (16). The MT-HGF mouse also displayed strong nuclear phosphorylated c-Myc which was interpreted as a readout for activation of the MAPK pathway, as also seen in the MMTV-Met tumors although neither MEK1/2 nor Erk1/2 was immunoblotted from the MT-HGF mice (16). With activation of both the MAPK and Akt pathways, the MMTV-Met tumors exhibit activation of both proliferative and survival pathways.

The M1250T mutation has been shown to permit direct binding of Stat3 to the Met receptor (47). The activation of Stat3 is not dependent upon the presence of M1250T, however, as studies have demonstrated that in leiomyosarcoma cells, HGF stimulation induces Stat3 phosphorylation and nuclear localization (76). Phosphorylation of Stat3 is observed in the MMTV-Met tumors, but it is not possible to know to what degree the phosphorylation is dependent on the presence of the M1250T mutation. The impact of the M1250T mutation in the Met receptor would have been best observed had there been Met-Y1003F or Met-wt tumors between which levels of phospho-Stat3 could have been contrasted.

Nuclear localization of p53 in the mixed lineage tumors reveals one potential cooperator with Met receptor signaling. Tumors arising in p53 mutant or p53 null mice often develop tumors of a basal subtype as demonstrated by the frequent presence of metaplasia (77-80). Additionally, the human basal tumor phenotype, contains more tumors with p53 mutations than the other classes (60, 62, 81). Thus, it seems unlikely that Met alone is responsible for the basal tumor phenotype, but rather

p53 dysregulation cooperates-affecting the stem/progenitor cell population. Still, data from the MMTV-Met tumors support a link between p53 and Met signaling, as was also seen in the p53/BRCA1 null tumors (58) and in tumors from Li Fraumeni patients (82).

The clustering of the microarray data supports the differences between the Met-M1250T and Met-YF/MT tumors as observed by immunohistochemistry. All tumors clustered separately from the normal tissue and the two Met-M1250T tumors formed a cluster distinct from the other Met-YF/MT tumors. Interestingly, the two Met-M1250T normal controls did not cluster together, indicating that the low levels of expression of the exogenous protein do not induce sufficient changes in normal nontransformed epithelium to generate a unique cluster. Human breast cancer microarray data has revealed that breast tumors that express high levels of Met fall within the basal cell type (Finak and Park, unpublished data). Additionally, studies of human breast cell lines have demonstrated that high levels of Met expression are found within basal lines (83). Thus, we plan to determine whether the Met-M1250T tumors and the few Met-YF/MT tumors that contained K14 positive cells are of the basal subtype and if the solid, luminal Met-YF/MT tumors cluster with the luminal subtype of breast cancer.

We have developed a novel murine model for Met receptor mediated mammary tumorigenesis. As Met expression is not an aggressive tumor-inducing oncogene, it will be important to determine if there are known oncogenic pathways such as p53 mutation or Ras overexpression that selectively cooperate with Met signals. By determining which pathways may cooperate with Met signaling, we may gain insight into how to specifically treat the 20% of breast tumors that show Met overexpression.

References

1. Reis, L. A. G., Eisner, M.P., Kosary, C.L., Hankey, B.F., Miller, B.A., Clegg, B.A., Mariotto, A., Feuer, E.J., Edwards, B.K., (eds). SEER Cancer Statistics Review, 1975-2002. Bethesda: National Cancer Institute, 2005.
2. Blume-Jensen, P. and Hunter, T. Oncogenic kinase signalling. Nature, *411:* 355-365, 2001.
3. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science, *235:* 177-182, 1987.
4. Ghoussoub, R. A., Dillon, D. A., D'Aquila, T., Rimm, E. B., Fearon, E. R., and Rimm, D. L. Expression of c-met is a strong independent prognostic factor in breast carcinoma. Cancer, *82:* 1513-1520, 1998.
5. Camp, R. L., Rimm, E. B., and Rimm, D. L. Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. Cancer, *86:* 2259-2265, 1999.
6. Beviglia, L., Matsumoto, K., Lin, C. S., Ziober, B. L., and Kramer, R. H. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int J Cancer, *74:* 301-309, 1997.
7. Lengyel, E., Prechtel, D., Resau, J. H., Gauger, K., Welk, A., Lindemann, K., Salanti, G., Richter, T., Knudsen, B., Vande Woude, G. F., and Harbeck, N. C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. Int J Cancer, *113:* 678-682, 2005.
8. Yamashita, J., Ogawa, M., Yamashita, S., Nomura, K., Kuramoto, M., Saishoji, T., and Shin, S. Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. Cancer Res, *54:* 1630-1633, 1994.
9. Sheen-Chen, S. M., Liu, Y. W., Eng, H. L., and Chou, F. F. Serum levels of hepatocyte growth factor in patients with breast cancer. Cancer Epidemiol Biomarkers Prev, *14:* 715-717, 2005.
10. Taniguchi, T., Toi, M., Inada, K., Imazawa, T., Yamamoto, Y., and Tominaga, T. Serum concentrations of hepatocyte growth factor in breast cancer patients. Clin Cancer Res, *1:* 1031-1034, 1995.
11. Maemura, M., Iino, Y., Yokoe, T., Horiguchi, J., Takei, H., Koibuchi, Y., Horii, Y., Takeyoshi, I., Ohwada, S., and Morishita, Y. Serum concentration of hepatocyte growth factor in patients with metastatic breast cancer. Cancer Lett, *126:* 215-220, 1998.
12. Toi, M., Taniguchi, T., Ueno, T., Asano, M., Funata, N., Sekiguchi, K., Iwanari, H., and Tominaga, T. Significance of circulating hepatocyte growth factor level as a prognostic indicator in primary breast cancer. Clin Cancer Res, *4:* 659-664, 1998.
13. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. Met, metastasis, motility and more. Nat Rev Mol Cell Biol, *4:* 915-925, 2003.
14. Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., and Kamalati, T. HGF/SF: a potent cytokine for

mammary growth, morphogenesis and development. Development, *121:* 2897-2908, 1995.

15. Pepper, M. S., Soriano, J. V., Menoud, P. A., Sappino, A. P., Orci, L., and Montesano, R. Modulation of hepatocyte growth factor and c-met in the rat mammary gland during pregnancy, lactation, and involution. Exp Cell Res, *219:* 204-210, 1995.
16. Gallego, M. I., Bierie, B., and Hennighausen, L. Targeted expression of HGF/SF in mouse mammary epithelium leads to metastatic adenosquamous carcinomas through the activation of multiple signal transduction pathways. Oncogene, *22:* 8498-8508, 2003.
17. Yant, J., Buluwela, L., Niranjan, B., Gusterson, B., and Kamalati, T. In vivo effects of hepatocyte growth factor/scatter factor on mouse mammary gland development. Exp Cell Res, *241:* 476-481, 1998.
18. Brinkmann, V., Foroutan, H., Sachs, M., Weidner, K. M., and Birchmeier, W. Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J Cell Biol, *131:* 1573-1586, 1995.
19. Soriano, J. V., Pepper, M. S., Nakamura, T., Orci, L., and Montesano, R. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. J Cell Sci, *108 (Pt 2):* 413-430, 1995.
20. Niemann, C., Brinkmann, V., Spitzer, E., Hartmann, G., Sachs, M., Naundorf, H., and Birchmeier, W. Reconstitution of mammary gland development in vitro: requirement of c-met and c-erbB2 signaling for branching and alveolar morphogenesis. J Cell Biol, *143:* 533-545, 1998.
21. Takayama, H., LaRochelle, W. J., Sharp, R., Otsuka, T., Kriebel, P., Anver, M., Aaronson, S. A., and Merlino, G. Diverse tumorigenesis associated with aberrant development in mice overexpressing hepatocyte growth factor/scatter factor. Proc Natl Acad Sci U S A, *94:* 701-706, 1997.
22. Liang, T. J., Reid, A. E., Xavier, R., Cardiff, R. D., and Wang, T. C. Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. J Clin Invest, *97:* 2872-2877, 1996.
23. Jeffers, M., Fiscella, M., Webb, C. P., Anver, M., Koochekpour, S., and Vande Woude, G. F. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci U S A, *95:* 14417-14422, 1998.
24. Schmidt, L., Duh, F. M., Chen, F., Kishida, T., Glenn, G., Choyke, P., Scherer, S. W., Zhuang, Z., Lubensky, I., Dean, M., Allikmets, R., Chidambaram, A., Bergerheim, U. R., Feltis, J. T., Casadevall, C., Zamarron, A., Bernues, M., Richard, S., Lips, C. J., Walther, M. M., Tsui, L. C., Geil, L., Orcutt, M. L., Stackhouse, T., Zbar, B., and et al. Germline and somatic mutations in the tyrosine kinase domain of the MET proto-oncogene in papillary renal carcinomas. Nat Genet, *16:* 68-73, 1997.
25. Abella, J. V., Peschard, P., Naujokas, M. A., Lin, T., Saucier, C., Urbe, S., and Park, M. Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol Cell Biol, *25:* 9632-9645, 2005.

26. Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell, *8:* 995-1004, 2001.
27. Kong-Beltran, M., Seshagiri, S., Zha, J., Zhu, W., Bhawe, K., Mendoza, N., Holcomb, T., Pujara, K., Stinson, J., Fu, L., Severin, C., Rangell, L., Schwall, R., Amler, L., Wickramasinghe, D., and Yauch, R. Somatic mutations lead to an oncogenic deletion of met in lung cancer. Cancer Res, *66:* 283-289, 2006.
28. Lee, J. H., Han, S. U., Cho, H., Jennings, B., Gerrard, B., Dean, M., Schmidt, L., Zbar, B., and Vande Woude, G. F. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene, *19:* 4947-4953, 2000.
29. Ma, P. C., Kijima, T., Maulik, G., Fox, E. A., Sattler, M., Griffin, J. D., Johnson, B. E., and Salgia, R. c-MET mutational analysis in small cell lung cancer: novel juxtamembrane domain mutations regulating cytoskeletal functions. Cancer Res, *63:* 6272-6281, 2003.
30. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci U S A, *94:* 11445-11450, 1997.
31. Rodrigues, G. A., Naujokas, M. A., and Park, M. Alternative splicing generates isoforms of the met receptor tyrosine kinase which undergo differential processing. Mol Cell Biol, *11:* 2962-2970, 1991.
32. Sgroi, D. C., Teng, S., Robinson, G., LeVangie, R., Hudson, J. R., Jr., and Elkahloun, A. G. In vivo gene expression profile analysis of human breast cancer progression. Cancer Res, *59:* 5656-5661, 1999.
33. Longati, P., Bardelli, A., Ponzetto, C., Naldini, L., and Comoglio, P. M. Tyrosines1234-1235 are critical for activation of the tyrosine kinase encoded by the MET proto-oncogene (HGF receptor). Oncogene, *9:* 49-57, 1994.
34. Rodrigues, G. A. and Park, M. Autophosphorylation modulates the kinase activity and oncogenic potential of the Met receptor tyrosine kinase. Oncogene, *9:* 2019-2027, 1994.
35. Tuck, A. B., Park, M., Sterns, E. E., Boag, A., and Elliott, B. E. Coexpression of hepatocyte growth factor and receptor (Met) in human breast carcinoma. Am J Pathol, *148:* 225-232, 1996.
36. Jin, L., Fuchs, A., Schnitt, S. J., Yao, Y., Joseph, A., Lamszus, K., Park, M., Goldberg, I. D., and Rosen, E. M. Expression of scatter factor and c-met receptor in benign and malignant breast tissue. Cancer, *79:* 749-760, 1997.
37. Follenzi, A., Bakovic, S., Gual, P., Stella, M. C., Longati, P., and Comoglio, P. M. Cross-talk between the proto-oncogenes Met and Ron. Oncogene, *19:* 3041-3049, 2000.
38. Faletto, D. L., Tsarfaty, I., Kmiecik, T. E., Gonzatti, M., Suzuki, T., and Vande Woude, G. F. Evidence for non-covalent clusters of the c-met proto-oncogene product. Oncogene, *7:* 1149-1157, 1992.
39. Nakajima, M., Sawada, H., Yamada, Y., Watanabe, A., Tatsumi, M., Yamashita, J., Matsuda, M., Sakaguchi, T., Hirao, T., and Nakano, H. The

prognostic significance of amplification and overexpression of c-met and c-erb B-2 in human gastric carcinomas. Cancer, *85:* 1894-1902, 1999.

40. Di Renzo, M. F., Olivero, M., Giacomini, A., Porte, H., Chastre, E., Mirossay, L., Nordlinger, B., Bretti, S., Bottardi, S., Giordano, S., and et al. Overexpression and amplification of the met/HGF receptor gene during the progression of colorectal cancer. Clin Cancer Res, *1:* 147-154, 1995.
41. Tsugawa, K., Yonemura, Y., Hirono, Y., Fushida, S., Kaji, M., Miwa, K., Miyazaki, I., and Yamamoto, H. Amplification of the c-met, c-erbB-2 and epidermal growth factor receptor gene in human gastric cancers: correlation to clinical features. Oncology, *55:* 475-481, 1998.
42. Kuniyasu, H., Yasui, W., Kitadai, Y., Yokozaki, H., Ito, H., and Tahara, E. Frequent amplification of the c-met gene in scirrhous type stomach cancer. Biochem Biophys Res Commun, *189:* 227-232, 1992.
43. Graveel, C., Su, Y., Koeman, J., Wang, L. M., Tessarollo, L., Fiscella, M., Birchmeier, C., Swiatek, P., Bronson, R., and Vande Woude, G. Activating Met mutations produce unique tumor profiles in mice with selective duplication of the mutant allele. Proc Natl Acad Sci U S A, *101:* 17198-17203, 2004.
44. Maroun, C. R., Holgado-Madruga, M., Royal, I., Naujokas, M. A., Fournier, T. M., Wong, A. J., and Park, M. The Gab1 PH domain is required for localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase. Mol Cell Biol, *19:* 1784-1799, 1999.
45. Fournier, T. M., Kamikura, D., Teng, K., and Park, M. Branching tubulogenesis but not scatter of madin-darby canine kidney cells requires a functional Grb2 binding site in the Met receptor tyrosine kinase. J Biol Chem, *271:* 22211-22217, 1996.
46. Ponzetto, C., Bardelli, A., Maina, F., Longati, P., Panayotou, G., Dhand, R., Waterfield, M. D., and Comoglio, P. M. A novel recognition motif for phosphatidylinositol 3-kinase binding mediates its association with the hepatocyte growth factor/scatter factor receptor. Mol Cell Biol, *13:* 4600-4608, 1993.
47. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. Central role of the threonine residue within the p+1 loop of receptor tyrosine kinase in STAT3 constitutive phosphorylation in metastatic cancer cells. Mol Cell Biol, *24:* 9390-9400, 2004.
48. Welm, A. L., Kim, S., Welm, B. E., and Bishop, J. M. MET and MYC cooperate in mammary tumorigenesis. Proc Natl Acad Sci U S A, *102:* 4324-4329, 2005.
49. Hiscox, S., Parr, C., Nakamura, T., Matsumoto, K., Mansel, R. E., and Jiang, W. G. Inhibition of HGF/SF-induced breast cancer cell motility and invasion by the HGF/SF variant, NK4. Breast Cancer Res Treat, *59:* 245-254, 2000.
50. Mahler, J. F., Stokes, W., Mann, P. C., Takaoka, M., and Maronpot, R. R. Spontaneous lesions in aging FVB/N mice. Toxicol Pathol, *24:* 710-716, 1996.

51. Khoury, H., Naujokas, M. A., Zuo, D., Sangwan, V., Frigault, M. M., Petkiewicz, S., Dankort, D. L., Muller, W. J., and Park, M. HGF converts ErbB2/Neu epithelial morphogenesis to cell invasion. Mol Biol Cell, *16:* 550-561, 2005.
52. Van Belle, E., Witzenbichler, B., Chen, D., Silver, M., Chang, L., Schwall, R., and Isner, J. M. Potentiated angiogenic effect of scatter factor/hepatocyte growth factor via induction of vascular endothelial growth factor: the case for paracrine amplification of angiogenesis. Circulation, *97:* 381-390, 1998.
53. Grant, D. S., Kleinman, H. K., Goldberg, I. D., Bhargava, M. M., Nickoloff, B. J., Kinsella, J. L., Polverini, P., and Rosen, E. M. Scatter factor induces blood vessel formation in vivo. Proc Natl Acad Sci U S A, *90:* 1937-1941, 1993.
54. Saucier, C., Khoury, H., Lai, K. M., Peschard, P., Dankort, D., Naujokas, M. A., Holash, J., Yancopoulos, G. D., Muller, W. J., Pawson, T., and Park, M. The Shc adaptor protein is critical for VEGF induction by Met/HGF and ErbB2 receptors and for early onset of tumor angiogenesis. Proc Natl Acad Sci U S A, *101:* 2345-2350, 2004.
55. Daniel, C. W., Strickland, P., and Friedmann, Y. Expression and functional role of E- and P-cadherins in mouse mammary ductal morphogenesis and growth. Dev Biol, *169:* 511-519, 1995.
56. Nagle, R. B., Bocker, W., Davis, J. R., Heid, H. W., Kaufmann, M., Lucas, D. O., and Jarasch, E. D. Characterization of breast carcinomas by two monoclonal antibodies distinguishing myoepithelial from luminal epithelial cells. J Histochem Cytochem, *34:* 869-881, 1986.
57. Dulbecco, R., Allen, W. R., Bologna, M., and Bowman, M. Marker evolution during the development of the rat mammary gland: stem cells identified by markers and the role of myoepithelial cells. Cancer Res, *46:* 2449-2456, 1986.
58. Smolen, G. A., Muir, B., Mohapatra, G., Barmettler, A., Kim, W. J., Rivera, M. N., Haserlat, S. M., Okimoto, R. A., Kwak, E., Dahiya, S., Garber, J. E., Bell, D. W., Sgroi, D. C., Chin, L., Deng, C. X., and Haber, D. A. Frequent met oncogene amplification in a Brca1/Trp53 mouse model of mammary tumorigenesis. Cancer Res, *66:* 3452-3455, 2006.
59. Korsching, E., Packeisen, J., Agelopoulos, K., Eisenacher, M., Voss, R., Isola, J., van Diest, P. J., Brandt, B., Boecker, W., and Buerger, H. Cytogenetic alterations and cytokeratin expression patterns in breast cancer: integrating a new model of breast differentiation into cytogenetic pathways of breast carcinogenesis. Lab Invest, *82:* 1525-1533, 2002.
60. Rakha, E. A., Putti, T. C., Abd El-Rehim, D. M., Paish, C., Green, A. R., Powe, D. G., Lee, A. H., Robertson, J. F., and Ellis, I. O. Morphological and immunophenotypic analysis of breast carcinomas with basal and myoepithelial differentiation. J Pathol, *208:* 495-506, 2006.
61. Kim, M. J., Ro, J. Y., Ahn, S. H., Kim, H. H., Kim, S. B., and Gong, G. Clinicopathologic significance of the basal-like subtype of breast cancer: a comparison with hormone receptor and Her2/neu-overexpressing phenotypes. Hum Pathol, *37:* 1217-1226, 2006.

62. Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Eystein Lonning, P., and Borresen-Dale, A. L. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A, *98:* 10869-10874, 2001.
63. White, D. E., Cardiff, R. D., Dedhar, S., and Muller, W. J. Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. Oncogene, *20:* 7064-7072, 2001.
64. Muller, W. J., Sinn, E., Pattengale, P. K., Wallace, R., and Leder, P. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. Cell, *54:* 105-115, 1988.
65. Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R., and Leder, P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell, *49:* 465-475, 1987.
66. Cardiff, R. D. Mouse models of human breast cancer. Comp Med, *53:* 250-253, 2003.
67. Lamorte, L., Royal, I., Naujokas, M., and Park, M. Crk adapter proteins promote an epithelial-mesenchymal-like transition and are required for HGF-mediated cell spreading and breakdown of epithelial adherens junctions. Mol Biol Cell, *13:* 1449-1461, 2002.
68. Zhu, H., Naujokas, M. A., and Park, M. Receptor chimeras indicate that the met tyrosine kinase mediates the motility and morphogenic responses of hepatocyte growth/scatter factor. Cell Growth Differ, *5:* 359-366, 1994.
69. Rong, S., Segal, S., Anver, M., Resau, J. H., and Vande Woude, G. F. Invasiveness and metastasis of NIH 3T3 cells induced by Met-hepatocyte growth factor/scatter factor autocrine stimulation. Proc Natl Acad Sci U S A, *91:* 4731-4735, 1994.
70. Jeffers, M., Rong, S., and Vande Woude, G. F. Enhanced tumorigenicity and invasion-metastasis by hepatocyte growth factor/scatter factor-met signalling in human cells concomitant with induction of the urokinase proteolysis network. Mol Cell Biol, *16:* 1115-1125, 1996.
71. Kohya, N., Kitajima, Y., Jiao, W., and Miyazaki, K. Effects of E-cadherin transfection on gene expression of a gallbladder carcinoma cell line: repression of MTS1/S100A4 gene expression. Int J Cancer, *104:* 44-53, 2003.
72. Stockinger, A., Eger, A., Wolf, J., Beug, H., and Foisner, R. E-cadherin regulates cell growth by modulating proliferation-dependent beta-catenin transcriptional activity. J Cell Biol, *154:* 1185-1196, 2001.
73. St Croix, B., Sheehan, C., Rak, J. W., Florenes, V. A., Slingerland, J. M., and Kerbel, R. S. E-Cadherin-dependent growth suppression is mediated by the cyclin-dependent kinase inhibitor p27(KIP1). J Cell Biol, *142:* 557-571, 1998.
74. Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., Tan, L. K., Rosen, J. M., and Varmus, H. E. Evidence that transgenes encoding components of the Wnt

signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci U S A, *100:* 15853-15858, 2003.

75. Xiao, G. H., Jeffers, M., Bellacosa, A., Mitsuuchi, Y., Vande Woude, G. F., and Testa, J. R. Anti-apoptotic signaling by hepatocyte growth factor/Met via the phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase pathways. Proc Natl Acad Sci U S A, *98:* 247-252, 2001.
76. Zhang, Y. W., Wang, L. M., Jove, R., and Vande Woude, G. F. Requirement of Stat3 signaling for HGF/SF-Met mediated tumorigenesis. Oncogene, *21:* 217-226, 2002.
77. Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat Genet, *29:* 418-425, 2001.
78. Reis-Filho, J. S., Milanezi, F., Steele, D., Savage, K., Simpson, P. T., Nesland, J. M., Pereira, E. M., Lakhani, S. R., and Schmitt, F. C. Metaplastic breast carcinomas are basal-like tumours. Histopathology, *49:* 10-21, 2006.
79. Wijnhoven, S. W., Zwart, E., Speksnijder, E. N., Beems, R. B., Olive, K. P., Tuveson, D. A., Jonkers, J., Schaap, M. M., van den Berg, J., Jacks, T., van Steeg, H., and de Vries, A. Mice expressing a mammary gland-specific R270H mutation in the p53 tumor suppressor gene mimic human breast cancer development. Cancer Res, *65:* 8166-8173, 2005.
80. Lin, S. C., Lee, K. F., Nikitin, A. Y., Hilsenbeck, S. G., Cardiff, R. D., Li, A., Kang, K. W., Frank, S. A., Lee, W. H., and Lee, E. Y. Somatic mutation of p53 leads to estrogen receptor alpha-positive and -negative mouse mammary tumors with high frequency of metastasis. Cancer Res, *64:* 3525-3532, 2004.
81. Shien, T., Tashiro, T., Omatsu, M., Masuda, T., Furuta, K., Sato, N., Akashi-Tanaka, S., Uehara, M., Iwamoto, E., Kinoshita, T., Fukutomi, T., Tsuda, H., and Hasegawa, T. Frequent overexpression of epidermal growth factor receptor (EGFR) in mammary high grade ductal carcinomas with myoepithelial differentiation. J Clin Pathol, *58:* 1299-1304, 2005.
82. Rong, S., Donehower, L. A., Hansen, M. F., Strong, L., Tainsky, M., Jeffers, M., Resau, J. H., Hudson, E., Tsarfaty, I., and Vande Woude, G. F. Met proto-oncogene product is overexpressed in tumors of p53-deficient mice and tumors of Li-Fraumeni patients. Cancer Res, *55:* 1963-1970, 1995.
83. Charafe-Jauffret, E., Ginestier, C., Monville, F., Finetti, P., Adelaide, J., Cervera, N., Fekairi, S., Xerri, L., Jacquemier, J., Birnbaum, D., and Bertucci, F. Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene, *25:* 2273-2284, 2006.

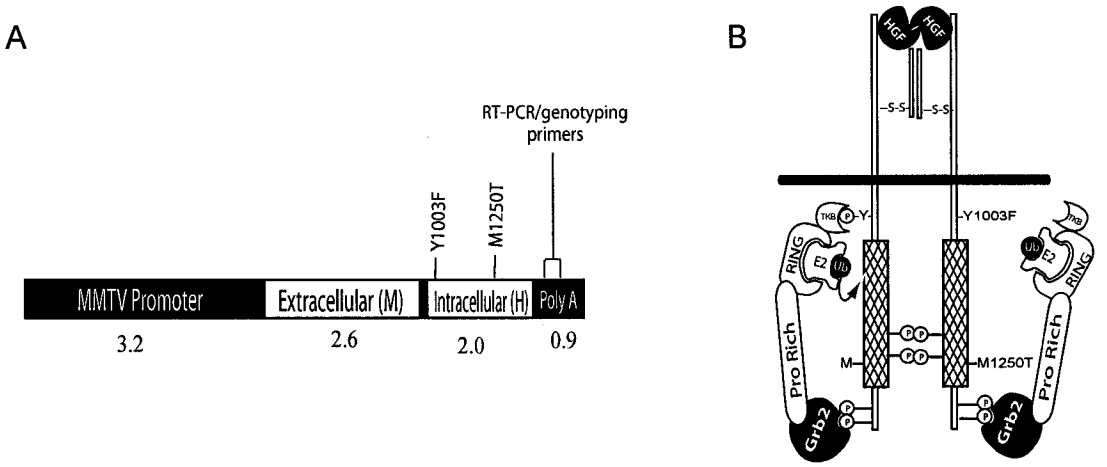


Figure 1. Schematics of Met receptor construct and receptor variants. A. Schematic of construct used to generate the transgenic mice. Mutations in the intracellular domain are indicated. B. Schematic of the Met receptor with the positions of the kinase domain M1250T mutation and the loss of negative regulation Y1003F juxtamembrane domain mutation that prohibits direct binding of the ubiquitin ligase, Cbl, thereby decreasing receptor ubiquitination.

