MET and MYC cooperate in mammary tumorigenesis

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In human breast cancer, overexpression of the protooncogene *MET* is strongly associated with poor prognosis and high risk of metastasis. It stands out as a reliable prognostic indicator of survival and defines a set of tumors exclusive of those that express *HER2* or hormone receptors. Studies have shown that overexpression of mutant forms of *MET* cause cancer in mice. However, *MET* mutations have not been found in human breast cancer, and the consequences of overexpression of normal *MET* are unknown. To investigate the role of *MET* and other putative oncogenes in breast cancer, we developed an experimental system that involves retroviral delivery of genes into primary mammary epithelial cells, followed by transplantation of the transduced cells into mammary fat pads. Using this approach, we found that overexpression of wild-type *MET* leads to the development of nonprogressive neoplasms. The lesions progressed to mammary adenocarcinoma when a second protooncogene, *MYC*, was overexpressed, indicating that *MET* and *MYC* cooperate in mammary tumorigenesis. Both the nonprogressive neoplasms and adenocarcinomas display characteristics consistent with transformation and expansion of mammary progenitor cells. The approach described here should provide a useful model with which to efficiently test effects of various genes on tumor development in the breast.

breast cancer | mouse models | retrovirus

The *MET* protooncogene encodes a transmembrane receptor tyrosine kinase, Met, that has been widely implicated in tumorigenesis. Mutations that result in constitutive activation of Met are common in hereditary papillary renal carcinoma and have also been reported in hepatocellular carcinoma, gastric cancer, ovarian cancer, and squamous cell carcinoma. In addition, the wild-type *MET* gene is amplified or overexpressed in many other types of human cancer, including breast cancer (1). Several studies have found that high levels of Met are associated with poor prognosis and high risk of metastasis in breast cancer (2–4). Overexpression of Met occurs in a group of tumors distinct from those that express estrogen and progesterone receptors and those that overexpress *HER2* (5), suggesting that inhibition of Met may provide an opportunity to treat tumors for which no targeted therapy is available.

Little is known about the tumorigenic function of *MET* *in vivo*. Overexpression of constitutively activated *MET* mutants under the metallothionein-1 promoter led to the development of metastatic mammary tumors in transgenic mice (6, 7), and replacement of the wild-type *MET* allele with various *MET* mutants resulted in the development of several types of tumors (8). Although these results provide insight into the tumorigenic capabilities of mutant Met proteins, our goal was to generate a mouse model in which the effects of wild-type *MET* overexpression could be studied, because *MET* is not mutated in human breast cancer.

Existing mouse models of breast cancer exhibit significant differences in pathology and metastasis from the human disease (9). This may be partly attributed to different breast anatomy and physiology between the two species. It is also possible that the majority of genetically engineered mouse models, which target expression of oncogenes to the mammary gland under promoters that are preferentially expressed in differentiated cells, do not recapitulate spontaneous transformation of various cell types in

the human breast. Thus, mouse models in which different cell types can be targeted for transformation might prove useful. A recent report described that overexpression of certain oncogenes, but not others, results in expansion of the mouse mammary progenitor cell population, and this correlates with heterogeneous tumor phenotypes (10). This phenomenon supports a current model for human breast cancer in which stem or progenitor cells become transformed, resulting in cellular heterogeneity within tumors as well as phenotypic diversity between tumors (11, 12). Effects of oncogene activation in mammary progenitor cells are difficult to examine, because mammary-specific promoters are highly responsive to steroid hormones (13, 14) and are highly expressed in more differentiated steroid receptor-positive cells. Specific targeting of transgene expression in mammary progenitor cells has not yet been described.

In the developing mammary gland, stem and progenitor cells are present within terminal end buds and along mature ducts and give rise to both myoepithelial and luminal epithelial lineages (15–17). Molecular characterization of mammary progenitor cells has revealed that they are contained within a population of cells that express Sca-1 (15). However, ≈20% of mammary epithelial cells (MEC) are Sca-1-positive, indicating that not all Sca-1-positive cells are progenitor cells. A second more restricted marker of these progenitor cells is cytokeratin 6 (CK6). CK6-positive cells are contained within the Sca-1-positive population (10). CK6 is normally expressed in the mammary gland during embryonic stages and within terminal end buds during ductal morphogenesis, but CK6-positive cells are rare in mature differentiated glands (18).