Tumor Latency and Penetrance in MMTV-Met Transgenic Mice

	Nulliparous			Multiparous		
Strain	mice with tumors/total mice	average latency	multifocal tumors	mice with tumors/total mice	average latency	multifocal tumors
FVB	0/15	N/A	0	4/21 (19%)	610	2/4 (50%)
MMTV-Met wt	0/25	N/A	0	3/13 (23%)	513	1/3 (33%)
MMTV-Met Y1003F	1/26 (4%)	821	0	3/17 (18%)	642	0
MMTV-Met M1250T	1/24 (4%)	614	0	3/14 (21%)	547	1/3 (33%)
MMTV-Met-YF/MT1	0/8	N/A	0	12/21 (57%)	409*	8/12 (67%)
MMTV-Met-YF/MT2	2/11 (18%)	441	1/2 (50%)	3/13 (23%)	460	1/3 (33%)

Table 1. Tumor incidence and latency
* statistically significant $p<0.05$ (student's t-test).

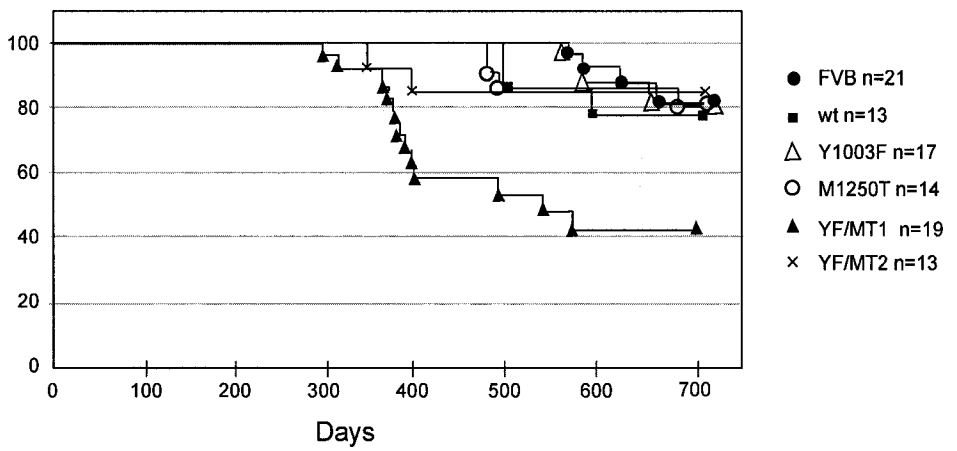


Chart 1. Percent tumor-free multiparous mice over time

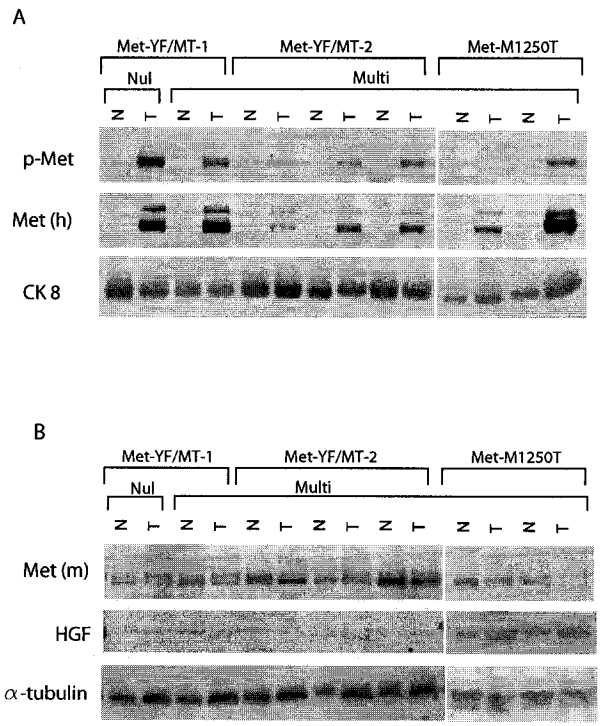


Figure 2. Tumors express elevated levels of exogenous Met protein. A. 40 μg of total protein lysates from normal and tumor tissue (N and T) resolved by SDS-PAGE and immunoblotted for phosphorylated Met receptor and exogenous human Met proteins. Cytokeratin 8 was used as a loading control for epithelial content of the lysates. B. Met receptor-related pathway proteins are not upregulated in tumor tissue. Protein lysates were immunoblotted for murine Met receptor, HGF and α-tubulin was used as a loading control Nul-nulliparous. Multi-multiparous. Met (h) is human Met; Met (m) is murine Met.

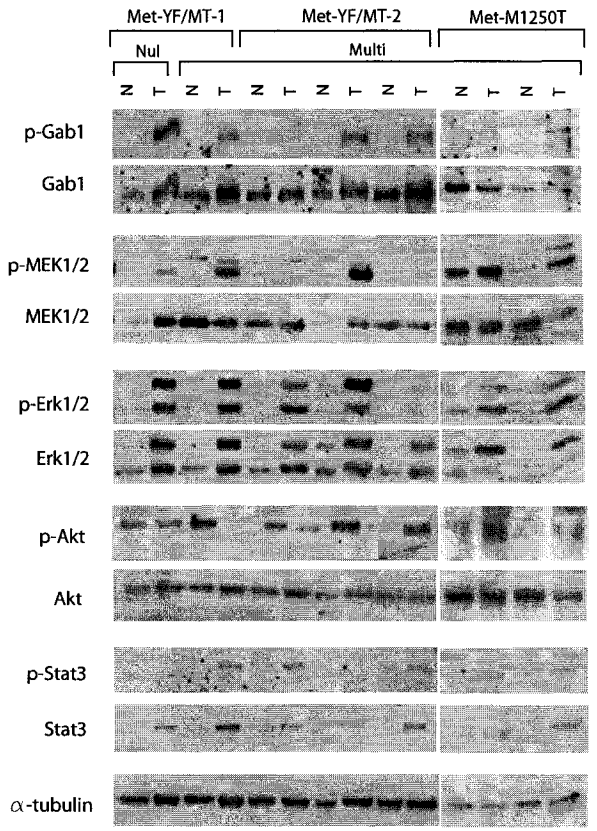


Figure 3. Tumors arising in MMTV-Met-YF/MT and MMTV-Met-M1250T transgenic animals display phosphorylation of proteins downstream of the Met receptor. 40 μg of total protein lysates from normal and tumor tissues (N and T) resolved by SDS-PAGE and immunoblotted for levels of MEK1/2, phospho-Ser217/221-MEK1/2, phospho-Thr202/Tyr204 Erk1/2, Erk1/2, PI3K pathway protein Akt and phospho-Ser473-Akt as well as the transcription factor Stat3 (phospho-Tyr705-Stat3). Levels of total protein were determined by stripping and reprobing the same membrane. α-tubulin was used as a loading control.

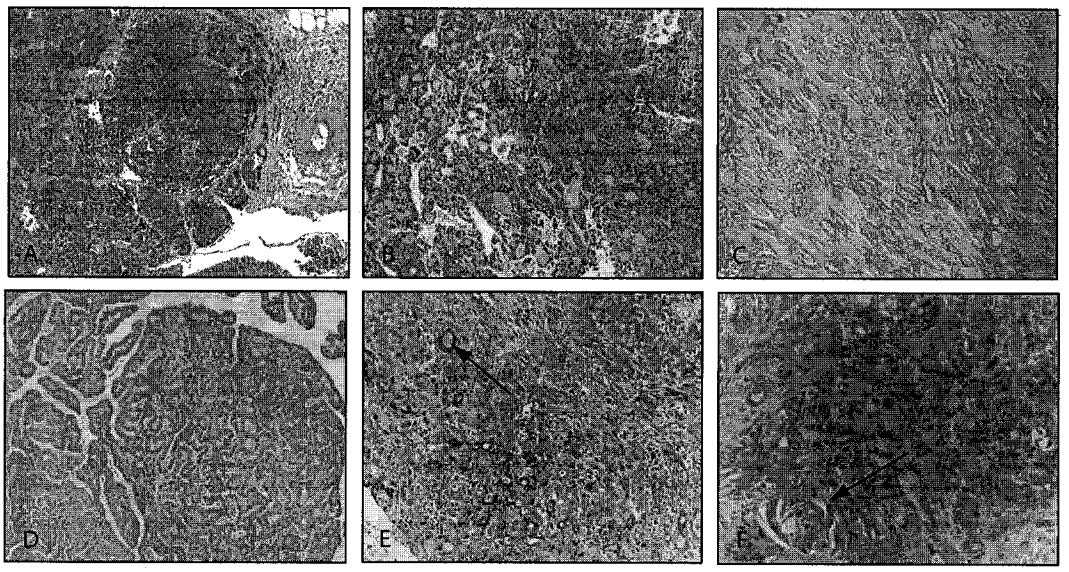


Figure 4. Histology of MMTV-Met tumors. A.-D.-10x original magnification. MMTV-Met-YF/MT tumors. E.-F. MMTV-Met-M1250T tumors. A. Solid, nodular adenocarcinoma . B. Solid, nodular adenocarcinoma with a secretory phenotype C. Well-differentiated invasive tubular adenocarcinoma invading muscle tissue adjacent to mammary fat pad. D. Well-differentiated tubulo-papillary adenocarcinoma E. 20x mag. Poorly differentiated Met-M1250T tumor retaining some small ductal structures (arrow). F. 40x mag. Met-M1250T tumor with squamous metaplasia (arrow).

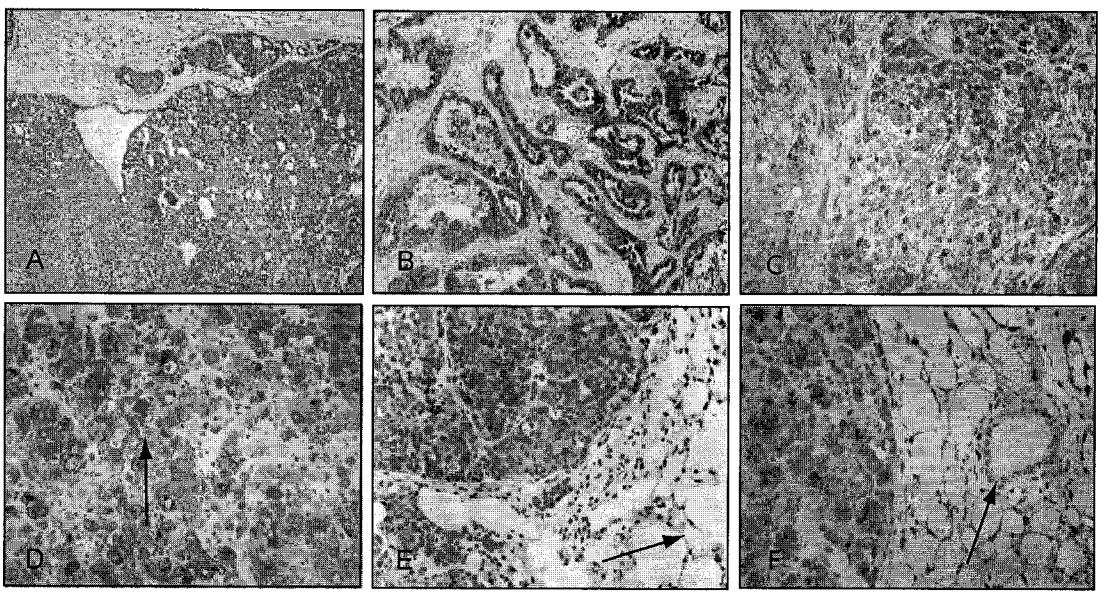


Figure 5. Exogenous Met receptor protein is expressed in cells of tumors and lung metastases. A, B, C- Original magnification 20x. Met-YF/MT Solid adenocarcinoma (A) and Met-YF/MT invasive tubular (B) tumors stain positively for exogenous protein with differences in intensity between cells. C-Met-M1250T tumor. D, E, F- Magnification 40x. D. High magnification view of the tumor cells that express exogenous Met protein in the Met-M1250T tumor. Note the ductal cells are not expressing exogenous Met (arrow). E. Extravascular lung metastasis stains positively for exogenous Met protein (lung parenchyma-arrow). F. Transplanted tumor stains for exogenous protein whereas adjacent host epithelium does not.

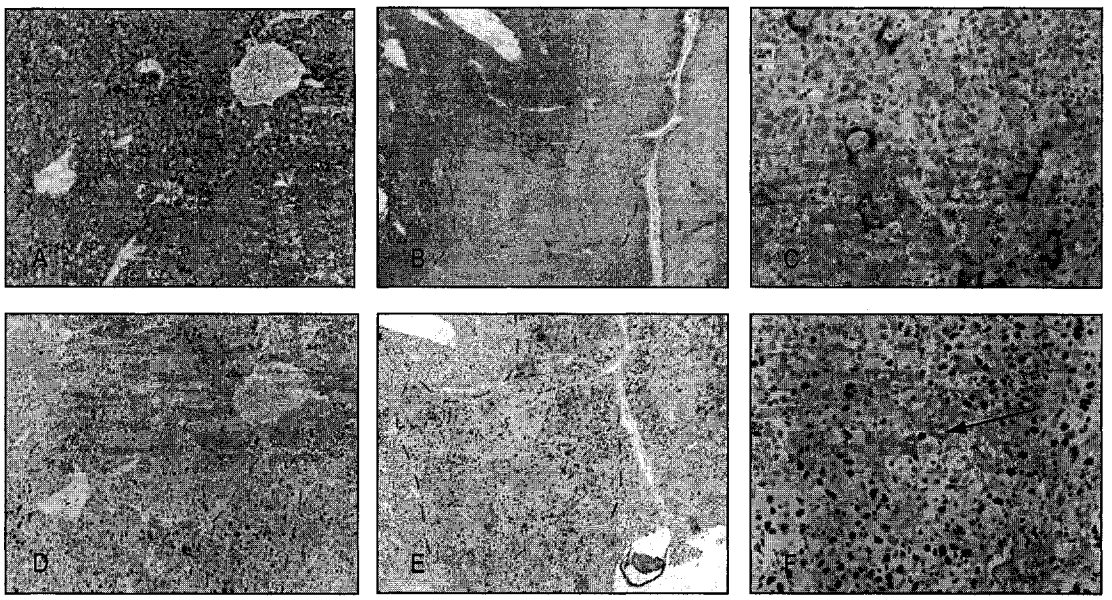


Figure 6. Immunohistochemical analysis of E-cadherin expression and proliferation by PCNA in serial sections. A.-C. E-cadherin. D.-F. PCNA. A,B,D,E- Met-YF/MT. Original magnification 20x. A. Strong expression of E-cadherin in a solid adenocarcinoma with clusters of PCNA positivity (D). B. Regions of solid nodular adenocarcinoma displaying strong and weak E-cadherin staining with inverse patterns of PCNA staining (F). C, F Met-M1250T Original magnification 40x. In non-serial sections E-cadherin staining is restricted to ductal structures (C). F. PCNA staining excluded from cells in ductal structures (arrow).

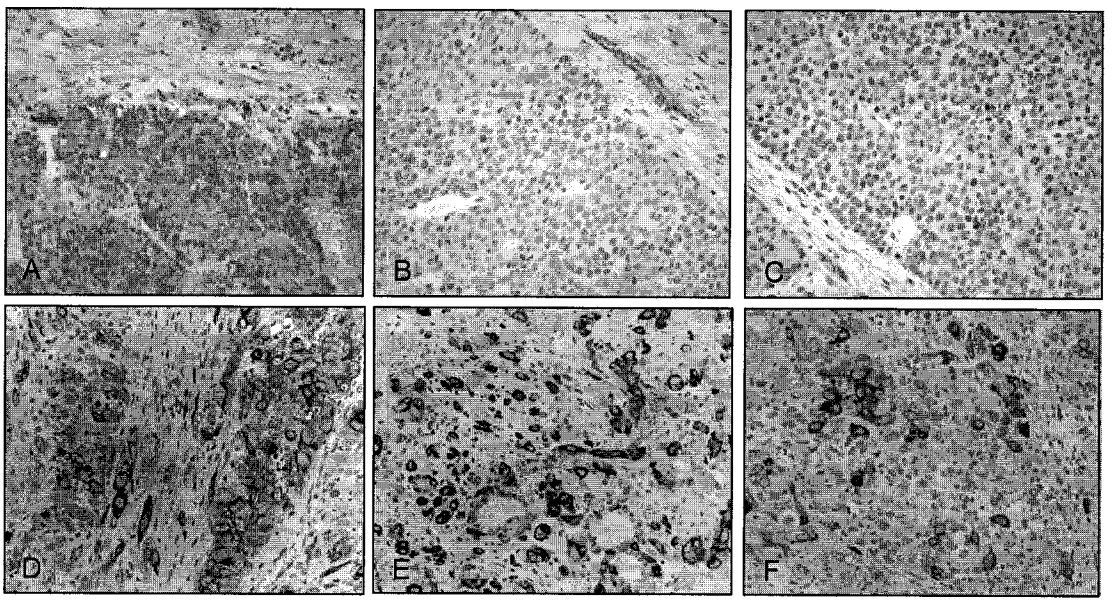


Figure 7. MMTV-Met-M1250T tumors contain progenitor cells. All images original magnification 40x. A.-C.-MMTV-Met-YF/MT solid adenocarcinoma D.-F. MMTV-Met-M1250T adenocarcinoma. A,D. Cytokeratin 8/18. B, E. Cytokeratin 14. C,F. Cytokeratin 6.

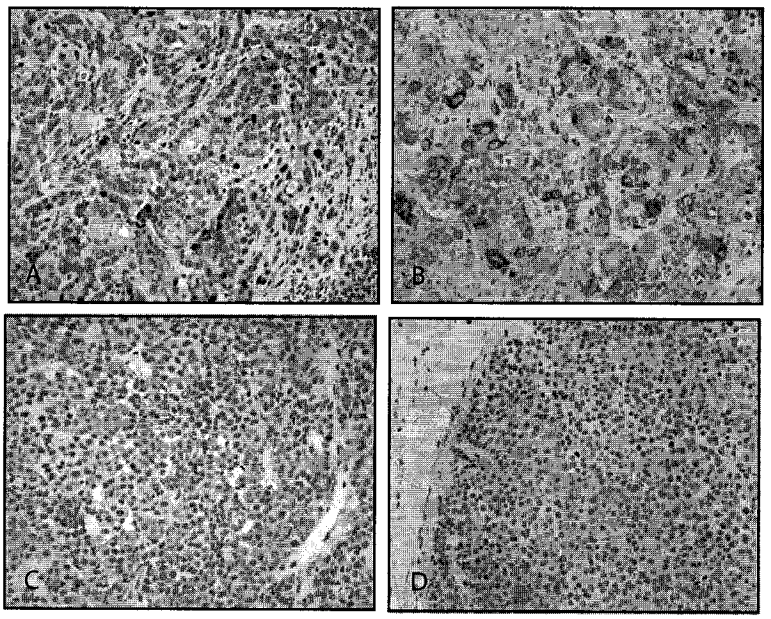


Figure 8- Tumors containing cells of mixed lineages display p53 nuclear localization. All images 40x original magnification. A,B- Met-M1250T tumor containing both p53 nuclear localization (A), and K14 positive cells (B). C, D- Met-YF/MT solid adenocarcinoma negative for both p53 nuclear localization (C), and K14 (D).

	# Tumors				# Tumors		
	Met-YF/MT	Met-M1250T	Microvessel Density	K8	K14	K6	p53
Solid-low microvessel	13	0	+	+++	2	0	0
Solid-high microvessel	9	0	+++	+++	1	0	0
Papillary	4	0	+++	+++	1	0	0
Invasive Tubular	3	0	++	++	3	2	2
Squamous Metaplasia	0	3	++	++	3	3	3

Table 2. Characteristics of MMTV-Met-M1250T and MMTV-Met-YF/MT tumors

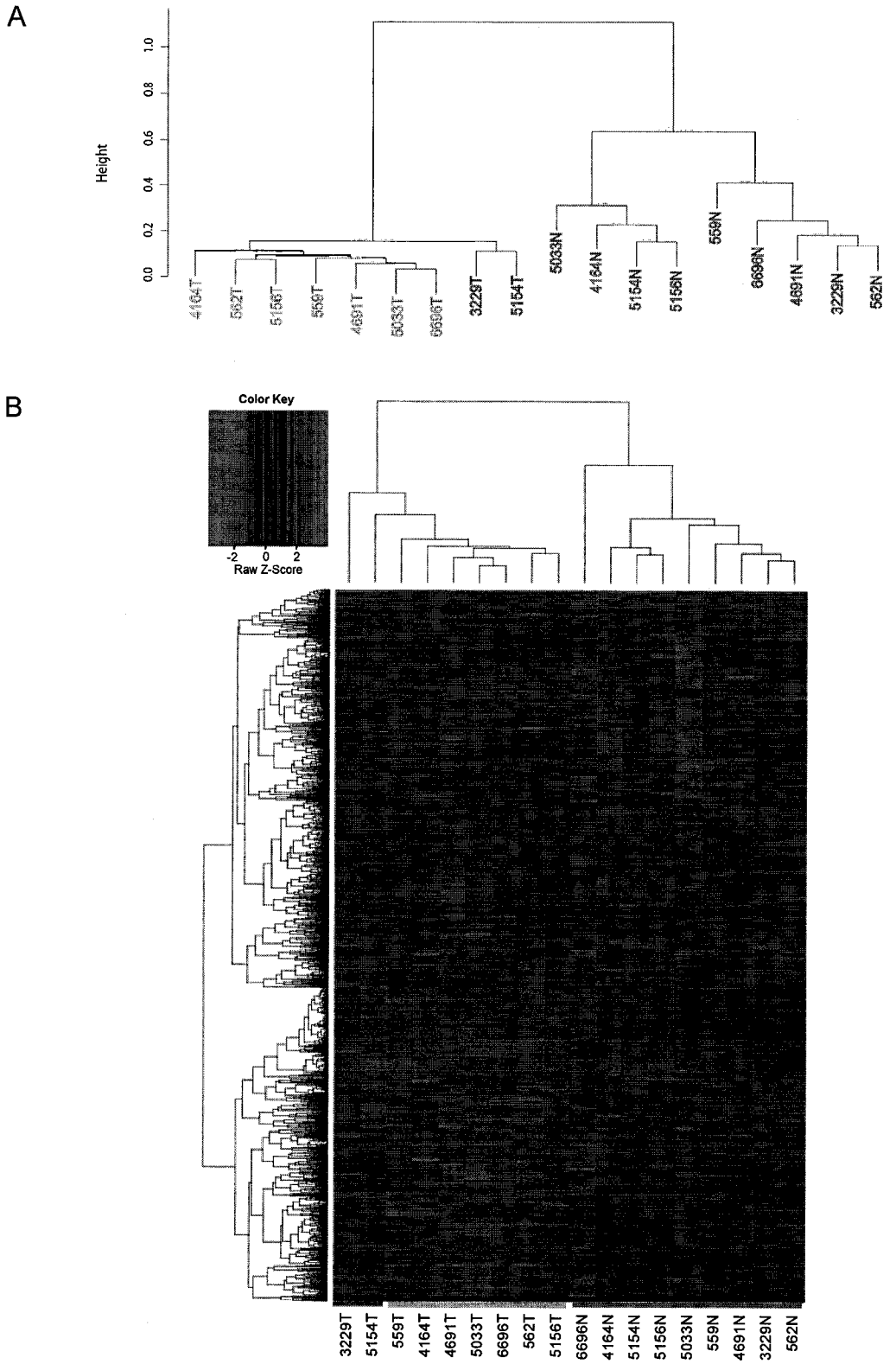
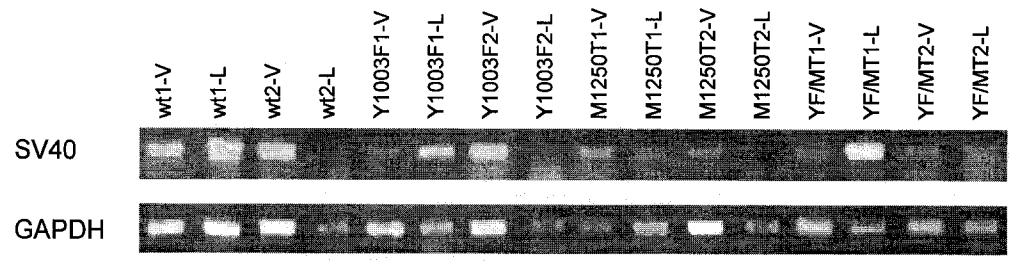


Figure 9. Hierarchical clustering and heatmap showing that Met-M1250T tumors cluster seperately from Met-YF/MT tumors and each segregates from matched-normal. A. Hierarchical clustering of Met tumors using all genome features shows segregation by Met receptor variant. B. Heatmap generated using the top 2000 genes shows Met-M1250T and Met-YF/MT gene expression clusters. Pink-Met-M1250T, Yellow-Met-YF/MT, Teal-normal. T=tumor, N=normal

A



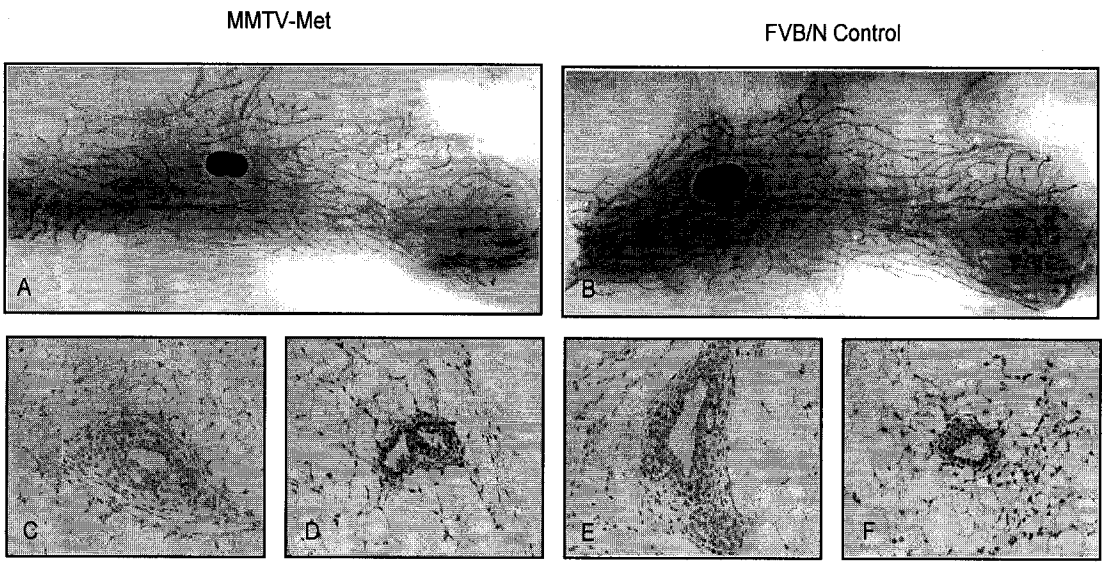
B

Met Variant		Copies	Tumors	Expressing Tumors
wt	wt1	4	2	0
	wt2	8	0	0
Y1003F	YF1	1	2	0
	YF2	9	1	0
M1250T	MT1	4	3	3
	MT2	6	1	0
Y100F/ M1250T	YF/MT1	5	12	21
	YF/MT2	2	3	7

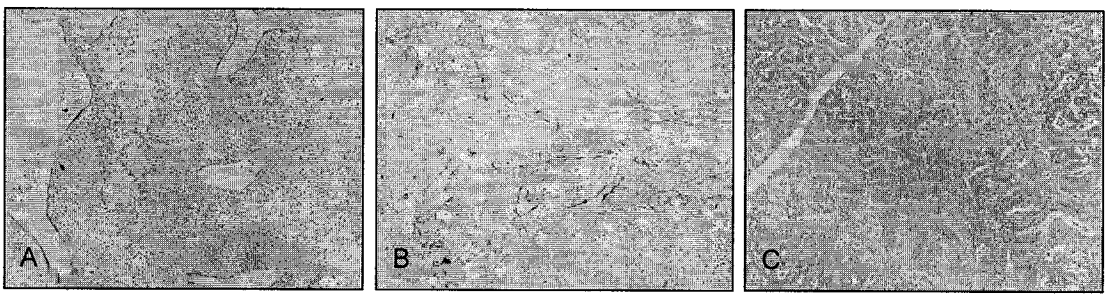
C

	Normal	Tumor	Fold Change
T1	2.48	1.07	0.43
T2	1.62	1	0.62
T3	2.45	1.58	0.64
T4	1.52	1.07	0.70
T5	1.43	1.29	0.90
T6	1.53	1.44	0.94
T7	2.02	2.12	1.05

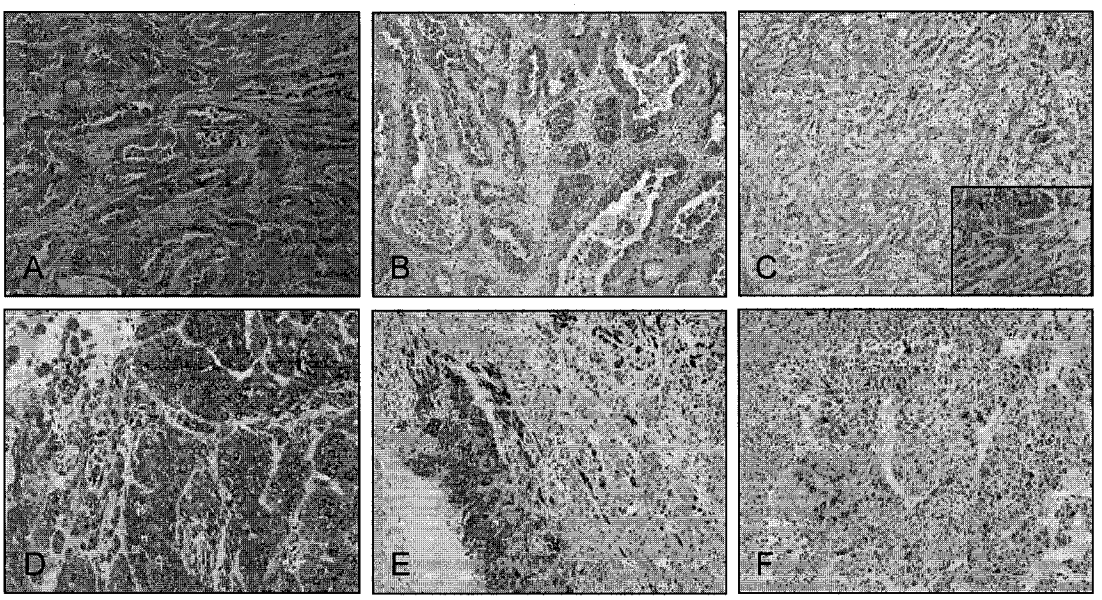
Supplemental figure 1. Quantitative data for transgenic lines. A. RT-PCR of RNA extracted from the mammary glands of virgin (V) and lactating d18 (L) females from each of the transgenic lines selected. B. Copy number as determined by qPCR of the transgene in the selected MMTV-Met lines C. Relative copies of transgene in tumor and normal tissues as determined by qPCR. T1, T2 etc. is tumor 1, tumor 2 etc..



Supplemental figure 2. MMTV-Met transgenics have normal mammary gland development. A, B-Whole mounts at 9 weeks from MMTV-Met mouse (A) and FVB/N control mouse (B). C-F- TUNEL staining of ductal sections from MMTV-Met mice (C,D) and FVB/N control mice (E,F)



Supplemental figure 3. CD 31 immunohistochemical staining of tumor sections displays different microvessel densities. All images taken at an original magnification of 20x. A. Low microvessel density solid tumor. B. Intermediate microvessel density. C. High microvessel density in tubulo-papillary tumor



Supplemental figure 4. Transplanted invasive tubular MMTV-Met-YF/MT adenocarcinomas display expansion of progenitor population. All images taken at 20x original magnification. A-C Original Met-YF/MT tumor. A. H&E staining. B. K8/18. C. K14. Inset is from a 40x image. D-F Transplanted tumor. D. K8/18 highlighting cohesive and invasive cells. E. K14 cells scattered and condensed. F. K6 progenitor marker.

Chapter 4

Expression of the Met Receptor Tyrosine Kinase is Required for Mammary Ductal Development

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Preface

Since 1995, it has been known that HGF treatment of mammary epithelial cells in a three-dimensional culture induces the formation of branching tubular structures, recapitulating the development of the mammary ductal tree *in vitro*. Various studies in the mammary gland have demonstrated that high doses of HGF, whether by treatment *in vitro*, infection of primary mammary epithelial cells followed by transplantation to cleared mammary fat pads, or by transgenesis, results in enhanced mammary ductal branching. *In vitro* assays have demonstrated that treatment of a developing mammary gland with antisense oligonucleotides to HGF blocks ductal branching, but no *in vivo* knock-out studies have been conducted. Both Met and HGF knock-out animals have been generated but are embryonic lethal, prohibiting the study of mammary gland development as the majority of ductal development occurs post-natally.

In order to knock out the Met receptor from the mammary epithelium, we have utilized floxed-Met (Met^{fl}) mice from Dr. Snorri Thorgeirsson, at the NIH, and directed Met excision to the mammary epithelium by crossing with transgenic mice expressing Cre recombinase under the control of a mammary-specific promoter. We have also crossed the Met^{fl} mice with a strain of reporter mice (GTRosa26) that express β-galactosidase following Cre expression in order to identify the cells in which Met has been deleted. In this manuscript, efforts towards generating Met-null mammary epithelial outgrowths are described.

This work is ongoing, and this chapter contains preliminary data, with the purpose of highlighting our understanding to date. Although studies are incomplete, it appears that Met-null cells are selected against, supporting a role for Met receptor signaling in mammary gland development.

Abstract

The development of a functional mammary gland is a complex process involving epithelial-stromal interactions as well as growth factor and hormonal signaling. Receptor tyrosine kinase signaling has been shown to play an important role in mammary gland development and tumorigenesis as exemplified by the transgenics and knock-out animals of the ErbB2 receptor and ligands of the EGFR family. Expression of the Met receptor tyrosine kinase and its ligand hepatocyte growth factor (HGF) is coordinately regulated in the mammary gland with higher levels of expression during puberty and early pregnancy followed by a dramatic drop during lactation and a resurgence during involution. Transgenic overexpression of HGF or treatment of excised mammary fat pads with HGF *in vitro* leads to excessive mammary ductal branching. Although HGF overexpression and treatment *in vitro* has been examined, the impact of loss of Met receptor signaling on the mammary gland has not been studied. Here we demonstrate that mammary-specific deletion of the Met receptor through Cre-mediated recombination does not have an overt effect on mammary gland development. Mammary glands isolated from homozygous floxed-Met mice were indistinguishable from the wild type Met glands, however the number of Cre-recombinase positive cells is fewer in $Met^{fl/fl}$ glands, indicating that Met-null cells are selected against, thereby supporting a role for Met in normal mammary gland development.

Abbreviations

EGFR	Epidermal Growth Factor Receptor
ErbB2	Erythroblastic leukemia viral oncogene homolog2
FVB/N	Friend's leukemia Virus B ($Fv1^{+}$)/NIH Strain
HGF	Hepatocyte Growth Factor
IHC	Immunohistochemistry
MDCK	Madin Darby Canine Kidney cells
Met^{fl}	Floxed Met
MMTV	Mouse Mammary Tumor Virus
MOI	Multiplicity of Infection
MFP	Mammary Fat Pad
RTK	Receptor Tyrosine Kinase

Introduction

Signaling through the Met receptor tyrosine kinase (RTK) has been shown to induce a morphogenic program of tubule formation in three dimensional culture in a number of epithelial cell types (1). Tubule formation/ductal morphogenesis is a complex process that requires the coordination of signals that stimulate cellular proliferation, migration, invasion through a matrix, polarization and, depending on the system, apoptosis in order to form a lumen (2). *In vivo*, although few knock-out studies have been performed, Met may play a role in tubule formation, as Met is expressed by the epithelial cells of organs such as breast (3), kidney (4), and lung (5) while nearby stromal fibroblasts secrete the ligand, hepatocyte growth factor (HGF) (6).

The Madin-Darby canine kidney (MDCK) cell line has been used to study the role of HGF in inducing tubule formation. Through studies in MDCK it was determined that HGF's unique ability to generate tubes in three-dimensional culture requires binding and full functionality of the docking protein Gab1, the primary downstream protein from the Met receptor (review (7)). HGF treatment of MDCK cells results in a prolonged phosphorylation of Gab1 and downstream MAPK proteins compared with the transient phosphorylation observed downstream of other RTKs that are unable to induce tubulogenesis, such as EGFR (8, 9). The effects of HGF on tubule formation in kidney cells are recapitulated in kidney culture assays; *ex vivo* treatment of a kidney rudiment with Met-blocking antibodies inhibits the development of the tubular structures (10). Kidney epithelial cells are not unique in their response to HGF. Mammary epithelial cell lines such as EpH4 (11), NMuMG (12), and MCF-10A (13) also undergo a morphogenic response to HGF stimulation by forming tubes when cultured in collagen or Matrigel.

Met and HGF expression levels are coordinately regulated in the mammary gland. Studies in rat (14) and mouse (3) have shown that levels of both receptor and ligand are highest during adolescence, early pregnancy and involution-periods of ductal development and glandular reorganization-but expression is nearly undetectable during late pregnancy and lactation-periods of cellular differentiation. Several *in vitro* and *in vivo* experiments have examined the role of the HGF/Met signaling in the mammary gland. Treatment of a mammary epithelial cell line with HGF in a three-dimensional culture assay resulted in the development of branched ductal structures whereas treatment

with the EGFR ligand neuregulin led to the formation of alveolar structures (15). Whole, excised mammary fat pads (MFP) treated with HGF developed highly branched ductal trees whereas the untreated glands had a poorly developed ductal system and blocking HGF with antisense oligonucleotides prohibited ductal branching (16). A similar enhanced branching phenotype was observed in MFPs transplanted with mammary epithelial cells overexpressing HGF (17) and in transgenic mice overexpressing HGF under the control of the mammary-specific whey acidic protein promoter (18).

The consequences of loss of Met receptor signals in the mammary epithelium have not been studied by means other than through the use of blocking antisense oligonucleotides (16). Met (19) and HGF (20, 21) knock-out mice are embryonic lethal at approximately day E14.5 and both display the same phenotypes of placental, liver and diaphragmatic defects. Rescue of HGF-null mice by *in utero* injection of HGF is possible, allowing them to develop to term, but they die immediately after birth as a result of still incomplete diaphragm development (22). In none of these studies has development of the mammary epithelium been examined. However, as the majority of mammary development occurs post-natally, only changes in the mammary bud would be observed by day E15 and only a rudimentary ductal tree would be present at birth (review (23)).

In order to study the effects of loss of expression of the Met receptor on the development of the mammary ductal tree we have utilized a conditional knock-out model in which the exon encoding the ATP-binding site in the kinase domain is flanked by *loxP* sequences (Met^{fl}) (gift of Dr. Snorri Thorgeirsson) thus allowing its excision following expression of Cre recombinase (24). We have utilized two different techniques to generate Met-null mammary epithelial cells- 1) infection of primary mammary epithelial cells *in vitro* with adenovirus expressing Cre followed by transplantion back into cleared mammary fat pads and 2) crossing Met^{fl} mice with mice that express Cre recombinase under the control of the mammary-specific mouse mammary tumor virus promoter/enhancer (MMTV-Cre) (25). The Met^{fl} mice have been crossed with transgenic mice carrying a Cre-responsive β-galactosidase (β-gal) reporter (GTRosa26) (26), permiting us to determine in which cells Cre had been expressed, and thus Met receptor

knocked out, as visualized by β-gal expression. The transplantation assays failed to yield Met-null outgrowths, however, the Met^{fl}/Cre/Rosa mice have been more successful. Both $Met^{fl/+}$ and $Met^{fl/fl}$ mice display normal, unretarded mammary ductal development, but have fewer β-galactosidase positive cells than $Met^{+/+}$/Cre/Rosa mice, indicating either negative selection for the $Met^{fl/fl}$ cells or a failure of the $Met^{fl/fl}$ to proliferate. Through these experiments we have begun to examine the role of Met receptor signaling in mammary ductal development.

Materials and Methods

Transgenic Mice:

Floxed Met transgenic mice were a generous gift of Dr. Snorri Thorgeirsson (National Institutes of Health) (24). The mice were derived on a mixed background of 129SV/C57BL/6. Both the GTRosa26 and MMTV-Cre (gift of Dr. William Muller) (25) mice were on the FVB/N background. Background strain is important in mammary gland studies, particularly when using the MMTV promoter, as it has been shown to be methylated in strains other than FVB/N (27, 28). Thus, we carried out 6 backcrosses of the Met^{fl} mice to establish the genetic background of FVB/N.

Met^{fl} mice were crossed with GTRosa26 and subsequently with MMTV-Cre mice in order to generate triple transgenics in which Met deleted cells will stain blue with x-gal treatment as a result of the Cre-mediated GTRosa26 beta-galactosidase activation.

Genotyping and Quantification of Met Excision

Mice were genotyped for presence of the floxed allele, MMTV-Cre and GTRosa26. DNA was isolated from tail clippings. Screening for the presence of the floxed allele: F-flox- 5'-TTAGGCAATGAGGTGTCCCAC-3', R-flox-5'-CCAGGTGGC TTCAAATTCTAAGG-3' (anneal 61°C, product 380 bp if floxed allele, 300 bp if wt allele). Cre expression: F-Cre-5'-AGGTGTAGAGAAGGCACTTAGC-3'. R-Cre -5'-CTAATCGCCATCTTCCAGCAGG-3' (anneal 63°C, product 411 bp).

Isolation of Primary Mammary Epithelial Cells

Primary mammary epithelial cells were isolated from 10-12 week old mice as described (29) with slight modifications. Briefly, glands 2, 3, and 4 were minced by hand using scissors and razor blades rather than a McIlwin Tissue Chopper. Minced tissue was digested with collagenase/trypsin in DMEM for 1 hour at 37°C. Tissue was washed several times in DMEM/FBS and centrifuged at low speed in order to decrease the number of fibroblasts. Alpha-hemolysin was utilized to lyse contaminating red blood cells. Organoids were plated for 1.5 hours in DMEM/FBS to permit adhesion of fibroblasts. Organoids were washed in PBS/0.02% EDTA in order to loosen cell-cell

adherens junctions and centrifuged at high speed. Cells were subsequently incubated in calcium/magnesium-free medium for 15 minutes at 37°C. Following this incubation, cells were re-suspended in culture medium (1:1 DMEM:F12, 10% FBS, 5 μg/mL insulin, 10 ng/mL EGF, 10 ng/mL cholera toxin) and passed through an 80 μm filter before plating in 6 cm dishes at 2 dishes per mouse sacrificed.

Infection and Injection of Primary Mammary Epithelial Cells

Three days after plating, 80% confluent primary mammary epithelial cells were infected with adenovirus expressing Cre at an MOI of ~50 following a protocol described by Rijnkels and Rosen (30) (also at: http://www.bcm.edu/rosenlab/protocols/adeno.pdf). Two days following infection cells were lifted from the dish by trypsinization; trypsin was neutralized with DMEM/FBS; cells were washed twice times with PBS and one plate of cells was counted. Approximately $1x10^6$ cells were suspended in a total volume of 10-20 μL and kept on ice until injected using a Hamilton syringe. Recipients of the mammary epithelial cells were 3 week old FVB/N mice. Mice were anaesthetised and endogenous mammary epithelium excised from MFP 4, leaving behind the lymph node. Cells were injected distal to lymph node and permitted to expand for 8 weeks, at which point the recipients were sacrificed.

Beta-galactosidase Activity Staining-Whole Mounts and Histological Sections

Mammary fat pads (transplanted or Met^{fl}/Rosa/Cre) were spread on bottom of 6 well tissue culture plates and allowed to dry until adherent. Whole mount staining was carried out as described at http://mammary.nih.gov/tools/histological/ Histology/index.html, however, tissue was stained in carmine alum for only 3 hours rather than overnight. For paraffin-embedded sections, glands were processed as for whole mounts, however, following overnight staining with x-gal and washing in PBS MFPs were transferred to 70% ethanol and embedded in paraffin and sectioned according to standard protocols. Paraffin sections were counter stained with nuclear fast red. Plates of cells were stained following the same x-gal staining protocol except that 5mM ferric cyanide compounds were used instead of 30 mM. Images were acquired using Axiovision and a Zeiss Axiovert digital camera.

Immunoprecipitations and Immnoblotting

Mammary fat pads 2 and 3 were excised from transgenic mice and snap frozen in liquid nitrogen. Protein lysates were made by crushing frozen tissue and lysing in 1% Triton buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Lysates were cleared by centrifugation and 2 mg of lysates was used to immunoprecipitate murine Met utilizing a rabbit polyclonal antibody made to a C-terminal peptide of murine Met (31). Cultured primary mammary epithelial cells were also lysed in 1% Triton buffer. For immunoprecipitations, lysates were incubated with specific antibodies for 2 hours at 4°C with gentle mixing. Antibody-bound proteins were collected on protein A-Sepharose beads and washed three times in their respective lysis buffers.

Proteins, whether whole cell lysate or immunoprecipitations were boiled in laemmeli buffer with 10% DTT and resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to a nitrocellulose membrane. Membranes were blocked in 3% bovine serum albumin in TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM EDTA, 0.1% Tween 20) for 1 h and incubated with primary and secondary antibodies in TBST for 2 h and 1 h, respectively. Antibody-bound proteins were visualized using an ECL detection kit (Amersham Biosciences, Buckinghamshire, UK).

Antibodies used were: Met (B2), Actin- Santa Cruz Biotechnologies (Santa Cruz, CA) and α-tubulin-Sigma Aldrich(St. Louis, MO). Secondary antibodies were HRP-conjugated anti-goat, anti-mouse from Amersham Bioscience (Buckinghamshire, UK).

Quantification of Deleted Met

The Triton-insoluble fraction of the lysates was utilized for DNA extraction by overnight digestion at 56°C in buffer containing proteinase K (100 mM NaCl, 10 mM Tris-Cl pH 8.0, 25 mM EDTA pH 8.0, 0.5% SDS, 0.1 mg/mL proteinase K). DNA was purified by phenol-chloroform extraction and resuspended in water. The polymerase chain reaction for determining Met exon 16 excision was carried out using the following primers: F-excise- 5'-CAGCCGTCAGACAATGGCAC-3'. Reverse is R-flox as written above (anneal 61°C, product 650 bp).

Results

In vitro Infection of Met^{fl} Mammary Epithelial Cells with Adenovirus-Cre Results in Loss of Met Receptor Protein

The technique of *in vitro* infection of primary mammary epithelial cells with adenovirus-Cre followed by transplantation to a cleared MFP has been utilized by other groups (30). Using an MOI of 50, 60-80% of epithelial cells should be infected, and this was observed in stained plates of cells (fig. 1a). In order to demonstrate that our *in vitro* infections were efficiently inducing excision of Met exon 16 we performed the infection and assayed the primary mammary epithelial cells for deletion of Met at the DNA and protein level two days following infection.

Immunoprecipitation and immunoblot detection of Met protein demonstrated that infection resulted in dramatically reduced levels of Met receptor protein in both $Met^{fl/+}$ and $Met^{fl/fl}$ epithelial cells (Fig. 1c). Despite the floxed exon 16 being an in-frame deletion, loss of the exon results in decreased total protein levels as has been observed by others utilizing this model (24, 32). Additionally, PCR analysis of DNA isolated from the infected mammary epithelial cells also revealed efficient recombination of Met DNA resulting in the truncated genomic sequence (Fig. 1b).

Transplantation of $Met^{fl/fl}$ Knock-Out Cells Fails to Yield Outgrowths

Following confirmation of exon 16 excision from the Met^{fl} cells infected with adenovirus-Cre, we could determine whether or not the recombined $Met^{fl/fl}$ cells could generate a normal mammary ductal tree. Adenovirus-Cre infects stem cells, thus stem cells that have undergone recombination will yield blue outgrowths if the Rosa allele is also present (30). Following infection, $Met^{+/+}$/Rosa, $Met^{fl/+}$/Rosa, and $Met^{fl/fl}$/Rosa cells were injected into cleared MFP 4 of syngeneic FVB/N mice. Eight weeks following transplantation, MFP 4 was removed and stained for β-galactosidase activity. Epithelial outgrowths were present in 63% of the transplanted glands, however, β-gal activity was present in only ~10% of outgrowths and never throughout the entire gland (fig. 2). Additionally, those ducts that did display β-gal positivity frequently ended in malformed terminal end buds regardless of the genotype of the cells (fig. 3A) whereas the non-β-gal

outgrowths displayed normal ductal outgrowth (fig. 3B). The failure to form ductal outgrowths was not dependent on the presence or absence of expression of the Met receptor because even outgrowths containing GTRosa26-alone with wt Met failed to express β-gal upon staining.

MMTV-Cre/Met$^{fl/fl}$ Mice Display Normal Mammary Ductal Trees

As a result of the failure of the transplanted epithelial cells to form β-gal positive outgrowths, we crossed the Met^{fl}/Rosa mice with MMTV-Cre transgenic in order to knock out Met expression *in situ*. The MMTV promoter is able to drive Cre expression in both luminal epithelial cells as well as myoepithelial cells (33) and may be able to induce expression in the stem cell population of cap cells in the terminal end bud (33), so all ductal cell populations should undergo DNA recombination. Gross examination of ductal trees from 6 week old mice revealed that the Met$^{fl/+}$/Cre and Met$^{fl/fl}$/Cre mammary glands, although they displayed homogenous staining of ducts, were indistinguishable from those of the control mice (fig. 4). Additionally, the terminal end buds did not display any morphological differences amongst genotypes.

Met$^{fl/fl}$ Glands Do Not Express Lower Levels of Met Protein

In order to confirm the genotypes of the mice sacrificed and to ensure that Met^{fl} was undergoing recombination, we made protein lysates and DNA from MFP 2/3 of the mice that were used for whole mount and histological analysis. Utilizing primers that detect the presence of the floxed allele, the genotypes of the mice were confirmed. The presence of the excision product was also confirmed in MFP 2/3, with less recombined product present in Met$^{fl/+}$ than in Met$^{fl/fl}$ (fig. 5A). Despite these indications that the floxed allele had undergone recombination, immunoprecipitation and Western blotting of endogenous Met revealed that in contrast to the *in vitro* assays, the Met$^{fl/+}$ and Met$^{fl/fl}$ mice still expressed nearly equivalent amounts of Met protein as the wild type control mammary glands (fig. 5B). Previous studies have utilized the Met^{fl} mice for Met ablation in pancreatic islet cells (32) and hepatocytes (24) and both studies demonstrated loss of Met protein following Cre expression.