To better understand the contribution of genes such as *MET* to breast cancer without restricting expression to differentiated cells, we optimized a retroviral system to introduce genes of interest into the mammary gland with a vector that was likely to express *MET* in stem and/or progenitor cells. Using this system, we found that overexpression of *MET* resulted in the development of multiple microscopic foci of neoplasia that failed to progress to full-blown malignancies. Although overexpression of *MET* alone did not result in the development of tumors, we found that an additional genetic stimulus, overexpression of *MYC*, cooperated with *MET* in mammary tumorigenesis. Overexpression of *MYC* alone resulted in epithelial hyperplasia as previously reported in studies of transgenic mice (19–21). The nonprogressive neoplasia (NPN) and tumors resulting from *MET* and *MET/MYC* overexpression, respectively, displayed cellular markers characteristic of mammary progenitor cells, suggesting that *MET* may play an important role in transformation and expansion of these cells.

Materials and Methods

Transgenic Mice. Mouse mammary tumor virus (MMTV)-rtTA transgenic mice were obtained from Lewis Chodosh (University of Pennsylvania School of Medicine, Philadelphia) (22). TRE-

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Abbreviations: CK6, cytokeratin 6; MEC, mammary epithelial cells; MMTV, mouse mammary tumor virus; NPN, nonprogressive neoplasia.

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Fig. 1. Overexpression of wild-type *MET* resulted in abnormal mammary gland development. Mammary gland outgrowths expressing either GFP alone (A, C, and E) or *MET* and GFP (B, D, and F) were evaluated by whole-mount microscopy 10 wk after transplantation. Infected cells were visualized by GFP fluorescence (A–D), and the entire gland was visualized by carmine alum staining (E and F).

Ten weeks after transplantation, mammary gland outgrowths that overexpressed *MET* displayed abnormal lobule-like structures (Fig. 1 B, D, and F). Glands arising from MEC expressing GFP alone had no abnormalities (see Figs. 1 A, C, and E and 2 A, C, and E). Hematoxylin/eosin staining of sections from mammary gland outgrowths revealed that the “lobules” in *MET*-expressing glands were actually disorganized clusters of epithelial cells (Fig. 2B). Immunohistochemical staining showed that the cytokeratin 18-positive luminal epithelial cells were abnormally clustered together, and often there was little or no discernable lumen (Fig. 2D). Likewise, the cytokeratin 14-positive myoepithelial cells were either scattered about or organized around filled islands of cells (Fig. 2F). Histological analysis confirmed that the epithelial cells were atypical with large nuclei, condensed chromatin, large nucleoli, and little cytoplasm (data not shown). TRE-*MET*/pMIG-tTA mammary outgrowths from mice that were treated with doxycycline to repress expression of *MET* were not distinguishable from those expressing GFP alone (Fig. 2G and data not shown).

Based on accepted nomenclature according to the Annapolis guidelines (www.nih.gov/Annapolis-guidelines) for mouse mammary pathology, the phenotype induced by overexpression of *MET* most closely resembles mammary intraepithelial neoplasia, a spectrum of intraluminal epithelial proliferations with cytologic atypia that includes *in situ* carcinomas (31). However, many of the lesions resulting from overexpression of *MET* do not have an intact myoepithelial layer, indicating the neoplasias are not strictly intraluminal. Because the lesions do not progress to tumors (see below), we refer to the *MET*-induced lesions as NPN. A total of 26 glands expressing *MET* were analyzed in five independent experiments. Multiple microscopic neoplastic foci

Fig. 2. Overexpression of *MET* resulted in ductal filling and loss of organization. Glands expressing GFP (A, C, and E) or *MET* and GFP (B, D, and F) were sectioned and stained with hematoxylin/eosin (A and B). Alternatively, sections were stained with antibodies specific for cytokeratin 18 to visualize luminal epithelial cells (C and D) or with antibodies against cytokeratin 14 to detect myoepithelial cells (E and F). Sections from mice that received MEC in which *MET* expression was repressed by treatment with doxycycline (G) or from mice that received MEC overexpressing *MET* (H) were stained with antibodies specific for tyrosine-phosphorylated Met.

were observed in $\approx 77\%$ (20/26) of the glands after successful transplantation. No green cells were detected in 6/26 of the glands despite successful mammary reconstitution. An additional nine glands were analyzed from mice maintained on doxycycline to repress *MET* expression. All nine glands appeared normal (as shown in Fig. 2G).