Cell type expressing Cre varies amongst genotypes

As a result of the lack of grossly visible differences and the failure to decrease Met protein levels, we examined histological sections of x-gal stained MFP 4 for differences in staining patterns, and thus Met knock-out, in the different cellular populations. Histological examination of x-gal/nuclear fast red stained MFP 4 revealed fewer x-gal stained cells in the $Met^{fl/+}$ and $Met^{fl/fl}$ glands than in the control glands. The Met wt glands showed strong staining of both luminal and myoepithelial cells (fig. 6A-C). $Met^{fl/+}$ and $Met^{fl/fl}$ glands contained fewer blue cells than Met wt but the $Met^{fl/+}$ glands did display staining in both the luminal and myoepithelial compartments but there was potentially failure of the cap cells to demonstrate expression of Cre-recombinase (Fig 6D-F) . Similarly, the $Met^{fl/fl}$ glands may have few cap cells staining blue, but strikingly, the majority of cells that had expressed Cre-recombinase were in the basal compartment, with few luminal cells staining (Fig 6. G-I), indicating that there is negative selection for $Met^{-/-}$ cells in the luminal compartment but the myoepithelial compartment can tolerate loss of Met.

Discussion

Mammary gland development is a complex process that requires appropriately timed and dosed signals between the stroma and the epithelium as well as from the endocrine glands to supply hormones. Receptor tyrosine kinase signaling plays an important role in the development of the mammary gland as demonstrated by the developmental defects seen in knock-out animals of colony stimulating factor 1 and its receptor (34), insulin-like growth factor-1 (35), and the ligand for the epidermal growth factor receptor- amphiregulin (36). The role of the Met RTK in mammary gland development has not been examined by knock-out studies as Met and HGF knock-out mice are embryonic lethal (19-21). High doses of HGF in the mammary gland lead to the development of a highly branched ductal tree (17, 18) and one report stated that *in vitro* blocking of Met signaling in the mammary gland resulted in a poorly branched tree (16).

Our assays to examine the role of ablation of Met receptor signaling on the development of the mammary gland initially were hampered by our model system. *In vitro* infection of primary mammary epithelial cells from wt, $Met^{fl/+}$ or $Met^{fl/fl}$ mice with adenovirus expressing Cre recombinase failed to yield ductal outgrowths that demonstrated expression of Cre and thus had not lost Met expression (fig 2). Cre recombinase, although a bacteriophage product that is specific for *loxP* sequences that are present in the prokaryotic genome, may possess some ability to induce DNA recombination in prokaryotic cells. Several papers have recently been published that demonstrated that cells expressing high levels of Cre subsequently fail to proliferate (37-39). Examination of DNA from the Cre-expressing cells revealed a high frequency of DNA damage and chromosomal breaks (37, 38). In order to overcome the difficulties associated with sustained high levels of Cre expression, several groups have designed self-excising vectors by surrounding part of the Cre coding sequence with *loxP* sequences. In this way, once Cre is expressed, the Cre coding sequence is removed, thereby inactivating itself, decreasing the cellular exposure to Cre and maintaining the integrity of the cellular DNA (38-40). It is possible that the adenovirus-Cre utilized for our *in vitro* infections induced excessively high levels of Cre, thereby damaging cellular DNA sufficiently to inhibit mammary epithelial cell proliferation, and leaving only the uninfected cells to repopulate the MFP. Additionally, if Cre expression did induce DNA

damage, this may explain the development of the abnormal β-galactosidase-expressing terminal end buds (fig. 3).

The MMTV-Cre transgenic mice have been used extensively, without incident, to induce mammary-specific knock-out of a number of proteins (25, 41, 42). The MMTV promoter perhaps induces lower levels of Cre expression than did the adenovirus-Cre, thereby limiting the amount of DNA damage that might occur. The grossly normal mammary glands we observed in the MMTV-Cre/Rosa mice indicates that the Cre-expressing cells were functionally normal and had no proliferative defects. The normal ductal development of the Met^{fl} glands was unexpected, particularly in light of the experiment that demonstrated that treatment of a mammary fat pad with HGF antisense oligonucleotide resulted in a failure of the ductal tree to develop branches (16). An important difference between these two Met knock-out assays is the duration of treatment with the agent that induces loss of Met signal. The HGF antisense oligonucleotides were applied for only 4 days, whereas MMTV-Cre expression is initiated as early as 3 weeks of age (William Muller, personal communication) and persists throughout the development of the mammary gland, thus the MMTV-Cre glands may continuously adapt to loss of Met expression in individual cells. Additionally, as was demonstrated by the immunoblot, Met protein levels are not decreased in the $Met^{fl/fl}$ mammary glands, despite the occurrence of DNA recombination (fig. 5). Again, this indicates either compensation by un-recombined cells to overexpress Met protein or failure of the Met knock-out cells to populate the mammary gland.

The β-gal staining pattern observed in the paraffin sections from the Met^{fl} mice may provide insight into why the $Met^{fl/fl}$ gland could develop normally. First, there are fewer cells in the Met^{fl} glands that have undergone recombination (fig. 6). The β-gal staining pattern observed in the Cre/Rosa/wt Met mice agrees with the strong staining pattern seen by others using the MMTV-Cre transgenics in which 50-100% of ductal cells expressed Cre (41, 42), whereas ducts from the $Met^{fl/fl}$ mice rarely contained 100% stained cells and tended to be more towards 50% staining. Second, the cellular population in the $Met^{fl/fl}$ glands that displayed a high percentage of β-gal staining cells appears to be the myoepithelial population. This observation must be confirmed by immunohistochemical (IHC) staining of paraffin sections with markers of myoepithelial

cells and luminal cells to quantify the percentage of cells of each population that is β-gal positive.

Niranjan et al. performed *in vitro* experiments examining the mitogenic effects of HGF on luminal epithelial and myoepithelial cells and found that myoepithelial cells did not display a proliferative response to treatment with HGF whereas luminal epithelial cells did (3). Thus, in the $Met^{fl/fl}$ mice the myoepithelial cells may be able to tolerate loss of Met expression and still proliferate to contribute to the development of a morphologically normal mammary gland. The luminal epithelial cells may be able to tolerate decreased levels of Met expression as seen by the β-gal positivity of luminal cells in the $Met^{fl/+}$ mammary gland, however Met-null cells may not be able to proliferate, thus leaving scattered single cells throughout the ducts. To test this, we will be performing immunohistochemical staining to determine if luminal cells from the $Met^{fl/fl}$ gland that express Cre are proliferating. Alternatively, recombined luminal cells may undergo apoptosis, and the developing mammary gland is sufficiently adaptable to compensate for the continuous loss of luminal cells, thereby avoiding a delay in ductal outgrowth. This could be assayed by performing IHC for apoptosing cells to determine if the β-gal positive luminal cells are dying.

An alternate explanation could be that progenitors committed to become myoepithelial cells tolerate Met loss better than luminal progenitors. Thus, when Met knock-out occurs, the myoepithelial progenitors behave normally, but luminal progenitors fail to contribute cells to the expanding ductal tree. Again, this could be assayed by examining which cell populations are proliferating, however this would not necessarily identify the myoepithelial progenitor as the IHC markers of this population are still unknown.

The role of Met expression in the stem/progenitor population should be explored further. Wagner et al. observed β-gal staining in the cap cells of the terminal end buds (33), however, he utilized a different MMTV-Cre construct. Although at this time the sample size is small, it appears as though loss of Met may not be tolerated in the stem cell compartment as indicated by the non-staining cap cells of the terminal end bud (fig. 6). If loss of Met expression induces stem cell death or senescence, there may be compensatory

hyperproliferation in that compartment by cells that have not lost Met expression. This can easily be assayed by IHC for markers of proliferation.

We have shown the Met receptor signaling is important for mammary gland development as demonstrated by the paucity of Met-null cells. The unparalleled ability of the body to adapt has thus-far foiled our attempts at generating a complete Met receptor knock-out in the mammary gland, perhaps indicating how critical Met is for proper mammary gland development.

References

1. Brinkmann, V., Foroutan, H., Sachs, M., Weidner, K. M., and Birchmeier, W. Hepatocyte growth factor/scatter factor induces a variety of tissue-specific morphogenic programs in epithelial cells. J Cell Biol, *131:* 1573-1586, 1995.
2. Pollack, A. L., Runyan, R. B., and Mostov, K. E. Morphogenetic mechanisms of epithelial tubulogenesis: MDCK cell polarity is transiently rearranged without loss of cell-cell contact during scatter factor/hepatocyte growth factor-induced tubulogenesis. Dev Biol, *204:* 64-79, 1998.
3. Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., and Kamalati, T. HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. Development, *121:* 2897-2908, 1995.
4. Di Renzo, M. F., Narsimhan, R. P., Olivero, M., Bretti, S., Giordano, S., Medico, E., Gaglia, P., Zara, P., and Comoglio, P. M. Expression of the Met/HGF receptor in normal and neoplastic human tissues. Oncogene, *6:* 1997-2003, 1991.
5. Tajima, H., Higuchi, O., Mizuno, K., and Nakamura, T. Tissue distribution of hepatocyte growth factor receptor and its exclusive down-regulation in a regenerating organ after injury. J Biochem (Tokyo), *111:* 401-406, 1992.
6. Sonnenberg, E., Meyer, D., Weidner, K. M., and Birchmeier, C. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. J Cell Biol, *123:* 223-235, 1993.
7. Gu, H. and Neel, B. G. The "Gab" in signal transduction. Trends Cell Biol, *13:* 122-130, 2003.
8. Maroun, C. R., Holgado-Madruga, M., Royal, I., Naujokas, M. A., Fournier, T. M., Wong, A. J., and Park, M. The Gab1 PH domain is required for localization of Gab1 at sites of cell-cell contact and epithelial morphogenesis downstream from the met receptor tyrosine kinase. Mol Cell Biol, *19:* 1784-1799, 1999.
9. Gual, P., Giordano, S., Williams, T. A., Rocchi, S., Van Obberghen, E., and Comoglio, P. M. Sustained recruitment of phospholipase C-gamma to Gab1 is required for HGF-induced branching tubulogenesis. Oncogene, *19:* 1509-1518, 2000.
10. Woolf, A. S., Kolatsi-Joannou, M., Hardman, P., Andermarcher, E., Moorby, C., Fine, L. G., Jat, P. S., Noble, M. D., and Gherardi, E. Roles of hepatocyte growth factor/scatter factor and the met receptor in the early development of the metanephros. J Cell Biol, *128:* 171-184, 1995.
11. Montesano, R., Soriano, J. V., Fialka, I., and Orci, L. Isolation of EpH4 mammary epithelial cell subpopulations which differ in their morphogenetic properties. In Vitro Cell Dev Biol Anim, *34:* 468-477, 1998.
12. Soriano, J. V., Pepper, M. S., Nakamura, T., Orci, L., and Montesano, R. Hepatocyte growth factor stimulates extensive development of branching duct-like structures by cloned mammary gland epithelial cells. J Cell Sci, *108 (Pt 2):* 413-430, 1995.
13. Montesano, R., Soriano, J. V., Malinda, K. M., Ponce, M. L., Bafico, A., Kleinman, H. K., Bottaro, D. P., and Aaronson, S. A. Differential effects of

hepatocyte growth factor isoforms on epithelial and endothelial tubulogenesis. Cell Growth Differ, *9:* 355-365, 1998.

14. Pepper, M. S., Soriano, J. V., Menoud, P. A., Sappino, A. P., Orci, L., and Montesano, R. Modulation of hepatocyte growth factor and c-met in the rat mammary gland during pregnancy, lactation, and involution. Exp Cell Res, *219:* 204-210, 1995.
15. Niemann, C., Brinkmann, V., Spitzer, E., Hartmann, G., Sachs, M., Naundorf, H., and Birchmeier, W. Reconstitution of mammary gland development in vitro: requirement of c-met and c-erbB2 signaling for branching and alveolar morphogenesis. J Cell Biol, *143:* 533-545, 1998.
16. Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K. M., Birchmeier, C., and Birchmeier, W. Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J Cell Biol, *131:* 215-226, 1995.
17. Yant, J., Buluwela, L., Niranjan, B., Gusterson, B., and Kamalati, T. In vivo effects of hepatocyte growth factor/scatter factor on mouse mammary gland development. Exp Cell Res, *241:* 476-481, 1998.
18. Gallego, M. I., Bierie, B., and Hennighausen, L. Targeted expression of HGF/SF in mouse mammary epithelium leads to metastatic adenosquamous carcinomas through the activation of multiple signal transduction pathways. Oncogene, *22:* 8498-8508, 2003.
19. Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., and Birchmeier, C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. Nature, *376:* 768-771, 1995.
20. Uehara, Y., Minowa, O., Mori, C., Shiota, K., Kuno, J., Noda, T., and Kitamura, N. Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. Nature, *373:* 702-705, 1995.
21. Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. Scatter factor/hepatocyte growth factor is essential for liver development. Nature, *373:* 699-702, 1995.
22. Uehara, Y., Mori, C., Noda, T., Shiota, K., and Kitamura, N. Rescue of embryonic lethality in hepatocyte growth factor/scatter factor knockout mice. Genesis, *27:* 99-103, 2000.
23. Dunbar, M. E. and Wysolmerski, J. J. Mammary ductal and alveolar development: lesson learned from genetically manipulated mice. Microsc Res Tech, *52:* 163-170, 2001.
24. Huh, C. G., Factor, V. M., Sanchez, A., Uchida, K., Conner, E. A., and Thorgeirsson, S. S. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. Proc Natl Acad Sci U S A, *101:* 4477-4482, 2004.
25. Andrechek, E. R., Hardy, W. R., Siegel, P. M., Rudnicki, M. A., Cardiff, R. D., and Muller, W. J. Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. Proc Natl Acad Sci U S A, *97:* 3444-3449, 2000.
26. Soriano, P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet, *21:* 70-71, 1999.

27. Chaillet, J. R., Bader, D. S., and Leder, P. Regulation of genomic imprinting by gametic and embryonic processes. Genes Dev, *9:* 1177-1187, 1995.
28. Zhou, H., Chen, W. D., Qin, X., Lee, K., Liu, L., Markowitz, S. D., and Gerson, S. L. MMTV promoter hypomethylation is linked to spontaneous and MNU associated c-neu expression and mammary carcinogenesis in MMTV c-neu transgenic mice. Oncogene, *20:* 6009-6017, 2001.
29. Smalley, M. J., Titley, J., and O'Hare, M. J. Clonal characterization of mouse mammary luminal epithelial and myoepithelial cells separated by fluorescence-activated cell sorting. In Vitro Cell Dev Biol Anim, *34:* 711-721, 1998.
30. Rijnkels, M. and Rosen, J. M. Adenovirus-Cre-mediated recombination in mammary epithelial early progenitor cells. J Cell Sci, *114:* 3147-3153, 2001.
31. Iyer, A., Kmiecik, T. E., Park, M., Daar, I., Blair, D., Dunn, K. J., Sutrave, P., Ihle, J. N., Bodescot, M., and Vande Woude, G. F. Structure, tissue-specific expression, and transforming activity of the mouse met protooncogene. Cell Growth Differ, *1:* 87-95, 1990.
32. Dai, C., Huh, C. G., Thorgeirsson, S. S., and Liu, Y. Beta-cell-specific ablation of the hepatocyte growth factor receptor results in reduced islet size, impaired insulin secretion, and glucose intolerance. Am J Pathol, *167:* 429-436, 2005.
33. Wagner, K. U., McAllister, K., Ward, T., Davis, B., Wiseman, R., and Hennighausen, L. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res, *10:* 545-553, 2001.
34. Sapi, E. The role of CSF-1 in normal physiology of mammary gland and breast cancer: an update. Exp Biol Med (Maywood), *229:* 1-11, 2004.
35. Ruan, W. and Kleinberg, D. L. Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. Endocrinology, *140:* 5075-5081, 1999.
36. Luetteke, N. C., Qiu, T. H., Fenton, S. E., Troyer, K. L., Riedel, R. F., Chang, A., and Lee, D. C. Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. Development, *126:* 2739-2750, 1999.
37. Loonstra, A., Vooijs, M., Beverloo, H. B., Allak, B. A., van Drunen, E., Kanaar, R., Berns, A., and Jonkers, J. Growth inhibition and DNA damage induced by Cre recombinase in mammalian cells. Proc Natl Acad Sci U S A, *98:* 9209-9214, 2001.
38. Silver, D. P. and Livingston, D. M. Self-excising retroviral vectors encoding the Cre recombinase overcome Cre-mediated cellular toxicity. Mol Cell, *8:* 233-243, 2001.
39. Pfeifer, A., Brandon, E. P., Kootstra, N., Gage, F. H., and Verma, I. M. Delivery of the Cre recombinase by a self-deleting lentiviral vector: efficient gene targeting in vivo. Proc Natl Acad Sci U S A, *98:* 11450-11455, 2001.
40. Mahonen, A. J., Airenne, K. J., Lind, M. M., Lesch, H. P., and Yla-Herttuala, S. Optimized self-excising Cre-expression cassette for mammalian cells. Biochem Biophys Res Commun, *320:* 366-371, 2004.

41. Andrechek, E. R., White, D., and Muller, W. J. Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth. Oncogene, *24:* 932-937, 2005.
42. White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U., and Muller, W. J. Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell, *6:* 159-170, 2004.

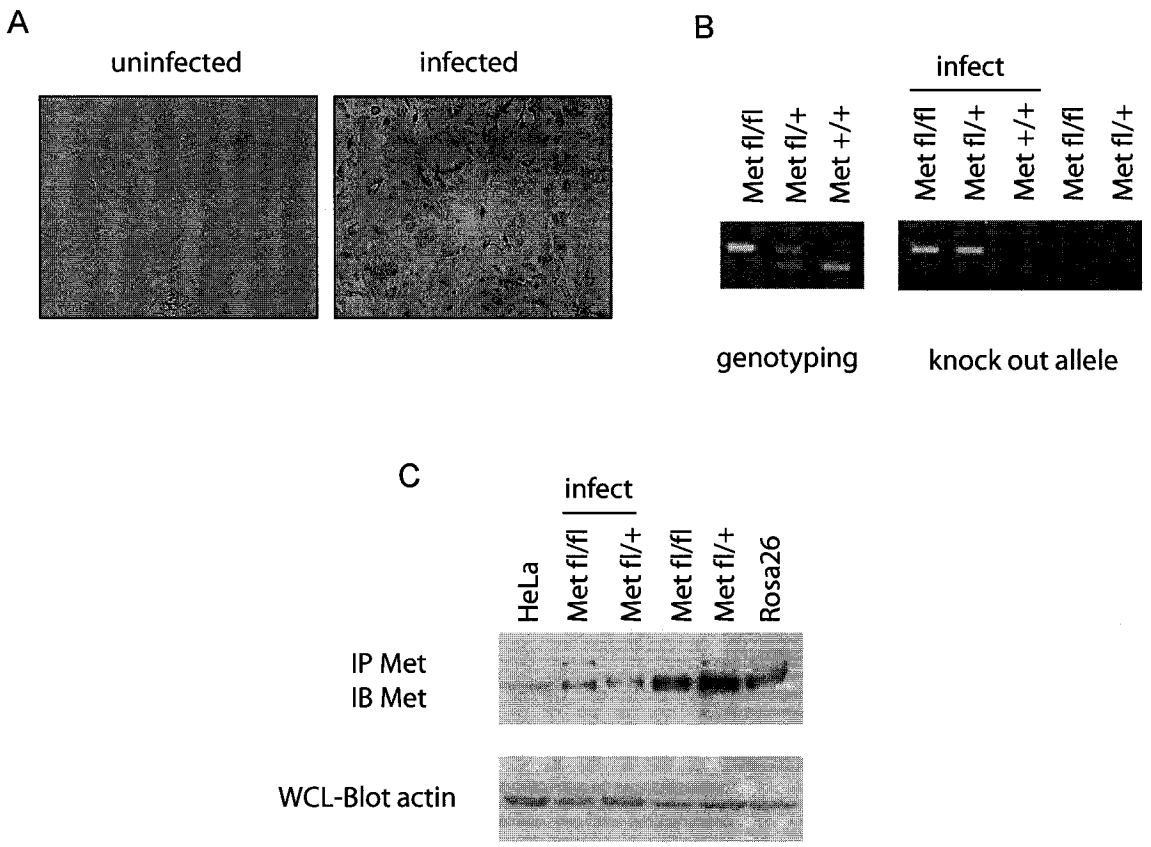

Figure 1. Mammary epithelial cells isolated from mice of the indicated genotypes infected in vitro with adenovirus-Cre results in efficient DNA recombination. A. Met^{fl}/Rosa cells in culture display DNA recombination by staining for β-galactosidase activity. B. Genotyping of cells in culture display homozygous, heterozygous, and wild type genotypes. Following infection a recombined DNA product is detected by PCR. C. Immunoblot for murine Met. Two mg of protein was immunoprecipated from infected and uninfected cellular lysates. Expression of Cre recombinase induces excision of exon 16 as well as decreased protein levels. Actin was used as a blotting control.

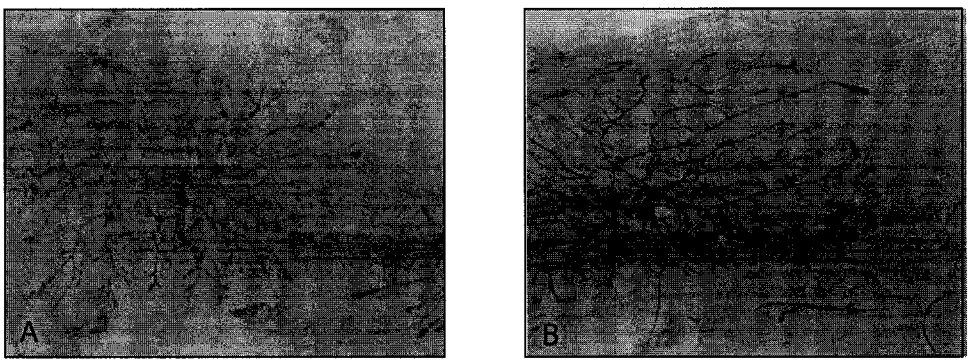

Figure 2. Stained and un-stained outgrowths of *in vitro* adenovirus-cre Infected Met^{fl} mammary epithelial cells. Transplanted primary mammary epithelial cells induced to express β-galactosidase by infection with adenovirus-Cre. Images taken at 2.5x magnification. Epithelial outgrowths either did not express β-galactosidase (A) or had only partial expression (B) as visualized by staining with x-gal.

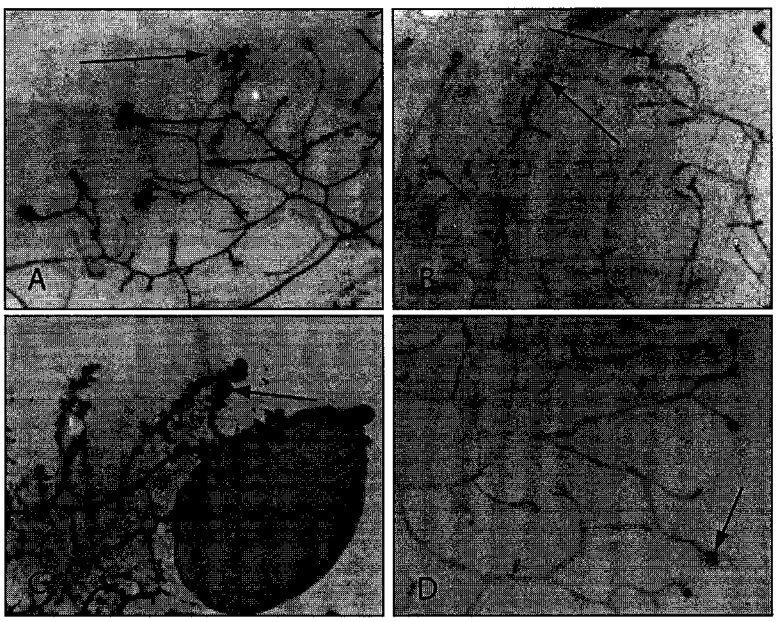

Figure 3. Abnormal terminal end buds develop in outgrowths that express β-galactosidase. All images at 5x original magnification A.-B. Transplanted Met wt-Rosa26 cells. C.-D. Transplanted $Met^{fl/+}$ cells. A. ,C.-Cells expressing β-galactosidase develop deformed terminal end buds. (arrow) B., D.-Terminal end buds appear normal (arrow) when no b-galactosidase expression is detected.

Figure 4. Met^{fl}-Cre mammary glands develop normally. X-gal stained whole mounts of mammary fat pad 4 from Met wt-Rosa (A), $Met^{fl/+}$ (B), and $Met^{fl/fl}$ (C) mice at 6 weeks of age. Images taken at 0.6x original magnification.

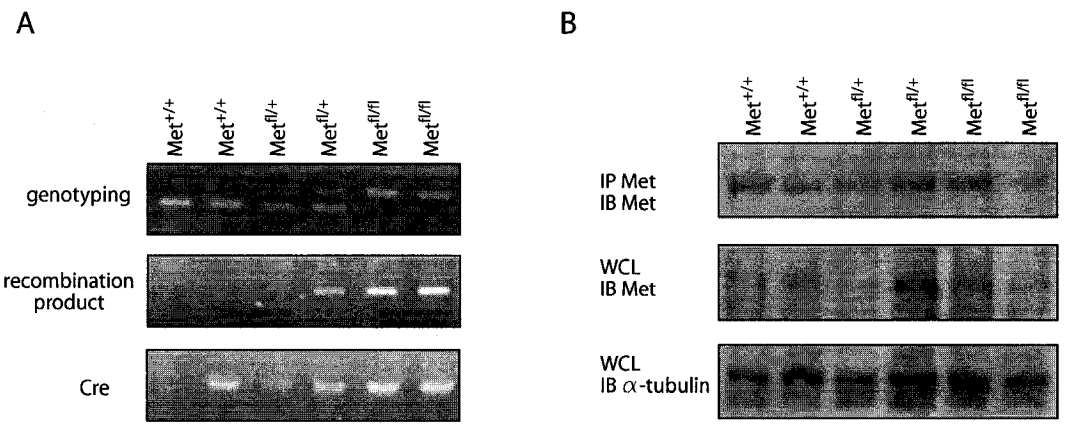

Figure 5. Met^{fl}-Cre mice retain normal Met protein levels. A. Genomic DNA extracted from the mammary fat pad of Met^{fl}-Cre mice was amplified using primers specific for (top) presence of floxed allele, (middle) recombined DNA, (bottom) Cre recombinase. B. Mammary fat pads were crushed and lysed in 1% Triton buffer. 2 mg of whole cell lysate was immunoprecipitated with α-Met antibody, separated by SDS-PAGE, and blotted for Met. 40 μg of whole cell lysate was separated by SDS-PAGE and blotted for Met and α-tubulin (loading control).

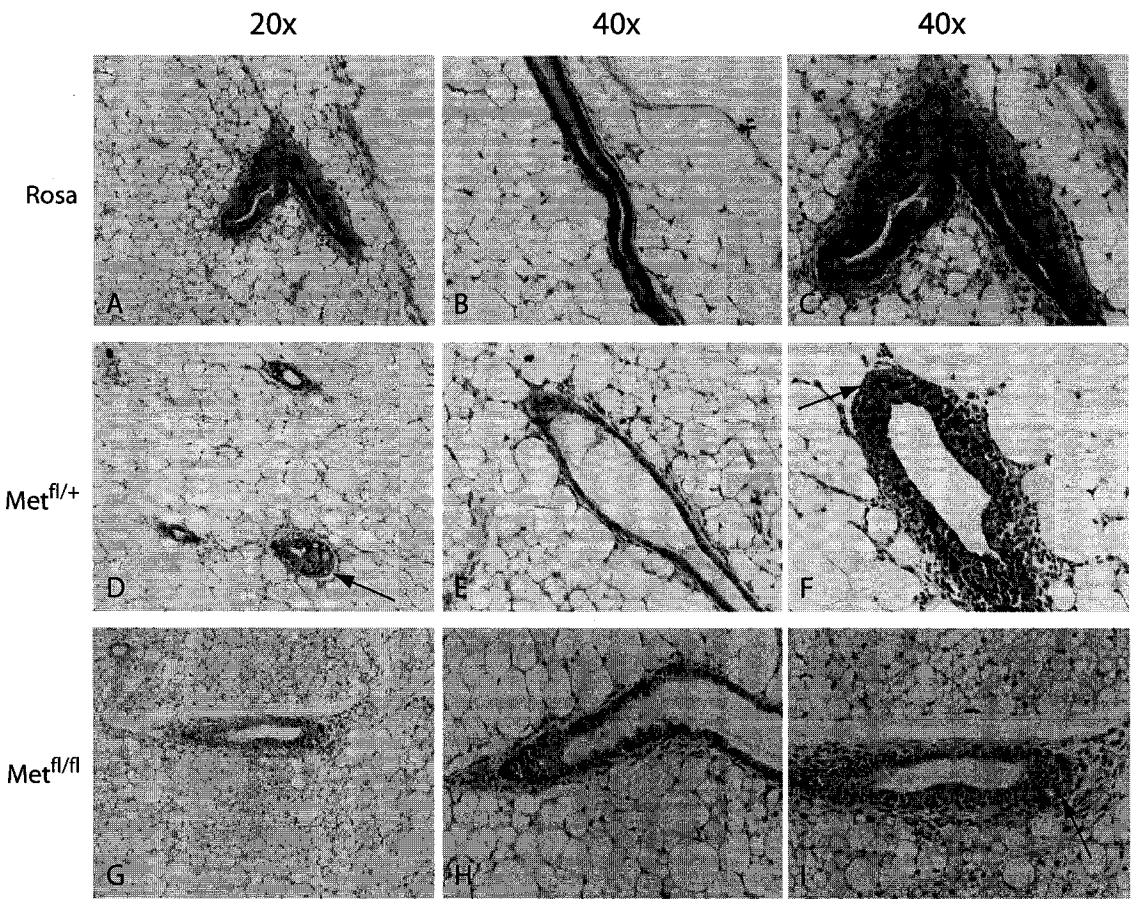

Figure 6. Met^{fl}-Cre ducts contain fewer cells that have undergone DNA recombination than do $Met^{+/+}$ ducts. Met wt glands stain darkly blue with nearly every cell expressing β-galactosidase (A-C). $Met^{fl/+}$ glands (D-F) contain many stained cells, but it appears that cap cells (arrow in F and D) are not stained. $Met^{fl/fl}$ glands contain fewer stained cells and also may have non-staining cap cells (arrow in I). Magnifications are 20x (A,D,G) and 40x (B,C,E,F,H,I).

Chapter 5

General Discussion

When I began these investigations, no animal models had examined the role of the Met receptor with naturally occurring mutations in the development of breast cancer. Nor had the effects of loss of Met receptor signaling on mammary gland development been studied. In this thesis I have characterised transgenic mice expressing a mutant Met receptor in the mammary epithelium under the control of the mouse mammary tumor virus promoter/enhancer (MMTV) and highlighted the importance of receptor ubiquitination in the control of Met receptor signaling. I have described the phenotypes of the mammary tumors resulting from the Met transgenic mice and demonstrated the presence of both luminal and basal cells in the tumors. I have also begun to examine the effects of loss of Met receptor signaling on the development of the mammary gland.

Loss of Ubiquitination of the Met Receptor Has an Additive Effect on Transformation When Combined with an Activating Mutation

Little was known about the role of the Y1003F juxtamembrane (JM) mutation compared to the M1250T activating mutation present within the catalytic domain. Tyrosine 1003 was initially found to have a biological role in 1995 when Weidner et al. substituted in a phenylalanine at that position and found that the altered receptor could induce a fibroblastic change in epithelial cells even in the presence of mutations affecting the multisubstrate binding sites of the Met receptor (1). From those studies it was concluded that Y1003 was involved in receptor negative regulation and substitution resulted in gain of function. Subsequent studies in our lab revealed that Y1003 is the binding site for the tyrosine kinase binding domain of the ubiquitin ligase Cbl, and the Y1003F substitution results in decreased receptor ubiquitination and prolonged downstream signaling (2). Despite the decreased receptor ubiquitination, the Met-Y1003F receptor still internalizes at the same rate as that of the wt receptor but is not efficiently degraded, hence it generates prolonged signals following ligand stimulation (3).

The importance of ubiquitination in regulating growth factor receptor signaling is demonstrated when decreased receptor ubiquitination induces cellular transformation. A number of studies of the epidermal growth factor receptor (EGFR)

family members have revealed that Cbl-mediated downregulation is critical for keeping the mitogenic signals downstream of the receptor in check. Expression of EGFR in which the Cbl-binding site has been mutated generates an enhanced mitogenic effect following ligand stimulation (4). The EGFR family member ErbB2 often forms heterodimers with EGFR but ErbB2 is resistant to Cbl-mediated degradation and confers this resistance on EGFR when part of a heterodimer (5). Additionally, sequestration of Cbl away from EGFR results in prolonged receptor signaling and cellular transformation (6) as seen with the Met-Y1003F. Thus, by these numerous mechanisms, loss of EGFR ubiquitination induces a stronger mitogenic effect and cellular transformation.

The JM domain of receptor tyrosine kinases is believed to be involved in generalized receptor negative regulation but the mechanism by which it performs this role varies between receptors. Studies of the Ephrin RTK have demonstrated that following dimerization, tyrosine residues in the JM domain must be phosphorylated preceding kinase activation (7). Structure studies have indicated that in its unphosphorylated form, the Ephrin JM domain interferes with the orientation of the domain involved in phosphoryl group transfer from ATP, thus JM domain phosphorylation is required for kinase activity (8). The JM domain of the Muscle-Specific Kinase is believed to inhibit kinase activity by binding a second protein that prevents kinase activation (9). Members of the Platelet Derived Growth Factor Receptor (PDGFR) family have been shown to have direct JM domain/kinase domain interactions, keeping the kinase domain in an inactive conformation (10). Phosphorylation of tyrosine residues within the JM domain of the PDGFR family results in a change in orientation of the JM domain, allowing the kinase domain to adopt an active conformation (10, 11). In the Met receptor, tyrosine 1003 provides a binding site for a ubiquitin ligase (12), thereby supplying a means of receptor regulation, but this does not exclude the JM domain from having additional negative regulatory functions based on structural changes.

One particularly well-studied RTK with JM mutations is the Kit receptor which is overexpressed in 90% of gastrointestinal stromal cell tumors (GISTs) (13). Mutations in the JM domain of Kit that disrupt the inhibitory α-helical structure of the

JM is present in the majority of these tumors (14). The α-helical structure directly interacts with the ATP-binding site of the kinase domain, thereby inhibiting kinase activity (15). Interestingly, the activated form of Kit, resulting from the mutant JM, can be inhibited by the kinase inhibitor STI571 (Gleevec) (16), a drug that was initially developed as an inhibitor of the common chronic myelogenous leukemia translocation product, Bcr-Abl (17). Treatment of GIST patients has been quite successful with up to 90% of patients treated experiencing symptom relief (18). This demonstrates the importance of identifying RTK JM mutations and provides hope for the potential to specifically treat the resulting cancers.

The JM domain of the Met receptor has not yet been crystallized, but there are several studies that suggest that it functions as more than simply a binding site for a ubiquitin ligase. Samples from gastric cancer led to the isolation of a Met receptor carrying a P1009S mutation in the JM domain (19). NIH3T3 cells expressing Met-P1009S grew in soft agar, but as was observed in my Met-Y1003F soft agar assay, formed much smaller colonies than the Met M1250T. Additionally, following HGF stimulation, Met-P1009S, like Met-Y1003F displayed prolonged receptor phosphorylation (19). Alanine-scanning mutagenesis performed in our lab indicated that P1009 is not involved in Cbl binding therefore, the role of P1009S in generating an oncogenic Met receptor may be different from that of Y1003F (12).

A recent study of human lung cancer samples found a sample that contained a deletion of the entirety of exon 14 which codes for the majority of the JM domain including Y1003 (20). Comparison of the proliferative enhancement Rat1 cells expressing Met-Y1003F or MetΔEx14 revealed that both mutations yielded the same increase in proliferation over wt or control vector, however, comparison of *in vivo* transformation efficiency was not performed as Met-Y1003F was omitted from the tumorigenesis assay (20). These studies of the transforming ability of the JM mutations indicate the complex role of the Met JM domain which may involve both structural changes and ubiquitination for downregulation of receptor signaling.

The M1250T mutation has been identified as an activating mutation in a number of studies (21, 22), however, assays in our lab have not indicated that the receptor has any greater kinase activity than the wt receptor (Lina Mussalam-

unpublished results). Recent studies have suggested that the M1250T mutation results in a change in Met substrate specificity (22, 23) and may provide a binding site for the transcription factor Stat3 (24). I was unable to co-precipitate Stat3 with CSF-Met-M1250T nor did I observe an increase Stat3 phosphorylation in Met-M1250T lysates when compared with Met-wt or Met-Y1003F (data not shown). The failure to observe strong Stat3 phosphorylation downstream of Met-M1250T may be a function of the cell lines used. I utilized the mammary epithelial cell lines EpH4 and T47D whereas Yuan et al. used transfected NIH3T3 and 293T cells (24).

Although elevated kinase activity was not observed, Met-M1250T expressed in Rat1 fibroblasts did induce focus formation as well as growth in soft agar, but notably, the M1250T mutation showed significant cooperation with the Y1003F substitution both *in vitro* and *in vivo* (fig. 2-3). In soft agar, Y1003F was able to induce limited proliferation of Rat1 fibroblasts, whereas the M1250T mutation could generate larger colonies. The Y1003F/M1250T colonies displayed a phenotype intermediate between the two individual mutants; the limited proliferation was observed in most colonies but a higher percentage of colonies was able to attain the larger diameter (fig. 2-3).

The injections of EpH4 Met variants into nude mice revealed another aspect of the transforming ability of the Met variants. The appearance of the Met-Y1003F/M1250T tumors at the same time as the Tpr-Met tumors was unexpected. In focus forming assays Tpr-Met is significantly more transforming than Met-wt or Met-Y100F (2). The simultaneous development of the Met-YF/MT and Tpr-Met tumors indicates the potency of the Met-YF/MT variant. Clearly, the soft agar and focus-forming assays should be repeated, including Tpr-Met as a positive control, as it would be interesting to compare the transforming ability of Met-M1250T and Met-YF/MT with Tpr-Met in these assays. The signals induced by the two variants may be similar because Tpr-Met is a cytoplasmic protein (25) and the Met-YF/MT receptor is not efficiently degraded, indicating that signaling could occur from the endosomal compartment, perhaps recruiting different signaling proteins than would be utilized at the plasma membrane (3). Alternatively, it may not be the location, but

rather the duration of the signal (fig. 2-2), as a result of inefficient degradation of both Tpr-Met and Met-YF/MT, that results in the similar abilities to transform.

The most dramatic difference in activities between the receptor variants is seen in the transgenic mice where mice expressing either of the single mutants developed tumors with long latency and low (or zero) penetrance whereas the Met-YF/MT transgenics displayed an average penetrance of 44% and a latency of 441 days (table 1-1). This could be a result of additive activating ability, or the difference in signal localization, or simply the duration of signaling. IHC for Met in both Met-M1250T and Met-YF/MT tumors did not indicate membranous localization of either receptor variant, however, high levels of expression and continuous signaling may make it difficult to observe a population at the membrane. Additionally, as there is no difference in internalization, perhaps a phospho-specific antibody such as those to Y1234/35 in the activation loop or Y1349/56 in the C-terminus would provide a better indication of where large pools of activated receptor lie.

Expression of a Dysregulated Met Receptor in the Mammary Epithelium Induces Mammary Tumorigenesis

The Met receptor tyrosine kinase is expressed at elevated levels when compared with normal tissue in 15-20% of human breast cancers (26-28). This overexpression is associated with decreased survival and a shorter disease-free interval in both lymph node positive and negative patients (26, 29-31). Elevated serum levels of HGF in women with breast cancer is associated with higher grade invasive tumors (32-35) and is associated with a shorter time to relapse following surgery (36). A number of Met-related transgenic models have been generated and they all develop mammary adenocarcinomas, albeit with different latencies and penetrances, however, none has specifically targeted expression of a dysregulated Met receptor to the mammary epithelium.

The Met receptor-related transgenic mice that have been generated include MT-Tpr-Met (37), MT-Met M1250T (38), and WAP-HGF (39). The WAP-HGF mice had the highest incidence of tumors, at 89% of multiparous mice (39), but the other models had more modest incidences from 25-60% of multiparous mice.

Between the two lines of Met-Y1003F/M1250T mice, I observed an average incidence of 40% by 600 days, including the non-exogenous Met expressing tumors. The incidence in the Met-M1250T lines is lower, and it will be necessary to increase the number of multiparous mice in order to obtain more tumor-bearing mice and better estimate the penetrance of tumor development. Other Met transgenic models had shorter latencies- 6 months for MT-Tpr-Met (37), less than 1 year for WAP-HGF (39) and the MT-Met M1250T mouse developed a mammary tumor at 10 months (38). The difference in latencies may be due to the differences in the activities of the promoters used to drive expression of the transgene or differences in the mammary cell populations in which they are expressed.

The latency of the MMTV-Met tumors necessitates caution when quantifying the tumors, because FVB/N mice frequently develop mammary adenocarcinomas at 18 months (550 days) and older (40, 41). This is exemplified by our FVB/N cohort in which 19% of multiparous mice developed tumors, but at an average age of greater than 20 months (600 days). A common cause of mammary tumors in the FVB/N background is pituitary adenoma-it induces proliferation and lactational differentiation of the mammary epithelium as a result of excess prolactin production (41). In our colony, only one of the mice was found to have an overt pituitary adenoma, potentially driving tumorigenesis, however, only mice with a blatant lactational phenotype had their pituitary glands examined. Excessive prolactin also results in the development of squamous nodules with an inflammatory infiltrate (42),

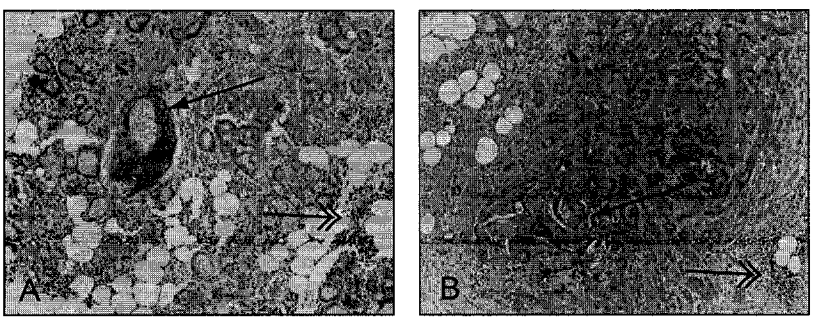

Figure 5-1. Squamous metaplasia in aged control mice and Met-M1250T tumors. Both images at 20x original magnification. A. Aged control mouse. B. Met-M1250T tumor. Single headed arrow-squamous metaplasia and keratin deposition. Double headed arrow-infiltrating inflammatory cells, they are also scattered throughout the stroma.

a common finding in many of our mice 600 days and older (fig. 5-1). Squamous nodules were also found in older mice from a separate colony that did not exhibit increased serum prolactin (40), suggesting that inflammatory squamous nodules may simply be a common finding in elderly mice. Squamous metaplasia was also found in the Met-M1250T tumors (fig. 3-4F, 5-1), and these tumors appeared with an average latency of 547 days. Thus determining whether or not the MMTV-Met tumors expressed exogenous Met protein became critical.

Determining expression of exogenous Met protein was facilitated by the use of the chimeric receptor and antibodies that bind only human Met. Expression of murine Met was not enhanced in the MMTV-Met tumors (fig. 3-2B), however, several tumors arose in both transgenic and control mice that did show overexpression of endogenous Met protein, but not exogenous Met protein (data not shown). Assaying exogenous Met protein expression could have been problematic if MMTV-Met expression had resulted in the development of spindle cell carcinomas. The spindle cell phenotype is coincident with repression of the MMTV promoter, as was observed in the MMT-ILK mice (43). MMTV repression by this means was not a concern for us as the majority of the tumors had a glandular phenotype. One difference between the ILK and the Met mice that should be noted was elevated ILK protein could be detected in non-transformed tissue, whereas in the MMTV-Met mice protein has been consistently undetectable except in transformed tissue (fig. 3-2A) (43).

Low expression of transgenes under the MMTV promoter in untransformed tissue with high expression in tumors is observed in other mammary tumor models. The MMTV-protein kinase CK2 mice display low/undetectable expression in normal mammary glands but a strong band is apparent in tumor lysate by immunoblotting (44). Interestingly, in the MMTV-CK2 mouse, both adenosquamous and spindle cell tumors developed, with expression of cytokeratin 14, indicating possible involvement of the basal/progenitor population (44). Similarly, the MMTV-heregulin (45) and MMTV-hEGFR (46) mice had low transgene expression in normal tissue but enhanced expression in tumor tissue.

MMTV-Met is Expressed at Only Low Levels in Normal Tissue but Upregulated in Transformed Tissue

Selecting transgenic lines at the beginning of the project was challenging due to the low expression of the transgene in the mammary epithelium and the absence of an overt phenotype. Western blot analysis failed to detect expression of the exogenous Met protein and RT-PCR revealed low to undetectable levels of expression. As the MMTV/SV40 construct utilized for the generation of these mice has been used successfully in multiple other transgenic animals both before and after the cloning of the Met transgenics, it seemed unlikely that there was a defect in the construct that resulted in failure to express. Additionally, the background strain, FVB/N, is less likely to methylate the promoter than other mouse strains, so promoter methylation should not have been the cause of low expression levels (47, 48). However, tumors expressed high levels of the exogenous protein, demonstrating the integrity and functionality of the construct. This raised the possibility that a few, scattered cells in the normal ductal epithelium might be expressing the Met transgene, however, examination of virgin glands by immunofluorescence for exogenous Met expression did not reveal any pockets of expression (data not shown).

Another possible reason for the failure to detect expression was that exogenous Met expression under MMTV resulted in premature death of cells. In order to determine if this was the cause of low expression, I examined transgenic mice and control littermates at 3 weeks, 6 weeks and 9 weeks for delays in ductal outgrowth and increased frequency of apoptotic cells. There was no obvious delay in ductal outgrowth nor was there an increase in number of apoptotic cells in ducts nor terminal end buds in the developing mammary gland (Supp. fig. 3-2). Alternatively, it was possible that exogenous Met expression resulted in an epithelial-mesenchymal transition, thereby suppressing expression from the MMTV promoter as seen in the MMTV-ILK mice (43). Again, examination of glands from the early stages of development as well from older mice, revealed no loss of epithelial integrity nor apparent increase in the number of fibroblasts in the transgenic animals compared with their control littermates.

Observing expression of exogenous Met protein in the tumors but not in the normal mammary tissue from the same animal raised the question of why could the tumors cells express high levels of Met when non-transformed cells showed no expression at all. If only transformed cells could express exogenous Met, it indicated that cells needed to be "tolerized" to high levels of Met expression. Alternatively, there could be a small population of cells that did express exogenous Met and these cells were selected during tumor initiation.

The "tolerization" hypothesis was supported by the expression levels observed in the various stable cell lines generated. Three breast-derived cell lines that were used to generate stable lines expressing the Met receptor variants. The T47D lines were generated first and had very high levels of expression of exogenous Met, especially the variants containing the Y1003F mutation (3). The EpH4 lines were generated next, quickly followed by NMuMGs. Both EpH4 and NMuMG cells are considered normal, non-tumorigenic murine mammary cell lines. Western blots for exogenous Met in the EpH4 and NMuMG cell lines showed higher levels of protein in the NMuMG cells than in the EpH4 cells (fig. 5-2). T47D cells were derived from

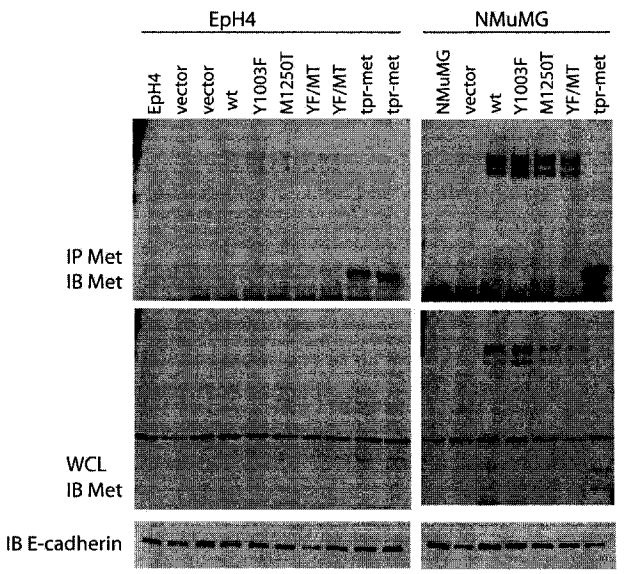

Figure 5-2. Expression levels of Met variants in EpH4 and NMuMG cells lines. Both experiments were performed at the same time. 1mg of protein was immunoprecipitated and 40 µg of whole cell lysates was loaded onto the polyacrylamide gel. Proteins were transferred and blotted at the same time. Met receptor variant are not detectable in EpH4 cells by whole cell lysates

pleural effusions of a woman with ductal carcinoma (49) and will form tumors in nude mice in the presence of estrogen (50). NMuMG cells, although considered non-transformed, will form benign cystadenomas upon reinjection into the MFP, indicating that they are not entirely normal (51). EpH4, however, do not form tumors upon injection into cleared fat pads through 6 months of observation (52, 53) and in our hands, we obtained tough, cartilaginous scar tissue after 6 months in the vector control cells but only in one mouse. These observations indicate that EpH4 cells are less transformed than the NMuMG or T47D cells and for this reason may not be able to support as high levels of expression of Met.

An alternate explanation for the tolerated expression levels hinges upon the lineage from which the cell lines are derived. T47D cells are considered to be luminal epithelial cells, as determined by microarray studies (50). EpH4 cells are a myoepithelial population with strong expression of cytokeratin 14 in all cells, as was determined by immunohistochemical staining of the EpH4-Met tumors (data not shown). The NMuMG cells may contain both luminal and basal populations as evidenced by the ability to subclone populations that were epitheliod or fibroblastic (54), however, neither microarray analysis nor immunohistochemistry has been carried out on these cells. It is possible that immortalized luminal cells tolerate higher levels of expression than do myoepithelial cells, again explaining the low levels of expression seen in the EpH4 but higher levels in the T47D and NMuMG cells.

MMTV-Met Tumors Display a Range of Histological Phenotypes

The principle of Pathway Pathology asserts that the specific signal transduction pathway activated in a tumor determines the phenotype of the tumor. This concept is supported by groups of transgenic mice, such as the ErbB2 transgenics, that despite being generated by slightly different ErbB2 cDNAs, all develop remarkably similar solid, nodular adenocarcinomas with a gradient of differentiation within each nodule (55). Likewise, mice expressing Wnt-pathway proteins, Ras, Myc, or even knock-out animals for PTEN display similar tumor phenotypes in genetically modified mice (56). Exceptionally, the MMTV-Met-

YF/MT tumors are not constrained to one particular phenotype. The most prevalent tumor phenotype is the solid adenocarcinoma, but we also observed papillary and invasive tubular adenocarcinomas. Even amongst the solid adenocarcinomas, there were differences in size and number of vascular channels and how much of an inflammatory reaction there was, but in general, the nuclei were regular with an open chromatin pattern and, if present, a single nucleolus. The solid tumors often had regions of densely packed and looser cells. When compared with the other Met-related transgenic models, the similarities they share in phenotypes is that they all display a range of phenotypes. The WAP-HGF model developed tumors that were described as adenosquamous, with nodular glandular units (39) and the MT-Tpr-Met mice developed tumors that were papillary, scirrous or nodular (37).