Immunohistochemical staining with antibodies specific for Met (not shown) and tyrosine-phosphorylated Met (Fig. 2H) verified that Met protein was present and actively signaling in epithelial cells that filled the ducts but not in the surrounding stroma. There was evidence for an inflammatory response, manifested by the presence of increased numbers of CD45⁺ leukocytes compared to the control outgrowths (data not shown); however, these cells represented only a small portion of the stroma in *MET*-expressing outgrowths. Our data show that overexpression of wild-type *MET* in the mouse mammary gland leads to NPN with loss of ductal organization. The phenotype was observed by 8 wk after transplantation but did not progress; no tumors or noticeable worsening of the neoplastic lesions was detected over a period of 8 months.

To rule out the possibility that the observations made with

overexpression of *MET* via retroviral transduction/transplantation were an artifact of the procedure, we tested whether MEC expressing *MET* under control of the MMTV promoter (which has no effect in the intact mammary gland; Fig. 7) would develop NPN upon infection and transplantation. For these experiments, we isolated MEC from 10- to 12-wk-old MMTV-rtTA/TRE-*MET* transgenic mice, infected them with pMIG, and transplanted them into cleared fat pads of 21-day-old mice that were treated with doxycycline throughout the experiment to induce *MET* expression. Ten weeks after transplantation, the resulting glands did not show any abnormalities by fluorescent whole-mount analysis (data not shown). These results show that the retroviral infection/transplantation technique does not promote abnormal ductal outgrowth.

***MET* and *MYC* Cooperate in Mammary Gland Tumorigenesis.** Because mammary outgrowths expressing *MET* via retroviral transduction do not progress to tumors over the course of several months, we speculated that the transplants might represent an ideal model in which to directly test cooperation of *MET* with other oncogenes in tumor progression. It has been demonstrated that multiparous transgenic mice expressing the ligand for Met, hepatocyte growth factor/scatter factor develop mammary tumors with an average latency of 8 months, and among other alterations, the tumors were shown to contain excessive amounts of activated Myc protein (32). *MYC* has been implicated in many types of human cancer, and it is overexpressed in a subset of breast cancers (33). Although *MET* and *MYC* loci are coamplified in some gastric cancers (34, 35), it is not known whether *MET* and *MYC* are cooverexpressed or whether these genes cooperate in the genesis of breast cancer.

To test whether overexpression of *MYC* would cause the *MET*-induced NPN to advance to tumors, we infected MEC from TRE-*MET* transgenic mice with pMIG-tTA alone or pMIG-tTA and pMIG-*MYC*. Alternatively, we infected MEC from non-transgenic mice with pMIG-*MYC* alone. Eight weeks later, we examined the mammary outgrowths. Although *MET* expression alone induced NPN (Fig. 3 A–C), expression of *MYC* alone caused increased branching and hyperplastic lateral budding (Fig. 3 D–F), as previously described in transgenic mouse models (19, 21). Coexpression of *MET* and *MYC* resulted in high-grade neoplasia in which ductal organization was no longer recognizable by 8 wk after transplantation (Fig. 3 G–I). Glands expressing both *MET* and *MYC* displayed a highly disorganized clustering of cells, demonstrated by staining for cytokeratin 14 (Fig. 3 C, F, and I). Whereas myoepithelial cells could still be detected surrounding ductal structures in outgrowths overexpressing *MYC* alone (Fig. 3F), outgrowths expressing either *MET* alone (Fig. 3C) or *MET* and *MYC* (Fig. 3I) were no longer organized and often did not contain an intact myoepithelial cell layer.

Although no tumors were observed in glands expressing either *MET* or *MYC* alone, mammary glands coexpressing *MET* and *MYC* progressed to focal palpable tumors by 10 wk. Histological analysis of tumors at 4 months revealed mammary adenocarcinoma (Fig. 4A), with features typical of highly proliferating lesions including prominent nucleoli and high mitotic indices (Fig. 4B). A total of 25 glands in two independent experiments were analyzed for cooperation between *MET* and *MYC*. Tumor penetrance was 100% after successful transplantation. An additional 17 glands were analyzed from mice that received doxycycline to repress *MET* expression (*MYC* was expressed alone). All of these glands developed various degrees of hyperplasia, as shown in Fig. 3 D–F.