The long latency and low transgene expression could contribute to an explanation for this range of phenotypes. The latency to tumor development implies that expression of dysregulated Met is insufficient to transform on its own, but requires additional oncogenic events for tumor initiation. The contributing oncogenic events may be what determines the tumor phenotype. It has been shown that when crossing two transgenic animals there is always a dominant oncogenic pathway that determines tumor phenotype rather than each contributing to a hybrid phenotype (57). We may be observing a dominant oncogene effect or loss of a tumor suppressor. The solid phenotype could be a manifestation of an activated Ras-MAPK pathway as observed in ErbB2, Ras, and Polyoma Middle T-driven tumors-all of which display a similar, solid phenotype (55, 58, 59). Strong activation of the MAPK pathway can be seen by immunoblotting MMTV-Met tumor lysates (fig. 3-3), but this does not exclude activation of other pathways. Papillary and invasive tubular may have activation of other pathways or loss of tumor suppressors such as PTEN in the papillary tumors (60). Activation or dysregulation of specific pathways could be examined by performing immunoblots for proteins within specific pathways. Alternatively, tissue sections could be used to determine the localization of activated forms of critical proteins. The forthcoming microarray data may also provide insight into which pathways may be dysregulated.

MMTV-Met Tumors Rarely Metastasize

Met receptor signaling *in vitro* has been shown to result in increased cellular motility and invasion (61). As a result of this *in vitro* behavior, it was expected that the MMTV-Met-derived tumors would become invasive and form distant metastases. Surprisingly, only two of the tumor-bearing mice were found to have lung metastases (fig. 3-5) and no metastases were found in other tissues, despite examining liver and bone. Even when tumors were transplanted, allowed to grow for 2 months, resected and allowed to re-grow, no lung metastases developed. In some histological sections, tumor cells were observed within vascular channels (fig. 5-3), however, it appears that the cells were unable to survive in a foreign microenvironment.

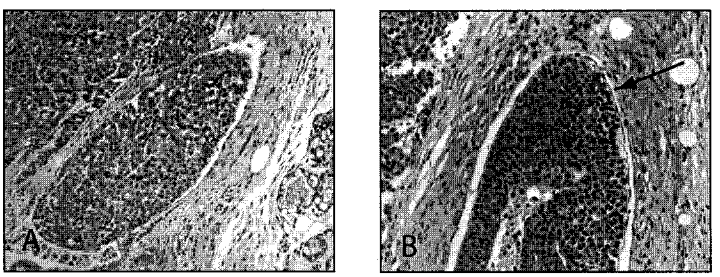

Figure 5-3 Intravascular tumor cells. A. Tumor cell embolus without endothelium. B. Endothelium-covered (arrow) tumor cells.

In many cases, the tumor cells were locally invasive, however, as observed in histological sections, especially those stained for exogenous Met protein or cytokeratin 8/18. Using either of these staining methods, single cells and groups of cells could be observed migrating into the stroma adjacent to the tumor mass (examples-fig.3-5A and Supp. fig. 3-4). Thus, the Met-expressing cells were capable of invading into the stroma, but generally, not able to colonize the lung.

The failure to metastasize highlights the different characteristics that are required for metastases to develop. Enhanced invasive ability is only one aspect of the metastatic cascade and may not actually be as large a contributor to metastases as originally thought. The ability to survive and then proliferate in other organs seems to be a more stringent qualification for metastatic ability. As recently described by Anne Chambers and colleagues, cells from primary tumors can be found in multiple

organs, but they remain quiescent, unable to expand until some as yet undetermined signal permits them to proliferate (62). This has also been demonstrated by other studies in which even in early stages of tumor development, cytokeratin positive cells can be found scattered throughout the bone marrow, however, the cells have not proliferated to form metastases (63, 64).

The identification of multiple scattered malignant, but non-proliferative, cells highlights the decreased importance of migratory/invasive ability of tumor cells. It is believed that tumors regularly "shed" cells into circulation not necessarily by cells invading into blood vessels, but by growing surrounded by leaky neovasculature, spreading into the lumen, and being carried to other organs (65). Clumps of tumor cells may enter the blood stream surrounded by a layer of endothelium as seen adjacent to the tumor sections (65, 66) (figure 5-3B).

The downregulation of adhesion molecules and the progression through an epithelial-mesenchymal transition (EMT) does not seem to increase the risk of metastases as seen in human tumors with consistent loss of E-cadherin expression. The MMTV-Met-M1250T tumors contained very few E-cadherin positive cells (fig. 3-6C), however, these tumors and their transplants did not give rise to distal metastases. In humans, the highly invasive E-cadherin negative breast tumor phenotype of invasive lobular adenocarcinoma (ILC) does not metastasize any more frequently than invasive ductal carcinoma no special type (67-69). Loss of E-cadherin expression is also found in a class of gastric cancers within a few families that carry a germline mutation in *cdh1*. Again, although the carriers of the mutant *cdh1* are predisposed to gastric and breast cancer, the tumors do not metastasize with any greater frequency than the more common types of gastric cancer (70).

MMTV-Met Tumors Contain Progenitor Cells

Although it has not been examined, two other Met-related transgenic models present data suggesting that stem cell/progenitor cells may have been affected. Both the MT-Tpr-Met (37) and the WAP-HGF (39) mouse tumors are described as having an adenosquamous component. The presence of squamous metaplasia is indicative of progenitor cell involvement as there is positive staining for CK6 and CK14 around

keratin pearls (71)-also personal observation (fig. 5-4). Transdifferentiation of mammary epithelium to other ectodermal derivatives indicates the plasticity of progenitor cells. Wnt pathway transgenic mice frequently develop adenosquamous tumors, and several studies have demonstrated a high percentage of putative stem cells present in these tumors by staining for CK5 or CK6 as well as FACS sorting of tumors for cells with stem cell markers (72, 73). The MMTV-Met-M1250T mice developed tumors with squamous metaplasia that demonstrated staining for progenitor markers adjacent to keratin pearls as well as in other areas of the tumor (fig. 3-7F), indicating that Met-M1250T may be inducing transformation of the progenitor population. Support for the role of Met in the progenitor population has also come from a recent study in which microarray analysis was performed on a panel of breast cancer cell lines and it was found that high Met expression was associated with a basal phenotype (74). Thus, progenitor cells may better tolerate elevated Met expression or they may have a growth advantage if Met is overexpressed.

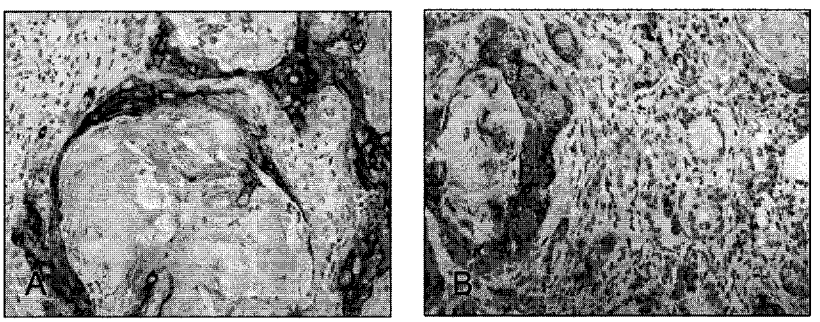

Figure 5-4 Cytokeratin expression around keratin pearls. Both images were taken at 40x magnification. A. K6 B. K14

Unlike the Met-M1250T tumors, the two invasive tubular adenocarcinomas that contained mixed lineage cells did not generate areas of squamous metaplasia. In humans, there appears to be no strong correlation between invasive tubular morphology and expression of basal markers unless the tumor is a mixed tubular adenocarcinoma (75). It would be of interest to examine a breast tissue bank for invasive tubular adenocarcinomas and perform staining for stem/progenitor cell

markers in order to determine if basal markers are frequently expressed within this histological subtype.

The finding that exogenous Met-M1250T expression can expand the progenitor population suggests that higher levels of Met expression are better tolerated in the stem/progenitor population and that subsequent mutations acquired by these cells over the lifetime of the mouse induce them to become the "tumor stem cells." This is supported by the latency to tumor development, which again, suggests that several genetic alterations are required for tumor development. With the formation of the tumor stem cell, it is possible that depending on the Met signal-whether it is Met-M1250T or Met-YF/MT-and other modifying factors, there could be differences in how the cells differentiate, as exemplified by the MMTV-Met-M1250T tumors being both K8 and K14 positive whereas the majority of the MMTV-Met-YF/MT tumors were solely K8 positive. Met-M1250T may enhance the self-renewal capacity of the progenitor cells and permit differentiation towards both luminal and basal lineages whereas the addition of Met-YF/MT drives the cells towards only luminal differentiation or is only tolerated by transformed committed luminal progenitors (fig. 5-5). The Met-YF/MT invasive tubular tumors that contain both basal and luminal cells may be expressing factors that modify the Met-YF/MT signaling to permit expansion of both populations. The forthcoming microarray data from the invasive tubular tumors will help to identify what distinguishes these Met-YF/MT tumors from the solid, luminal Met-YF/MT tumors.

The microarray data generated thus far supports the observation that Met-M1250T and Met-YF/MT tumors are different from each other. The presence of myoepithelial and progenitor cells in the Met-M1250T tumors indicates the possibility that these tumors may be classified as basal tumors, and the comparisons between the human basal profile and the Met-M1250T profile will be made. Microarray data from human breast cancers has shown that tumors expressing the highest levels of Met protein cluster with basal tumors (Finak, Park, unpublished data), thus, dysregulated Met signaling may play a role in transforming mammary progenitor cells in both mice and humans.

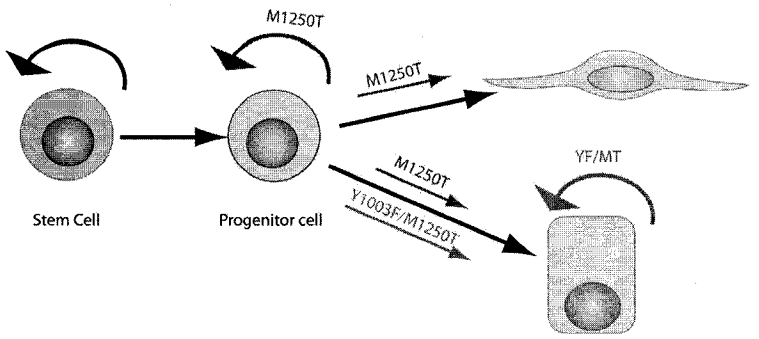

Figure 5-5 Hypothesis for Met variants driving tumorigenesis. Met-M1250T may enhance progenitor cell proliferation and permit differentiation towards both luminal and basal lineages. Met-YF/MT may drive progenitor cell differentiation towards luminal cells only or may enhance proliferation of a progenitor cell committed to the luminal lineage (represented by the luminal cell undergoing self-renewal).

Met Signals Cooperate with p53 in Formation of Mixed Lineage Tumors

Tumors arising in genetically modified mice that carry a deletion in p53 and a defective *BRCA1* frequently overexpress Met and have amplification of the Met locus (76). A much earlier study in Li-Fraumeni patients (hereditary *p53* mutation) had also found that in 3/4 human sarcoma samples examined and in 9/15 p53-null murine tumor samples, Met was highly expressed (77). With this evidence of p53/Met cooperation in tumorigenesis, we examined both the MMTV-Met tumors for p53 nuclear localization. We observed p53 nuclear localization in the Met-M1250T (fig. 8a) tumors and the Met-YF/MT invasive tubular carcinomas, tumors that contained both luminal and basal cells, whereas the luminal-only tumors did not have nuclear p53 staining. This finding is not unexpected as p53 nuclear localization is associated with the basal phenotype in human tumors (75, 78-80).

To augment these studies, it would be useful to examine the breast tumors in the human breast cancer bank that overexpress Met receptor. If loss of p53 function selects for Met overexpression, screening the bank for p53 mutants or tumors with p53 nuclear localization and co-ordinately examining Met receptor expression, could support or refute the possibility of cooperation. Perhaps p53 and Met cooperation occurs preferentially within tumors of a specific histological phenotype, again, another possibility to be explored by thoroughly examining the human breast tumor

bank. It is possible that the Met amplification in human tumors occurs preferentially in the context of *BRCA1* or *BRCA2* mutation as was observed in one mouse model (76). Screening the breast tumor bank for *p53* and *BRCA* mutations and assaying for Met amplification might yield a stronger association between genomic instability and Met signaling.

To test the hypothesis that Met and p53 cooperate in mammary tumorigenesis we have set up breeding pairs with p53-null mice and the MMTV-Met mice. If loss of p53 function is a "tolerizing" event, we may be able to detect Met expression in the mammary epithelium earlier in development. The range of tumor histologies will mostly likely be the same as for the p53-null alone, but with cooperation from the transgenic Met, the tumors should arise with a decreased latency for both p53-null alone and MMTV-Met alone. It will be necessary to examine these tumors for increased expression of endogenous Met, to determine if Met amplification occurs in these tumors, even in the presence of exogenous Met, as it does in the $p53^{-/-}$ $BRCA1^{-/-}$ tumors (76).

Met Receptor Plays a Role in Mammary Gland Development

The effect of loss of Met receptor expression on the development of the mammary gland has never been directly addressed. We have begun to examine the results of Met deletion in the mammary epithelium using floxed-Met (Met^{fl}) mice with mammary-directed Cre expression. Additionally, we have crossed the Met^{fl} mice with a strain of reporter mice (GTRosa26) that express β-galactosidase following Cre expression in order to identify the cells in which Met has been deleted.

It was surprising to find that $Met^{fl/fl}$ mice did not display any mammary gland developmental defects, neither at the gross nor at the microscopic level. Previous reports had demonstrated that treating MFPs in culture with antisense oligonucleotides to HGF blocked ductal branching during the period of treatment (81), therefore I expected to see fewer branches in the $Met^{fl/fl}$ glands. The lack of phenotype may be explained by the nearly normal levels of endogenous Met observed in the mammary gland by immunoblotting (fig. 4-5b). Histological examination of the glands revealed the reason for the normal levels of Met protein was that there

were very few Met-null luminal cells in the $Met^{fl/fl}$ glands compared with the $Met^{+/+}$ glands as determined by the presence of β-galactosidase expression.

Although the percentage of luminal cells that underwent DNA recombination was lower in the $Met^{fl/fl}$ mice, the percentage of Met-null (blue) myoepithelial cells was high. It is difficult to determine from the x-gal staining if the percentage of myoepithelial cells that underwent recombination in the $Met^{fl/fl}$ and $Met^{+/+}$ glands is similar, however this could be determined by immunofluorescence (IF) or immunohistochemistry (IHC), staining for markers of myoepithelial cells in the cells that express β-galactosidase. One report, that demonstrated that myoepithelial cells do not require HGF for proliferation (82), provides support for the hypothesis that Met-null myoepithelial cells may be able to continue to proliferate and contribute to the expanding ductal system. However, again, IF or IHC is required to positively identify the cells that have undergone recombination and that are proliferating, in order to determine if the myoepithelial cell populations from both the $Met^{+/+}$ and $Met^{fl/fl}$ mice display the same fraction of proliferating cells.

Met receptor signals may be required for survival or proliferation of luminal epithelial cells which is why there are so few Met-null luminal cells. If Met signals are required for survival, the Met-null luminal cells may be undergoing apoptosis; this can easily be assayed for by TUNEL staining. If Met is required for proliferation of luminal cells, then, staining for a proliferation antigen such as PCNA or Ki67 will reveal whether or not the Met-null luminal cells are proliferating. In both of these scenarios, the Met-competent luminal cells will have to compensate for the loss of proliferation of the Met-null luminal cells in order to avoid a delay in ductal outgrowth, thus we could expect a higher proliferative fraction in the $Met^{fl/fl}$ gland than in the $Met^{+/+}$ glands.

The paucity of recombined $Met^{fl/fl}$ luminal cells could also be explained by a failure of Met-null progenitor cells to differentiate towards luminal cells, whereas myoepithelial cell differentiation is unaffected. This could be assayed by culturing primary cells in conditions that permit both luminal and myoepithelial cells to proliferate such as demonstrated by Stingl et al. (83). In this way, we could determine if the Met-null progenitor cells preferentially generate myoepithelial cells.

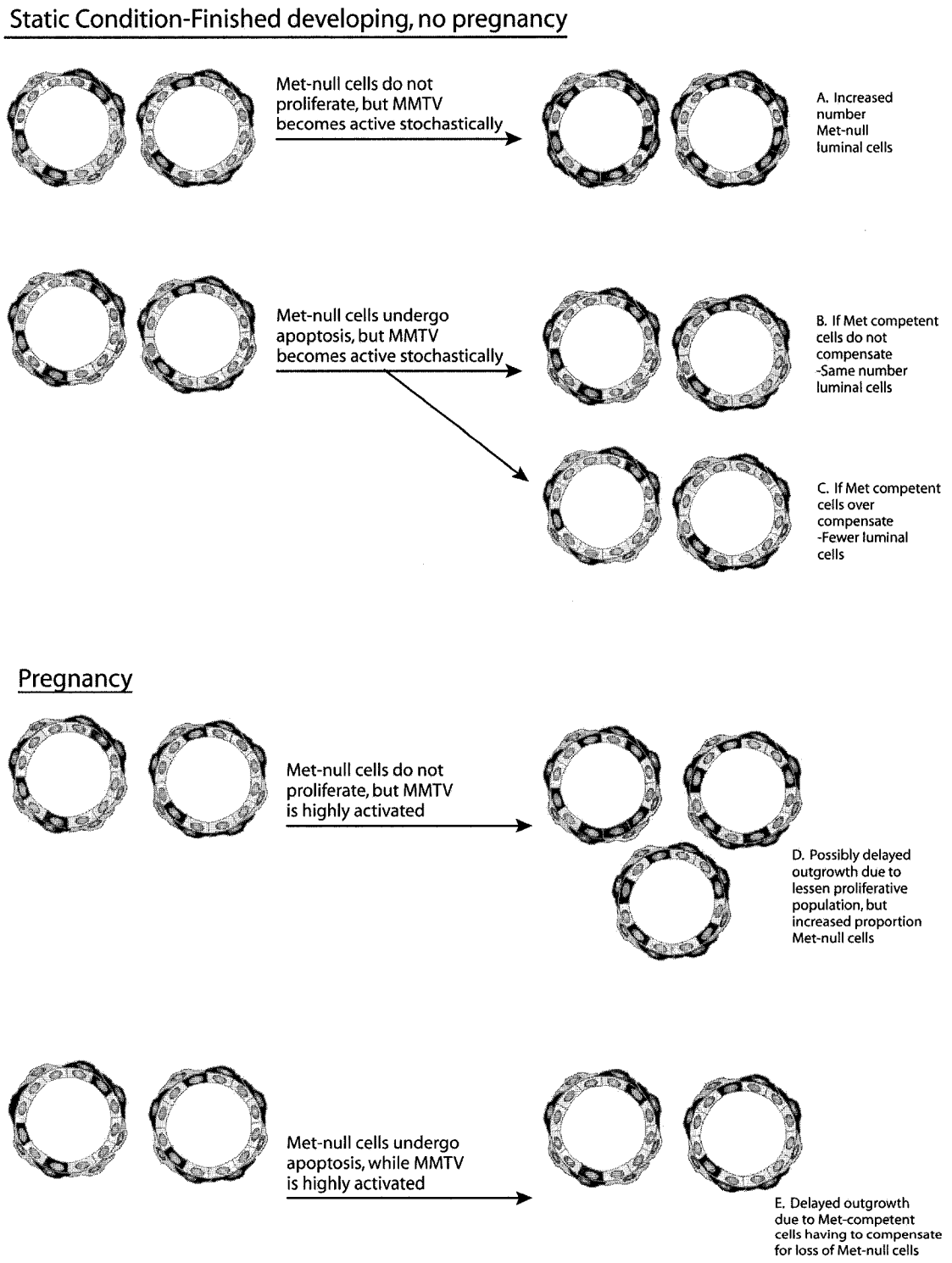

Figure 5-6 Scenarios for future time points in $Met^{fl/fl}$ experiments.

At the six week time point, there is no difference in mammary gland development, however, examination of both earlier and later time points is crucial. The MMTV promoter utilized to drive Cre expression is activated as early as 3 weeks of age, thus it is possible that at an earlier time point a difference in phenotypes may be observed if all the luminal cells that have undergone recombination fail to proliferate or undergo apoptosis. It will also be of interest to examine whether or not the proportion of recombined cells changes. MMTV becomes active in a stochastic manner (84-86), thus, if Met-null cells fail to proliferate, there should be an incremental increase in the percentage of blue cells as the gland matures because proliferation becomes less important so there would not need to be a compensation by the non-recombined cells for normal growth to occur (fig. 5-6a). If the Met-null cells undergo apoptosis, then the rates of apoptosis of Met-null and compensatory proliferation of Met-competent cells should balance, leaving the gland with a constant percentage of Met-null luminal cells (fig. 5-6b). Alternatively, there could be an over-compensation for the lost Met-null cells, leaving fewer blue cells in the luminal population (fig. 5-6c). In all of these scenarios, the myoepithelial population should continue to undergo recombination, but this will not affect the function nor growth of the gland, if myoepithelial cells do not require Met for proliferation nor function.

The next question to be address is: how do the Met-null cells respond to the need to proliferate, expand, and differentiate during pregnancy and early lactation? Met and HGF levels are elevated during early pregnancy (82, 87) but decrease during late pregnancy and lactation, implying that the early stages of pregnancy-induced proliferation require Met signaling. If Met-null luminal cells (or Met-null progenitors) fail to proliferate, there may be delayed ductal outgrowth but the number of Met-null cells will increase as a result of the increased activity from the MMTV promoter during pregnancy (fig. 5-6d). If Met-null luminal cells (or progenitors) undergo apoptosis, it is expected that there would be delayed outgrowth during pregnancy and that the proportion of Met-null cells will decrease as the Met-competent cells compensate for the apoptosing population (fig. 5-6e). Finally, Met/HGF expression is again elevated during involution (82, 87), so the consequences of loss of Met expression during involution must be examined.

Thus, there are a number of questions still to be addressed in this project, but the preliminary results do indicate that, although the mammary gland is able to compensate for loss of Met expression, there is negative selection for Met-null luminal epithelial cells.

Conclusion

From this work, the role of the Met receptor in the mammary gland has been further explored. Its importance in the progenitor population has been revealed through the generation of tumors that contain both luminal and myoepithelial cells and through the studies of the Met-null mammary glands.

Additionally, the importance of negative regulation of a receptor tyrosine kinase has been shown. The combination of an activating mutation with a mutation that results in loss of receptor ubiquitination yields a transformative ability that greater than either mutation alone.

Abbreviations

Cbl	Casitas B-lineage Lymphoma
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial Mesenchymal Transition
ErbB2	Erythroblastic leukemia viral oncogene2
FVB/N	Friend's leukemia VirusB ($Fv1^{+}$)/NIH strain
GIST	Gastrointestinal Stromal Tumor
IF	Immunofluorescence
IHC	Immunohistochemistry
JM	Juxtamembrane
K6	Cytokeratin 6
K8/18	Cytokeratin 8/18
K14	Cytokeratin 14
Ki67	proliferation antigen discovered at University of Kiel
Met^{fl}	Floxed Met mouse
MFP	Mammary Fat Pad
MMTV	Mouse Mammary Tumor Virus
PCNA	Proliferating Cell Nuclear Antigen
PDGFR	Platelet-Derived Growth Factor Receptor
RTK	Receptor Tyrosine Kinase
TUNEL	Terminal deoxynucleotidyl Transferase mediated-dUTP Nick End Labeling

References

1. Weidner, K. M., Sachs, M., Riethmacher, D., and Birchmeier, W. Mutation of juxtamembrane tyrosine residue 1001 suppresses loss-of-function mutations of the met receptor in epithelial cells. Proc Natl Acad Sci U S A, *92:* 2597-2601, 1995.
2. Peschard, P., Fournier, T. M., Lamorte, L., Naujokas, M. A., Band, H., Langdon, W. Y., and Park, M. Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein. Mol Cell, *8:* 995-1004, 2001.
3. Abella, J. V., Peschard, P., Naujokas, M. A., Lin, T., Saucier, C., Urbe, S., and Park, M. Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation. Mol Cell Biol, *25:* 9632-9645, 2005.
4. Waterman, H., Katz, M., Rubin, C., Shtiegman, K., Lavi, S., Elson, A., Jovin, T., and Yarden, Y. A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling. Embo J, *21:* 303-313, 2002.
5. Muthuswamy, S. K., Gilman, M., and Brugge, J. S. Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers. Mol Cell Biol, *19:* 6845-6857, 1999.
6. Wu, W. J., Tu, S., and Cerione, R. A. Activated Cdc42 sequesters c-Cbl and prevents EGF receptor degradation. Cell, *114:* 715-725, 2003.
7. Binns, K. L., Taylor, P. P., Sicheri, F., Pawson, T., and Holland, S. J. Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors. Mol Cell Biol, *20:* 4791-4805, 2000.
8. Wybenga-Groot, L. E., Baskin, B., Ong, S. H., Tong, J., Pawson, T., and Sicheri, F. Structural basis for autoinhibition of the Ephb2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region. Cell, *106:* 745-757, 2001.
9. Till, J. H., Becerra, M., Watty, A., Lu, Y., Ma, Y., Neubert, T. A., Burden, S. J., and Hubbard, S. R. Crystal structure of the MuSK tyrosine kinase: insights into receptor autoregulation. Structure, *10:* 1187-1196, 2002.
10. Griffith, J., Black, J., Faerman, C., Swenson, L., Wynn, M., Lu, F., Lippke, J., and Saxena, K. The structural basis for autoinhibition of FLT3 by the juxtamembrane domain. Mol Cell, *13:* 169-178, 2004.
11. Mol, C. D., Lim, K. B., Sridhar, V., Zou, H., Chien, E. Y., Sang, B. C., Nowakowski, J., Kassel, D. B., Cronin, C. N., and McRee, D. E. Structure of a c-kit product complex reveals the basis for kinase transactivation. J Biol Chem, *278:* 31461-31464, 2003.
12. Peschard, P., Ishiyama, N., Lin, T., Lipkowitz, S., and Park, M. A conserved DpYR motif in the juxtamembrane domain of the met receptor family forms an atypical c-Cbl/Cbl-b tyrosine kinase binding domain binding site required

for suppression of oncogenic activation. J Biol Chem, *279:* 29565-29571, 2004.

13. Lennartsson, J., Jelacic, T., Linnekin, D., and Shivakrupa, R. Normal and oncogenic forms of the receptor tyrosine kinase kit. Stem Cells, *23:* 16-43, 2005.
14. Ma, Y., Cunningham, M. E., Wang, X., Ghosh, I., Regan, L., and Longley, B. J. Inhibition of spontaneous receptor phosphorylation by residues in a putative alpha-helix in the KIT intracellular juxtamembrane region. J Biol Chem, *274:* 13399-13402, 1999.
15. Chan, P. M., Ilangumaran, S., La Rose, J., Chakrabartty, A., and Rottapel, R. Autoinhibition of the kit receptor tyrosine kinase by the cytosolic juxtamembrane region. Mol Cell Biol, *23:* 3067-3078, 2003.
16. Heinrich, M. C., Griffith, D. J., Druker, B. J., Wait, C. L., Ott, K. A., and Zigler, A. J. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. Blood, *96:* 925-932, 2000.
17. Druker, B. J., Tamura, S., Buchdunger, E., Ohno, S., Segal, G. M., Fanning, S., Zimmermann, J., and Lydon, N. B. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med, *2:* 561-566, 1996.
18. Logrono, R., Jones, D. V., Faruqi, S., and Bhutani, M. S. Recent advances in cell biology, diagnosis, and therapy of gastrointestinal stromal tumor (GIST). Cancer Biol Ther, *3:* 251-258, 2004.
19. Lee, J. H., Han, S. U., Cho, H., Jennings, B., Gerrard, B., Dean, M., Schmidt, L., Zbar, B., and Vande Woude, G. F. A novel germ line juxtamembrane Met mutation in human gastric cancer. Oncogene, *19:* 4947-4953, 2000.
20. Kong-Beltran, M., Seshagiri, S., Zha, J., Zhu, W., Bhawe, K., Mendoza, N., Holcomb, T., Pujara, K., Stinson, J., Fu, L., Severin, C., Rangell, L., Schwall, R., Amler, L., Wickramasinghe, D., and Yauch, R. Somatic mutations lead to an oncogenic deletion of met in lung cancer. Cancer Res, *66:* 283-289, 2006.
21. Jeffers, M., Schmidt, L., Nakaigawa, N., Webb, C. P., Weirich, G., Kishida, T., Zbar, B., and Vande Woude, G. F. Activating mutations for the met tyrosine kinase receptor in human cancer. Proc Natl Acad Sci U S A, *94:* 11445-11450, 1997.
22. Maritano, D., Accornero, P., Bonifaci, N., and Ponzetto, C. Two mutations affecting conserved residues in the Met receptor operate via different mechanisms. Oncogene, *19:* 1354-1361, 2000.
23. Miller, M., Ginalski, K., Lesyng, B., Nakaigawa, N., Schmidt, L., and Zbar, B. Structural basis of oncogenic activation caused by point mutations in the kinase domain of the MET proto-oncogene: modeling studies. Proteins, *44:* 32-43, 2001.
24. Yuan, Z. L., Guan, Y. J., Wang, L., Wei, W., Kane, A. B., and Chin, Y. E. Central role of the threonine residue within the p+1 loop of receptor tyrosine kinase in STAT3 constitutive phosphorylation in metastatic cancer cells. Mol Cell Biol, *24:* 9390-9400, 2004.

25. Rodrigues, G. A. and Park, M. Autophosphorylation modulates the kinase activity and oncogenic potential of the Met receptor tyrosine kinase. Oncogene, *9:* 2019-2027, 1994.
26. Ghoussoub, R. A., Dillon, D. A., D'Aquila, T., Rimm, E. B., Fearon, E. R., and Rimm, D. L. Expression of c-met is a strong independent prognostic factor in breast carcinoma. Cancer, *82:* 1513-1520, 1998.
27. Camp, R. L., Rimm, E. B., and Rimm, D. L. Met expression is associated with poor outcome in patients with axillary lymph node negative breast carcinoma. Cancer, *86:* 2259-2265, 1999.
28. Beviglia, L., Matsumoto, K., Lin, C. S., Ziober, B. L., and Kramer, R. H. Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. Int J Cancer, *74:* 301-309, 1997.
29. Tsarfaty, I., Alvord, W. G., Resau, J. H., Altstock, R. T., Lidereau, R., Bieche, I., Bertrand, F., Horev, J., Klabansky, R. L., Keydar, I., and Vande Woude, G. F. Alteration of Met protooncogene product expression and prognosis in breast carcinomas. Anal Quant Cytol Histol, *21:* 397-408, 1999.
30. Lengyel, E., Prechtel, D., Resau, J. H., Gauger, K., Welk, A., Lindemann, K., Salanti, G., Richter, T., Knudsen, B., Vande Woude, G. F., and Harbeck, N. C-Met overexpression in node-positive breast cancer identifies patients with poor clinical outcome independent of Her2/neu. Int J Cancer, *113:* 678-682, 2005.
31. Lee, W. Y., Su, W. C., Lin, P. W., Guo, H. R., Chang, T. W., and Chen, H. H. Expression of S100A4 and Met: potential predictors for metastasis and survival in early-stage breast cancer. Oncology, *66:* 429-438, 2004.
32. Toi, M., Taniguchi, T., Ueno, T., Asano, M., Funata, N., Sekiguchi, K., Iwanari, H., and Tominaga, T. Significance of circulating hepatocyte growth factor level as a prognostic indicator in primary breast cancer. Clin Cancer Res, *4:* 659-664, 1998.
33. Sheen-Chen, S. M., Liu, Y. W., Eng, H. L., and Chou, F. F. Serum levels of hepatocyte growth factor in patients with breast cancer. Cancer Epidemiol Biomarkers Prev, *14:* 715-717, 2005.
34. Taniguchi, T., Toi, M., Inada, K., Imazawa, T., Yamamoto, Y., and Tominaga, T. Serum concentrations of hepatocyte growth factor in breast cancer patients. Clin Cancer Res, *1:* 1031-1034, 1995.
35. Maemura, M., Iino, Y., Yokoe, T., Horiguchi, J., Takei, H., Koibuchi, Y., Horii, Y., Takeyoshi, I., Ohwada, S., and Morishita, Y. Serum concentration of hepatocyte growth factor in patients with metastatic breast cancer. Cancer Lett, *126:* 215-220, 1998.
36. Yamashita, J., Ogawa, M., Yamashita, S., Nomura, K., Kuramoto, M., Saishoji, T., and Shin, S. Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. Cancer Res, *54:* 1630-1633, 1994.
37. Liang, T. J., Reid, A. E., Xavier, R., Cardiff, R. D., and Wang, T. C. Transgenic expression of tpr-met oncogene leads to development of mammary hyperplasia and tumors. J Clin Invest, *97:* 2872-2877, 1996.

38. Jeffers, M., Fiscella, M., Webb, C. P., Anver, M., Koochekpour, S., and Vande Woude, G. F. The mutationally activated Met receptor mediates motility and metastasis. Proc Natl Acad Sci U S A, *95:* 14417-14422, 1998.
39. Gallego, M. I., Bierie, B., and Hennighausen, L. Targeted expression of HGF/SF in mouse mammary epithelium leads to metastatic adenosquamous carcinomas through the activation of multiple signal transduction pathways. Oncogene, *22:* 8498-8508, 2003.
40. Nieto, A. I., Shyamala, G., Galvez, J. J., Thordarson, G., Wakefield, L. M., and Cardiff, R. D. Persistent mammary hyperplasia in FVB/N mice. Comp Med, *53:* 433-438, 2003.
41. Wakefield, L. M., Thordarson, G., Nieto, A. I., Shyamala, G., Galvez, J. J., Anver, M. R., and Cardiff, R. D. Spontaneous pituitary abnormalities and mammary hyperplasia in FVB/NCr mice: implications for mouse modeling. Comp Med, *53:* 424-432, 2003.
42. Huseby, R. A., Soares, M. J., and Talamantes, F. Ectopic pituitary grafts in mice: hormone levels, effects on fertility, and the development of adenomyosis uteri, prolactinomas, and mammary carcinomas. Endocrinology, *116:* 1440-1448, 1985.
43. White, D. E., Cardiff, R. D., Dedhar, S., and Muller, W. J. Mammary epithelial-specific expression of the integrin-linked kinase (ILK) results in the induction of mammary gland hyperplasias and tumors in transgenic mice. Oncogene, *20:* 7064-7072, 2001.
44. Landesman-Bollag, E., Romieu-Mourez, R., Song, D. H., Sonenshein, G. E., Cardiff, R. D., and Seldin, D. C. Protein kinase CK2 in mammary gland tumorigenesis. Oncogene, *20:* 3247-3257, 2001.
45. Weinstein, E. J. and Leder, P. The extracellular region of heregulin is sufficient to promote mammary gland proliferation and tumorigenesis but not apoptosis. Cancer Res, *60:* 3856-3861, 2000.
46. Brandt, R., Eisenbrandt, R., Leenders, F., Zschiesche, W., Binas, B., Juergensen, C., and Theuring, F. Mammary gland specific hEGF receptor transgene expression induces neoplasia and inhibits differentiation. Oncogene, *19:* 2129-2137, 2000.
47. Chaillet, J. R., Bader, D. S., and Leder, P. Regulation of genomic imprinting by gametic and embryonic processes. Genes Dev, *9:* 1177-1187, 1995.
48. Zhou, H., Chen, W. D., Qin, X., Lee, K., Liu, L., Markowitz, S. D., and Gerson, S. L. MMTV promoter hypomethylation is linked to spontaneous and MNU associated c-neu expression and mammary carcinogenesis in MMTV c-neu transgenic mice. Oncogene, *20:* 6009-6017, 2001.
49. Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., Chaitcik, S., and Brenner, H. J. Establishment and characterization of a cell line of human breast carcinoma origin. Eur J Cancer, *15:* 659-670, 1979.
50. Lacroix, M. and Leclercq, G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat, *83:* 249-289, 2004.
51. Owens, R. B. Glandular epithelial cells from mice: a method for selective cultivation. J Natl Cancer Inst, *52:* 1375-1378, 1974.

52. Pinkas, J., Martin, S. S., and Leder, P. Bcl-2-mediated cell survival promotes metastasis of EpH4 betaMEKDD mammary epithelial cells. Mol Cancer Res, *2:* 551-556, 2004.
53. Pinkas, J. and Leder, P. MEK1 signaling mediates transformation and metastasis of EpH4 mammary epithelial cells independent of an epithelial to mesenchymal transition. Cancer Res, *62:* 4781-4790, 2002.
54. Van den Broecke, C., Vleminckx, K., De Bruyne, G., Van Hoorde, L., Vakaet, L., Van Roy, F., and Mareel, M. Morphotypic plasticity in vitro and in nude mice of epithelial mouse mammary cells (NMuMG) displaying an epithelioid (e) or a fibroblastic (f) morphotype in culture. Clin Exp Metastasis, *14:* 282-296, 1996.
55. Rosner, A., Miyoshi, K., Landesman-Bollag, E., Xu, X., Seldin, D. C., Moser, A. R., MacLeod, C. L., Shyamala, G., Gillgrass, A. E., and Cardiff, R. D. Pathway pathology: histological differences between ErbB/Ras and Wnt pathway transgenic mammary tumors. Am J Pathol, *161:* 1087-1097, 2002.
56. Cardiff, R. D., Munn, R. J., and Galvez, J. J. The tumor pathology of genetically engineered mice: a new approach to molecular pathology. *In:* J. G. Fox, M. T. Davisson, S. W. Quimby, C. E. Barthold, C. E. Newcomer, and A. L. Smith (eds.), The mouse in biomedical research: Experimental biology and oncology, Vol. 2, pp. 581-622. New York: Elsevier Inc., 2006.
57. Cardiff, R. D. Mouse models of human breast cancer. Comp Med, *53:* 250-253, 2003.
58. Cardiff, R. D., Sinn, E., Muller, W., and Leder, P. Transgenic oncogene mice. Tumor phenotype predicts genotype. Am J Pathol, *139:* 495-501, 1991.
59. Cardiff, R. D. and Wellings, S. R. The comparative pathology of human and mouse mammary glands. J Mammary Gland Biol Neoplasia, *4:* 105-122, 1999.
60. Freeman, D. J., Li, A. G., Wei, G., Li, H. H., Kertesz, N., Lesche, R., Whale, A. D., Martinez-Diaz, H., Rozengurt, N., Cardiff, R. D., Liu, X., and Wu, H. PTEN tumor suppressor regulated p53 protein levels and activity through phosphatase-dependent and -independent mechanisms. Cancer Cell, *3:* 117-130, 2003.
61. Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G. F. Met, metastasis, motility and more. Nat Rev Mol Cell Biol, *4:* 915-925, 2003.
62. Townson, J. L. and Chambers, A. F. Dormancy of solitary metastatic cells. Cell Cycle, *5:* 1744-1750, 2006.
63. Braun, S., Pantel, K., Muller, P., Janni, W., Hepp, F., Kentenich, C. R., Gastroph, S., Wischnik, A., Dimpfl, T., Kindermann, G., Riethmuller, G., and Schlimok, G. Cytokeratin-positive cells in the bone marrow and survival of patients with stage I, II, or III breast cancer. N Engl J Med, *342:* 525-533, 2000.
64. Gebauer, G., Fehm, T., Merkle, E., Beck, E. P., Lang, N., and Jager, W. Epithelial cells in bone marrow of breast cancer patients at time of primary surgery: clinical outcome during long-term follow-up. J Clin Oncol, *19:* 3669-3674, 2001.

65. Weidner, N. New paradigm for vessel intravasation by tumor cells. Am J Pathol, *160:* 1937-1939, 2002.
66. Sugino, T., Kusakabe, T., Hoshi, N., Yamaguchi, T., Kawaguchi, T., Goodison, S., Sekimata, M., Homma, Y., and Suzuki, T. An invasion-independent pathway of blood-borne metastasis: a new murine mammary tumor model. Am J Pathol, *160:* 1973-1980, 2002.
67. Arpino, G., Bardou, V. J., Clark, G. M., and Elledge, R. M. Infiltrating lobular carcinoma of the breast: tumor characteristics and clinical outcome. Breast Cancer Res, *6:* R149-156, 2004.
68. Sastre-Garau, X., Jouve, M., Asselain, B., Vincent-Salomon, A., Beuzeboc, P., Dorval, T., Durand, J. C., Fourquet, A., and Pouillart, P. Infiltrating lobular carcinoma of the breast. Clinicopathologic analysis of 975 cases with reference to data on conservative therapy and metastatic patterns. Cancer, *77:* 113-120, 1996.
69. Mersin, H., Yildirim, E., Gulben, K., and Berberoglu, U. Is invasive lobular carcinoma different from invasive ductal carcinoma? Eur J Surg Oncol, *29:* 390-395, 2003.
70. Polkowski, W. P., Skomra, D. G., Mielko, J., Wallner, G. T., Szumilo, J., Zinkiewicz, K., Korobowicz, E. M., and van Lanschot, J. J. E-cadherin expression as predictive marker of proximal resection line involvement for advanced carcinoma of the gastric cardia. Eur J Surg Oncol, *30:* 1084-1092, 2004.
71. Li, Y. and Rosen, J. M. Stem/progenitor cells in mouse mammary gland development and breast cancer. J Mammary Gland Biol Neoplasia, *10:* 17-24, 2005.
72. Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., Tan, L. K., Rosen, J. M., and Varmus, H. E. Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc Natl Acad Sci U S A, *100:* 15853-15858, 2003.
73. Liu, B. Y., McDermott, S. P., Khwaja, S. S., and Alexander, C. M. The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc Natl Acad Sci U S A, *101:* 4158-4163, 2004.
74. Charafe-Jauffret, E., Ginestier, C., Monville, F., Finetti, P., Adelaide, J., Cervera, N., Fekairi, S., Xerri, L., Jacquemier, J., Birnbaum, D., and Bertucci, F. Gene expression profiling of breast cell lines identifies potential new basal markers. Oncogene, *25:* 2273-2284, 2006.
75. Rakha, E. A., Putti, T. C., Abd El-Rehim, D. M., Paish, C., Green, A. R., Powe, D. G., Lee, A. H., Robertson, J. F., and Ellis, I. O. Morphological and immunophenotypic analysis of breast carcinomas with basal and myoepithelial differentiation. J Pathol, *208:* 495-506, 2006.
76. Smolen, G. A., Muir, B., Mohapatra, G., Barmettler, A., Kim, W. J., Rivera, M. N., Haserlat, S. M., Okimoto, R. A., Kwak, E., Dahiya, S., Garber, J. E., Bell, D. W., Sgroi, D. C., Chin, L., Deng, C. X., and Haber, D. A. Frequent

met oncogene amplification in a Brca1/Trp53 mouse model of mammary tumorigenesis. Cancer Res, *66:* 3452-3455, 2006.

77. Rong, S., Donehower, L. A., Hansen, M. F., Strong, L., Tainsky, M., Jeffers, M., Resau, J. H., Hudson, E., Tsarfaty, I., and Vande Woude, G. F. Met proto-oncogene product is overexpressed in tumors of p53-deficient mice and tumors of Li-Fraumeni patients. Cancer Res, *55:* 1963-1970, 1995.
78. Shien, T., Tashiro, T., Omatsu, M., Masuda, T., Furuta, K., Sato, N., Akashi-Tanaka, S., Uehara, M., Iwamoto, E., Kinoshita, T., Fukutomi, T., Tsuda, H., and Hasegawa, T. Frequent overexpression of epidermal growth factor receptor (EGFR) in mammary high grade ductal carcinomas with myoepithelial differentiation. J Clin Pathol, *58:* 1299-1304, 2005.
79. Jacquemier, J., Padovani, L., Rabayrol, L., Lakhani, S. R., Penault-Llorca, F., Denoux, Y., Fiche, M., Figueiro, P., Maisongrosse, V., Ledoussal, V., Martinez Penuela, J., Udvarhely, N., El Makdissi, G., Ginestier, C., Geneix, J., Charafe-Jauffret, E., Xerri, L., Eisinger, F., Birnbaum, D., and Sobol, H. Typical medullary breast carcinomas have a basal/myoepithelial phenotype. J Pathol, *207:* 260-268, 2005.
80. Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Eystein Lonning, P., and Borresen-Dale, A. L. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A, *98:* 10869-10874, 2001.
81. Yang, Y., Spitzer, E., Meyer, D., Sachs, M., Niemann, C., Hartmann, G., Weidner, K. M., Birchmeier, C., and Birchmeier, W. Sequential requirement of hepatocyte growth factor and neuregulin in the morphogenesis and differentiation of the mammary gland. J Cell Biol, *131:* 215-226, 1995.
82. Niranjan, B., Buluwela, L., Yant, J., Perusinghe, N., Atherton, A., Phippard, D., Dale, T., Gusterson, B., and Kamalati, T. HGF/SF: a potent cytokine for mammary growth, morphogenesis and development. Development, *121:* 2897-2908, 1995.
83. Stingl, J., Eaves, C. J., Zandieh, I., and Emerman, J. T. Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. Breast Cancer Res Treat, *67:* 93-109, 2001.
84. Andrechek, E. R., White, D., and Muller, W. J. Targeted disruption of ErbB2/Neu in the mammary epithelium results in impaired ductal outgrowth. Oncogene, *24:* 932-937, 2005.
85. White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U., and Muller, W. J. Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell, *6:* 159-170, 2004.
86. Andrechek, E. R., Hardy, W. R., Siegel, P. M., Rudnicki, M. A., Cardiff, R. D., and Muller, W. J. Amplification of the neu/erbB-2 oncogene in a mouse model of mammary tumorigenesis. Proc Natl Acad Sci U S A, *97:* 3444-3449, 2000.

87. Pepper, M. S., Soriano, J. V., Menoud, P. A., Sappino, A. P., Orci, L., and Montesano, R. Modulation of hepatocyte growth factor and c-met in the rat mammary gland during pregnancy, lactation, and involution. Exp Cell Res, *219:* 204-210, 1995.

2. Approval Signatures

Principal Investigator/ Course Director		**Date:**
Chair, Facility Animal Care Committee		**Date:**
UACC Veterinarian		**Date:**
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*		**Date:**
Approved Animal Use Period	**Start:**	**End:**

3. Summary (in language that will be understood by members of the general public)

AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge ***(was section 5a in main protocol).***

The aim of this project is to understand the role of the Met receptor tyrosine kinase (RTK) and one of its downstream signaling molecules, Crk, in the development of the mammary gland and in the induction of epithelial mesenchymal transition (EMT) which is an aspect of both normal tissue development as well as tumorigenesis and metastasis. These studies will yield a better understanding of the role of Met and its downstream signals in inducing the cell scattering that is involved in the normal development of mammary epithelium as well as in breast cancer cell invasion.

Form version Dec. 13, 2004

4. Has an unanticipated problem occurred?	**YES** ☐ **NO** ☒ **if yes, supply details:**

5. If creating genetically modified animals or new combinations of genetic modifications, complete and attach a *Phenotype Disclosure form* (http://www.mcgill.ca/rgo/animal/forms/)

6. Procedures

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES ☐ **NO** ☐

IF NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol ***(include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):***

b) For D level of invasiveness,

Include here ALL procedures except transgenic procedures, including the ones described in the original protocol as well as new and changed procedures in CAPS ***(was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.***

NOTE : NO NEW PROCEDURE

Anaesthesia, Breeding transgenics, Euthanasia of adult and neonatal rodents

Transplantation of mammary epithelial cells to a cleared mammary fat pad (MFP): Mammary fat pads will be harvested from floxed Met mice and the epithelial cells isolated in vitro. The epithelial cells will be infected in vitro with a replication incompetent adenovirus expressing cre recombinase. The treated

epithelial cells will then be suspended in phosphate buffered saline and injected into a cleared MFP. The injection of the epithelial cells requires anaesthetizing a 3 week old female animal with the rodent cocktail given intramuscularly at a dose of 0.1mL/100g body weight. Following confirmation that the animal is insensible an inverted "Y" incision is made in the skin of the abdomen allowing for exposure of the the number four MFP. The cells to be tranplanted to the MFP are injected into a distal portion of the MFP and the proximal portion of the gland is removed using a cauterizer, thus removing any endogenous mammary epithelium. The incisions is closed using clips which are removed 10 days later. The procedure should take no longer than ten minutes starting from the first incision. Once the procedure is complete the mouse will be placed near a heat lamp (3-4 feet depending on bulb strength) and will be monitored until partial consciousness. For the 10 days following surgery the animals will be observed daily for signs of infection and distress and if either of these occur the animal will be sacrificed.

Tumorigenesis and metastasis assay: To assay the ability of Met-expressing cells to form tumors or experimental metastases mice will be injected with tumor cells (10^4-10^7 in PBS or DMEM) either intravenously via the tail vein or subcutaneously.

Generation of transgenic animals: Performed as a fee for service at the McIntyre Transgenic Facility (Dr. Michel Tremblay's protocol #4437).

Special Water : To induce transgene expression in nursing mothers MT-met lines and control mice will receive 10mM zinc sulfate (ZnSO4) in their drinking water. At weaning, all pups will be given 25mM ZnSO4. A group will be sacrificed at 35 days of age and an other group will be sacrificed at 8 months of age. Monitoring : daily

Hepatocarcinogenesis : we will administrate a single injection of diethylnitrosamine (DEN) to seleceted MT-met mice. DEN (from sigma Chemical Co., St-Louis, MO USA) will be injected IP in 15-days-old mice at a dose of 5ug/g of body weight. The mice will be sacrificed at 52 weeks of age. Monitoring : daily

7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☐ NO☐

IF NO, supply new endpoints that are different from the original protocol:

b) For D level of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

Experimental Endpoints :

Aging Mice : For physiological reasons, the mice will be kept no longer than 2 years. All mice will be sacrificed when they reach 2 years old. All animals are to be monitored weekly for the first 18 months, then daily during week days for the rest of the animal's life, for signs of distress. Any of the following criteria will warrant euthanasia: loosing motricity, unresponsive to touch, slow respiration, cold to the touch, hunched up with matted fur, signs of sudden weight loss, failure to eat* and drink* (leading to more than 20% weight loss), prominent appearing ribs and spine, sunken hips. Other signs of distress are writhing, hyper- and hypoactivity, vocalizations, and isolation from the group. As soon as one of these criteria is met, the mouse

will be euthanized immediately. The cages are identified with a unic designed sticker as "retirement community". We used to have a calendar to take notes about the monitoring but it will be change to a log book.

Mice receiving the epithlial transplant will be sacrificed at 13 weeks of age; this is 10 weeks following the MFP injection. Floxed-Met mice crossed with the MMTV-Cre expressing mice will either be sacrificed at 10 weeks of age in order to observe any mammary gland developmental defects or they will be bred and sacrificed mid-pregnancy or during lactation to observe any differentiation or lactational defects incurred by the loss of Met RTK expression. There will be no groups of aging mice, however, a few homozygous mice of floxed-Met, mmtv-cre, floxed-lacZ and the combined floxed Met/floxed lacZ will be kept for breeding purposes.

MT-met mice : - there's 2 endpoints for the mice on special water : 35 days old and 8 months old.
- Hepatocarcinogenesis : The mice will be sacrificed at 52 weeks of age.

Nude mice : We did not work on this part yet, but once we have established a time frame that is optimal for our experiment (tumor formation or metastasis) we will establish a protocol with specific endpoints (see clinical endpoints section about tumor -bearing mice). Tumor tissue will be collected for histopathological evaluation after sacrificing the mice by cervical dislocation with anaesthesia.

Clinical endpoints :
No adverse effects are anticipated from the mammary manipulations involving the floxed-Met gene. The mice carrying the floxed-Met alleles should be phenotypically normal as are the floxed-lacZ and MMTV-Cre mice. Following MFP injection of the transplanted epithelial cells the recipients will be monitored daily for signs of rejection or adverse effects of the surgery. These include such manifestations as inflammation, swelling, malaise, weight loss >20%, labored breathing, lack of grooming and abnormal defecation (e.g. diarrhea). Regarding the experiments affecting liver, there can be signs of liver dysfunction such as jaundice, ascites and behavioral abnormalities. Should any of these signs be observed, the mice will be sacrificed. Following the 10 days post surgery the clips will be removed and the monitoring of the mice will decrease to twice weekly.

For any cells that have not been investigated for their ability to induce experimental metastases (the growth of cancer cells in the lungs), we will monitor the animals daily, looking for signs of distress or discomfort, weight loss greater than 20%, abnormal defecation, or respiratory distress. In ALL cases for all the procedures, animals exhibiting any of the signs listed above will be sacrificed immediately. Animals will be monitored at least twice per week and tumor-bearing animals will be sacrificed before tumor ulceration or before the tumor reaches a volume of 1.0 cm3.

8. Hazards (click here if none are used: ☐)

a) Are the hazards different from original protocol? (infectious, radioactive, toxic, carcinogen, tumours)

YES ☐ NO ☒ if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES:☒ NO:☐ None used:☐

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6

Species	mouse	mouse	mouse	mouse	mouse	mouse
Supplier/Source	CR	In House (IH)	IH	IH	Dr W. Muller	CR
Strain	FVB	c-met (floxed Met)	mmtv Cre	GT Rosa	Neu / floxed-lacZ (activated ErbB2)	CD1 nu/nu
Sex	M/F	M/F	M/F	M/F	M	F
Age/Wt	3-12 weeks	varies	varies	varies	6-12 weeks	3-12 weeks
# To be purchased	max of 20	0	0	0	2	72
# Produced by in-house breeding	max of 100	max of 300	max of 200	max of 200	max of 100	0
# Other (e.g.field studies)	none	none	none	none	none	none
TOTAL# /YEAR	**120**	**300**	**200**	**200**	**102**	**72**

10. Justification of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

Strain 1 - FVB

120 FVB mice is the number needed. The FVB mice are used for the mammary fat pad surgeries and the maintaining for all our lines. The maximum to be purchased is 20 because we will buy new mice only to refresh the genetic of our FVB colony (to avoid cosanguinity). All produced FVBs (100 mice) will be use such as control, as breeder or as a MFP surgery patient, none will be discarded at weaning unless it is sick.
20 + 100 = 120

Strain 2 - c-met

Last year, we accomplished the 7 back-crosses to FVB Background. Now, we have operational heterozygotes (+/-) c-met mice. Our experiments requires homozygotes (-/-) so from now on we will keep only the -/- mice. In the best situatuion, about 30% of the pups of a litter are -/- (often, it is only 10-20%). We need to produces several litters at begining of the year (here goes the first 150 mice but about 30 will be kept). From those 30 -/- mice, we will get progeny (30 mice) as backup for the colony. 10 mice will be used to cross with the Cre mice (see strain 3 for details). 10 mice will be used to cross with GT Rosa mice (See strain 4 for details). The last 100 mice goes for the experiments spreaded over the year. 10 or 20 mice are required at the time. They are euthanised at 12 or 16 weeks old and their mammary fat pads are harvested.
150 + 30 + 10 + 10 + 100 = 300

Strain 3 - mmtv Cre

100 mice will be used for the pure Cre colony maintenance. All negative mice will be euthanised at weaning. By the time we get strain 2 ready for breeding with Cre mice it will be in the second half of the year. From the crossing between mmtv Cre mice and c-met mice, we estimate a production of 100 mice. No experiment is plan for these mice until next year. We are producing them this year.
100+100= 200

Strain 4 - GT Rosa

100 mice will be used for the pure GT Rosa colony maintenance. All negative mice will be euthanised at weaning. By the time we get strain 2 ready for breeding with GT Rosa mice it will be in the second half of the year. From the crossing between GT Rosa mice and c-met mice, we estimate a production of 100 mice. No experiment is plan for these mice until next year. We are producing them this year.

100+100= 200

Strain 5 - Neu
The process of getting those mice is pending. The 2 males we got last year were negative. We will get replacements shortly. The number of mice requested reflects the requirement for identification and maintenance of the transgenic stocks. The number written is the number of mice to be generated, however, the majority of the mice will not be kept. Following genotyping between day 14 and 20, the unsuitable mice will be sacrificed at weaning.
2 + 100 = 102

Theory related
The homozygous floxed-Met mice will also be bred with the floxed-lacZ mice in order to create a line of mice that carries two alleles of both the floxed-Met and floxed-lacZ so that when these mice are crossed with the MMTV-Cre mice the Met RTK will be knocked out in the mammary epithelium and the lacZ reporter gene will become functional in the epithelium. This will allow the knock out cells to be identified upon tissue harvest.

Strain 6 - CD1 nu/nu
In order to establish the role of Crk, a downstream signaling molecule from the Met RTK, in the metastatic and tumorigenic phenotype of human breast cancer cell lines, human breast cancer cell lines (MCF7 and T47D) will be transfected with plasmids to establish RNAi ablation of Crk expression. Cell lines where Crk is successfully ablated will be injected once subcutaneously or via tail vein into nude mice. For each injection 6 mice will be used. Total 12 mice per cell line repeated 3 times 36 mice per cell line.
(3 x 12) + (3 x 12) = 72
SC IV

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 7	Sp/strain 8	Sp/strain 9	Sp/strain 10	Sp/strain 11	Sp/strain 12
Species	mouse	mouse	mouse	mouse	mouse	mouse
Supplier/Source	In House (IH)	IH	IH	IH	IH	IH
Strain	Crk I	Crk II	Met wt	Met Y1003 F	Met M1268T	Met Y1003F/M1268T
Sex	M/F	M/F	M/F	M/F	M/F	M/F
Age/Wt	varies	varies	varies	varies	varies	varies
# To be purchased	0	0	0	0	0	0
# Produced by in-house breeding	Approx 150	300	60	60	60	100
# Other (e.g.field studies)	none	none	none	none	none	none
TOTAL# /YEAR	**150**	**300**	**60**	**60**	**60**	**100**

10. Justification of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

Strains 7 and 8 - Crk I and II

The involvment of the Crk adapter protein in mammary tumorigenesis and mammary development will be observed in transgenic mice expressing Crk I or Crk II adapter proteins under the control of the mammary specific MMTV promoter.MMTV/Crk transgenic MICE WILL BE GENERATED as a fee for service at the McIntyre Transgenic Core facility and mice will be transferred to the H3 facility by the staff of the McGill H3 facility. In the H3 facility the mice will be bred and progeny observed for tumor formation by palpation or sacrifice. At the moment, we are working on the construct for Crk I. The Crk II mice are generated and started to breed in our animal facility. The number of mice requested reflects the necessity of identification and maintenance of transgenic stocks (approx. a max of 300 mice). Using our previous experience with the McIntyre facility as a guide, we should obtain 10 transgenic animals from 30 injections for the Crk I construct. This should be done in the last third of the year. We estimate the production of crk I mice as a maximum of 150. We will initially breed 10 CrkII and 10 CrkI transgenics and F1s will be examined for transgene expression. Total of 10 male/female mice for breeding purposes. Two or three lines for each transgene will be selected and kept for maintenance of each line. MMTV/CrkII transgenic (5 founders) MMTV/CrkI transgenic (5 founders) Total 10 transgenic breeding animals.

Strain 9 - 10 - 11 - 12 : Met wt, Y1003F, M1268T and Y1003F/M1268T

Regarding the following strains : Met wt, Met Y1003F and Met M1268T, we are completing the required number of mice to get a portion of the colony as an aging colony and an other portion to breed for tissue colletion and to maintain it as well. (40 aging mice + 60 young mice = 100 mice per line to keep).

The double mutant mice (Met Y1003F/M1268T) requires to produce more mice because we need to determine which of the ten founders transmit the transgene and get its progeny expressing the transgene in their mammary epithelium. For that purpose, some breeding females will be sacrifice at lactation stage (since the expression is present in the lactating mammary glands), and we need to breed FVB to get Foster parents. As other Met strain mentionned earlier, the double mutant strain requires aging and maintaining mice. So, the maximum to keep at all time for any of these lines is 200 mice (including aging mice from last year).

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 13	Sp/strain 14	Sp/strain 15	Sp/strain 16	Sp/strain 17	Sp/strain 18
Species	mouse	mouse				
Supplier/Source	In House (IH)	IH				
Strain	MT-met wt	MT-met Y1003F				
Sex	M/F	M/F				
Age/Wt	varies	varies				
# To be purchased	0	0				
# Produced by in-house breeding	200	200				
# Other (e.g.field studies)	none	none				
TOTAL# /YEAR	**200**	**200**				

10. Justification of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates. For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

Strain 13 -14 : MT-met wt and Y1003F

The metallothionein section (the MT-met mice). We have developed ten founders expressing an activated Met allele as well as 10 founder animals expressing a wild type Met allele under either the MMTV (mouse mammary tumor virus) or MT (metallothionein) promoter. Founders that transmit the transgene and whose progeny express the transgene in their mammary epithelium will be used to maintain lines. We have to select two to three lines per transgene to maintain and focus our work on. In addition, a group of 60 mice (30 mice per line) to induce transgene expression, nursing mothers of Mt lines and control (non-transgenic FVB) mice will receive a zinc treatment in their drinking water. An other group of MT mice from the selected founders will receive a single injection of diethylnitrosamine (DEN) to promote hepatocarcinogenesis. A part of the colony must age (positive and negative mice) without the treatment, and an other group must receive both treatments which is harmless to the animal. The number of mice requested for strains 1 and 2 reflects the requirement for identification and maintenance of the transgenic stocks as well. We estimate our mouse production at 200 mice to cover all conditions for this year. We already have a total of 100 mice in the room (some are aging, some are stocks and 30 are receiving the zinc sulfate water.)

200 + 100 = 300

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.

Dr. Morag Park	Principle investigator	Will not handle live animals online training course	Yes
Anie Monast	Animal Technician	DEC III Animal Health Science Calas Certification RLAT All McGill training completed Hepatocyte collection (training at Quebec city, collaboration with Dr M.Tremblay since 2003) Partial Hepatectomy surgery	Yes
Dr. Caroline Saucier	Research Associate	McGill mouse workshop McGill on-line training course	Yes
Grigorios Paliouras	Graduate Student	McGill mouse workshop McGill on-line training course Partial Hepatectomy surgery	Yes
Stephanie Petkiewicz	Graduate Student	McGill mouse workshop McGill on-line training course	Yes
Veena Sangwan	Graduate Student	McGill mouse workshop McGill on-line training Hepatocyte collection (training at Quebec city, collaboration with Dr M.Tremblay since 2003)	Yes

*** Indicate for each person, if participating in the local OHP Program, see http://www.mcgill.ca/rgo/animal/occupational/ for details.**

Approved by:

2. Approval Signatures

Principal Investigator/ Course Director		**Date:**
Chair, Facility Animal Care Committee		**Date:**
UACC Veterinarian		**Date:**
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*		**Date:**
Approved Animal Use Period	**Start:**	**End:**

3. Summary (in language that will be understood by members of the general public)

AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge *(was section 5a in main protocol).*

We have shown that the Met receptor tyrosine kinase (RTK) induces epithelial-mesenchymal (E-M) transition in breast epithelium, in addition to the production of angiogenic factors. We have identified signalling pathways crucial to these processes, which are hallmarks of malignant cancer cells. Since the Met receptor tyrosine kinase is deregulated in many human tumor types it is important to develop animal model to understand the role of this receptor in tumorigenesis, tumor angiogenesis, cell invasion and metastasis. These studies would help to identify new potential therapeutic targets in the treatment of cancer harboring deregulated Met receptor in addition to develop animal models for pre-clinical studies

4. Has an unanticipated problem occurred?	**YES** ☐ **NO** ☒	**if yes, supply details:**

5. If creating genetically modified animals or new combinations of genetic modifications, complete and attach a *Phenotype Disclosure form* (http://www.mcgill.ca/rgo/animal/forms/)

6. Procedures

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☐ NO☐

IF NO, complete the following:
Detail new procedures that are different from section 10a of the original protocol *(include a copy of the entire revised procedure section 10a of the original protocol with the changes and/or new procedures in CAPS):*

b) For D level of invasiveness,

Include here ALL procedures except transgenic procedures, including the ones described in the original protocol as well as new and changed procedures in CAPS *(was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.*

Tumorigenesis assay: To assay for the ability of tumor cells (fibroblast or epithelial cells) to form tumor(s) in mice we will be injected subcutaneously with 104-107 of cells [in phosphate buffered saline (PBS) or Dulbecco's modified Eagle's media (DMEM) in a final volume ~100ul]. Animals will be monitor daily and tumor vol measure. Tumor(s) bearing animals will be sacrificed before tumors reach a volume >1.0 cm3 or before the appearance of any tumor ulceration.

In vivo angiogenesis Matrigel-plug assays: To assess the ability of tumor cells to induce angiogenesis, the same procedure as for tumorigenesis assays described above will be performed with the exception that cells will be resuspended with Matrigel (250µl total) rather than PBS or DMEM for their subcutaneous injection. Matrigel is liquid at 4°C but forms a solid gel plug at 37°C trapping the cells. Animals will be monitored applying the same criteria as described for tumorigenesis assays. The animals will be sacrificed between 3-15 days post their injection, at which time the extent of angiogenesis to the Matrigel plugs containing cells will be examined.

Pharmacological Intervention: The ability of VEGF inhibitors to inhibit tumor formation or angiogenesis induced by Met receptor (or Met mutant) expressing cells might need to be tested. For this, cells will be implanted as described previously for tumorigenesis and angiogenesis in vivo Matrigel-plug assays (see above). Treatment will begin 2-4 days after cells implantation by daily-weekly administration via s.c. (at the nape of the neck) or i.p. or i.v. (tail vein) injection of VEGF inhibitor or appropriate vehicle control (e.g. 0.5% glycerol, or saline buffer). VEGF inhibitor expected to be used is well know reagent used extensively in the past in preclinical animal studies : it is VEGF-Trap (Regeneron). The dose, 25 mg/kg, will be injected SC or IV (tail vein) twice a week. The volume to be injected at once will be a maximum of 100ul per mouse (if injected IV) and a maximum of 25ul (if injected SC), depends on dilutions. Animals will be monitored daily and weighted twice a week. Any animals showing any sign of distress or discomfort i.e. weight losses greater than >20%, diarrhea, or respiratory distress will be sacrificed immediately. Otherwise tumor(s) bearing animals will be sacrificed before tumors reach a volume >1.0 cm3 or before the appearance of any tumor ulceration, and primary tumors will be collected for histopathological evaluation. All animals will be sacrificed by cervical dislocation with anesthesia (inhalation of isoflurane).

All cells that we expect to use have been tested for the presence of pathogens (MAP tested). If cells not previously MAP tested for the presence of pathogens is required for preliminary experiments, the quarantine facility will be used at the McIntyre.

Intranipple injection: It will be performed in order to boost the expression of the transgene in the mammary epithelial cells for our poor expressing mouse lines. Ten day pregnant animals will be anaesthetized using the rodent cocktail (ket/xyl/ace) at a dose of 50/5/1 mg/kg body weight (which is 0.1mL/100g body weight). A pulled glass capillary needle will be inserted into the number four teat under a dissecting microscope and approximately 50 uL of retrovirus will be microinjected into the ductal system. The procedure should take no longer than ten minutes starting once the animal is insensible. Once the procedure is complete the mouse will be placed near a heat lamp (3-4 feet depending on bulb strength) and will be monitored until partial consciousness. During the week following the procedure the mice will be monitored daily for signs of inflammation, discomfort or other adverse effects of the surgery. After the first week the mice will be monitored twice weekly for any abnormal mammary gland phenotypes such as mastitis, inflammation or other signs of animal discomfort. Should any of these signs present, the animals will be immediately euthanized. All pups resulting from the experimental animals will be sacrificed as neonates as per SOP.

Partial hepatectomy : Two Thirds Partial Hepetectomy surgery is as desscribed below :
- Anesthetize mouse with avertin (15 ul / g body weight, 2.5% avertin in o.9% saline, ip)
- Shave abdomen
- Paint abdomen with iodovet scrub solution, then wipe with EtOH wipe
- Make a 1 cm longitudinal incision in the skin, then in the peritoneal membrane
- Externalize and separately ligate the median and lateral hepatic lobes with sterile silk or cotton suture material. Remove the lobes leaving a small stump of tissue to prevent the ligature from slipping off. Will use a cautery when required.
- Rinse the peritoneal cavity with approx. 0.5 ml warm sterile saline, then fill the cavity with an additional 0.3 – 0.4 ml to compensate for loss of blood and hepatic tissue mass.
- Suture the peritoneal membrane (continuous suturing), then the skin (discontinuous sutures), using surgical silk (5-0).
- Keep the mouse under a heat lamp, and monitor temperature with a thermometer continuously, until mouse wakes up and becomes ambulatory.

This technic was approved in 2004, a letter was provided with last renewal, Grigorios Paliouras and Anie Monast are approuved
Any complications, adverse results and mortalities associated with this procedure will be reported to the University veterinarian.

Hepatic portal vein (h.p.v.) injection : Mouse is anaesthetized with a dosage of 0.5mg/g of Avertin via an IP injection. 70% ethanol is sprayed on the abdomen and chest. Then the mouse is surgically opened through a ventral midline incision to expose the portal vein and allow for the drug injection. All mice will be under anaesthesia for a maximum of 15 minutes, after which time, they will be sacrificed via cervical dislocation while still under anaesthesia.

Liver perfusion : Mouse is anaesthetized with a dosage of 0.5mg/g of Avertin via an IP injection. Depth of anaethesia is merued until there is no response to pain. Then it is tapped in a tray and 70% ethanol is sprayed on the abdomen and chest. The mouse is surgically opened through a ventral midline incision to expose liver and the hepatic portal vein. We insert a 25g needle into the vein. That needle is connected to a pump. All the perfusion mediums (liver perfusion medium, collagenase and PBS) are placed in a water bath so the their temperature is 42C when it goes into the vein. All mice will be under anaesthesia for a

maximum of 10 minutes, they die when the perfusion starts. The speed of the perfusion is 5 ml per minute. So, the vein is tied behind the bevel of the needle and is cut in the chest. This way only the liver gets perfused. The perfusion starts with the calcium free liver perfusion medium. It will perfuse for 3,5 or 4,5 minutes : it depends on the liver texture. Then, it is 2 up to 3 minutes of collagenase. Once completed, liver is fragile : it is gently removed from the mouse and we collect the hepatocytes in Wash Medium. From here the procedure is a tissue culture one (spins, washes and platting).

7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☐ NO☐

IF NO, supply new endpoints that are different from the original protocol:

b) For D level of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

Experimental Endpoints :
For tumorigenesis assays, tumor(s) bearing animals will be sacrificed before tumors reach a volume >1.0 cm3 or before any tumor ulceration. For Matrigel Plug assays, animals will be sacrificed within 15 days after injection.

FVB STRAIN 2, 3
The males will be euthanised once their reproductive duty will be completed (52 females to fertilize). All females will be pregnant at one point in their life depending on the males activity (the intranipple microinjection is performed on day 10 of pregnancy). The experimental endpoint is 10 weeks following the microinjection of the tpr-met and tpr-met 2F retroviruses (positive and negative control vectors), at which point the animals will be sacrificed and their mammary glands removed in order to determine if there is a malignant change in the mammary gland epithelium. For the mice injected with the Met receptor variants, the endpoint will vary depending on the phenotype observed in the animals. It is expected that some of the mice will develop mammary tumors longer than 10 weeks following the intranipple injection; thus the mice will be allowed to age (for a maximum survival time of a year old) and once the mice display some signs of discomfort or a mammary gland phenotype they will be sacrificed (clinical endpoint, see below). All pups resulting from the experimental animals will be sacrificed as neonates (on date of birth).

STRAIN 4
We will look at 2 different endpoints approximately 40 and 58 hours following PHx. STRAIN 5 :For each substance that will be administered, there is 10 endpoints to look at : 0, 15 sec, 30 sec, 45 sec, 1 min, 2 min, 4 min, 6 min, 10 min, or 15 min. The liver will be removed and flash frozen for removal of protein lysate.

Clinical Endpoints :
For tumorigenesis assays, tumor(s) bearing animals will be sacrificed before tumors reach a volume >1.0 cm3 or before any tumor ulceration. In order to prevent animal suffering, the mice will be monitored daily during critical periods of the experiments and then twice weekly for signs of distress or discomfort, diarrhea or respiratory distress. For all procedures described, animals exhibiting any of the above signs will be immediately euthanized.

Strain 2 : It is anticipated that some of the mice will develop mammary tumors. Additionally, if mice are otherwise healthy, if a tumor mass reaches a volume of 1 cm3 or if there are indications of impending tumor ulceration, the animal will be sacrificed.

Monitoring: To prevent any animals suffering, FOR EVERY PROCEDURES DESCRIBED ABOVE we will look for any sign of distress or discomfort, weight losses greater than >20%, diarrhea, or respiratory distress. Animals exhibiting any of the sign listed above will be sacrificed immediately. Tumors or Matrigel-plug potential metastases tissues will be collected for monitoring and histopathological evaluation after sacrificing the mice by cervical dislocation with anesthesia (inhalation of isoflurane).

8. Hazards (check here if none are used: ☐)

a) Are the hazards different from original protocol? (infectious, radioactive, toxic, carcinogen, tumours)

YES ☐ NO ☒ if yes, supply details (material, risks, precautions):

b) Have the cell lines been tested for human and animal pathogens? YES:☒ NO:☐ None used:☐

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	mouse	mouse	mouse	mouse	mouse	mouse
Supplier/Source	CR	CR	CR	CR	CR	In House
Strain	CD1 nu/nu	FVB	FVB	FVB	FVB	PTP1B Balb/c
Sex	F	F	M	F/M	F/M	F/M
Age/Wt	varies	3 months	3 months	4-5 weeks	Various : 6 to 12 weeks	varies
# To be purchased	480	52	5	18-30	80	0
# Produced by in-house breeding	0	416 pups but will be sacrificed as neonates	0	0	0	60
# Other (e.g.field studies)	none	none	none	none	none	none
TOTAL# /YEAR	**480**	**468**	**5**	**max of 30**	**80**	**max of 60**

10. Justification of Animal Numbers:

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Strain 1 - CD1 nu/nu :
# of cell lines: 6
# of mice/group/cell line tested: 10
# of assays (tumor, metastasis, angiogenesis and pharmacological intervention): 4
# of endpoints: 1

# of repetitions: 2
Total # of mice/year: 6 x10 x 4 x 1x 2= 480

Strain 2 and 3 - FVB F and M 3 months old:
Six (6) retroviral constructs will be used for infecting the mammary epithelium of the female mice mid-pregnancy by intranipple injections. These six contructs include the positive and negative controls that will be used for the preliminary studies to establish and confirm our technique. Six mice will be used for both the positive and negative controls making a total of 12 mice for the preliminary studies. The four Met RTK variant constructs will each be injected into ten (10) mice making a total of 40 mice. These ten mice allow for a failure rate of 30% which would still leave us with a sufficient number of mice carrying each construct to analyze. Thus, adding the 12 mice for the preliminary studies and the 40 mice for the final studies, this generates a total of 52 mice. The five males to be purchased from Charles River are necessary for impregnating the females. Concerning the pups, all pups resulting from the experimental animals will be sacrificed as neonates as per SOP. We can estimate the production to be approximately 416 pups (52 females X 8 pups). Total : 52 + 416 = 468.

Strain 4 - FVB 4-5 weeks :
The Met RTK signalosome will be examined during liver regeneration by performing a two thirds partial hepatectomy (PHx). In the two thirds PH model, the left and medial hepatic lobes are ligated and excised, resulting in removal of 65% to 70% of the liver. It has already been shown that there is elevated levels of HGF up to 72 hours following PHx. During mouse liver regeneration, the first peak of DNA synthesis occurrs in the parenchymal cells (hepatocytes and bile duct epithelial cells) 40 hours after resection. Another peak of DNA synthesis occurs 12-24 hours later in the nonparenchymal cells. Therefore we would be interested in looking at 2 different time point during liver regeneration, approximately 40 and 58 hours following PHx. So, in theory we will do 3 groups of 3 mice for each time points (40 hrs, 58 hrs). According to the results, the number of mice required should start with 18 but we want to get a larger approuved limit in case we need to do more groups. In any case, the maximal number of mouse to be used would be 30.

Strain 5 - FVB 6-12 weeks:
To examine protein-protein complex formation at the Met tyrosine kinase receptor in the liver, mice will be injected with hepatocyte growth factor (33.75 ng/ul), InlB (150 nM), or BpW(phen) (0.08 ug/g) (in PBS in a final wolume of 0.2 or 0.5 ml) using one of the following routes, p.v. or i.p.. For each substance that will be administered, 10 mice will be needed to allow us to eveluate the recruitment of proteins to the Met tyrosine kinase receptor. We will be performing non-survival surgery, such that at the end of each time point (0, 15 sec, 30 sec, 45 sec, 1 min, 2 min, 4 min, 6 min, 10 min, or 15 min) (Khan et. al. 1989) the liver will be removed and flash frozen for removal of protein lysate. The experiment will be done twice. We will use 10 mice as control for each time point. So, the total of mice is (30 experimental mice +10 control mice) x 2 = 80 mice.

Strain 6 - PTP1B :
The PTP1B mice are wild type (wt) and knockout (ko). We have a breeding of each. First we want to breed them to get stock of at least 10 mice for each line. Then, all the females progeny will be use in pairs for liver perfusions (1 wt and 1 ko). The males are used only in the colony maintnance. The liver perfusion will be performed on 3-5 weeks old mice. We estimate the number of experiment at 20.
(10 wt + 10 ko) + (2 types x 20 exp) = 60

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.
This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.