Overexpression of *MET* in Neoplasms Correlates with an Abundance of the Early Developmental Marker CK6. Tumors resulting from overexpression of *MET* and *MYC* were remarkable in that they contained cells from both myoepithelial and luminal epithelial

Fig. 3. *MET* cooperates with *MYC* to promote progression of neoplasia in the mammary gland. Whole mounts of mammary gland outgrowths harvested 8 wk after transplantation were viewed by fluorescent microscopy (A, D, and G) and by carmine alum staining and light microscopy (B, E, and H). Sections from mammary glands isolated at this stage were also stained with antibodies against the myoepithelial marker cytokeratin 14 (C, F, and I). Glands that expressed *MET* alone (A–C), *MYC* alone (D–F), or *MET* and *MYC* (G–I) are shown.

cell lineages, as illustrated by staining for cytokeratins 14 and 18, respectively (Fig. 4 C and D). A recent report revealed that mammary hyperplasias and tumors that are composed of heterogeneous cell types also express early progenitor cell markers such as Sca-1 and CK6 (10). To determine whether tumors arising from *MET*/*MYC* overexpression might be a result of progenitor cell expansion, we examined the expression of CK6 by immunohistochemistry. Fig. 5A shows that *MET*/*MYC*-induced tumors contained numerous CK6-positive cells and thus fit into a class of tumors in which heterogeneity of cell types correlates with aberrant expression of the early developmental marker CK6.

Fig. 4. *MET* and *MYC* cooperate in mammary tumorigenesis. Sections of tumors resulting from expression of both *MET* and *MYC* were stained with hematoxylin/eosin (A and B). The tumors were isolated 4 months posttransplantation. Sections were also stained with antibodies against cytokeratin 14 (C) and cytokeratin 18 (D).

Fig. 5. Neoplastic lesions induced by *MET* express the mammary progenitor cell marker CK6. Immunohistochemistry with antibodies specific for CK6 was carried out on sections from the following samples: adenocarcinoma harvested 4 months after coexpression of *MET* and *MYC* (A), *MET*-induced neoplasia harvested 10 wk after transplantation (B), 10-wk mammary outgrowths that expressed GFP alone (C), and mammary glands from 10-wk-old virgin wild-type (D) or MMTV-rtTA/TRE-*MET* (E) mice. A mammary gland from a 1-year-old MMTV-rtTA/TRE-*MET* mouse containing a rare focal hyperplasia is shown in F.

Because *MET*/*MYC* tumors display characteristics consistent with expansion of a progenitor cell population, we investigated whether NPN might be the result of *MET* overexpression in progenitor cells. We found that lesions resulting from overexpression of *MET* contained large numbers of CK6-positive cells (Fig. 5B), whereas mammary outgrowths expressing only GFP and mammary glands from unmanipulated mice contained few or no CK6-positive cells (Fig. 5C and D). Mammary glands from most MMTV-rtTA/TRE-*MET* transgenic mice also had virtually no CK6-positive cells and were not distinguishable from wild-type controls (Fig. 5E). Rare hyperplasias observed in MMTV-rtTA/TRE-*MET* mice >1 year of age were CK6-positive (Fig. 5F). Because *MET* is overexpressed and activated in glands from all MMTV-rtTA/TRE-*MET* mice (Fig. 7), we conclude that expression of CK6 is not merely a result of *MET* activity.

Discussion

Development of Breast Cancer Models Using Ecotropic Retroviral Infection and Transplantation of Primary MEC. We report the optimization of a system to efficiently overexpress genes of interest in the mouse mammary gland. This method has allowed us to generate mouse models of breast cancer that are different from the commonly used transgenic models. Overexpression of wild-type *MET* using our system resulted in multiple microscopic foci of neoplasia that did not progress to malignancy in the time frame of our experiments (>8 months) unless *MYC* was also overexpressed. This model should be useful to study progression of cancer and for the identification of additional oncogenic insults that can cooperate with *MET* in tumorigenesis.

In contrast to the results obtained by overexpression of wild-type *MET* into MEC via retroviral transduction and transplantation, overexpression of wild-type *MET* under the MMTV promoter did not result in abnormal mammary glands. It is formally possible that the MMTV promoter does not elicit *MET* expression at the exact developmental time or at levels above a required threshold to achieve a neoplastic phenotype. Because *MET* overexpression and activation are readily detectable at similar time points in both systems by parallel immunohistochemistry, however, it is more likely that the difference in outcomes lies in the cell types in which the promoters are expressed. Overexpression of *MET* by the MMTV promoter may occur mainly in differentiated, hormone-responsive cells, and that may not result in cell transformation. In contrast, the retroviral vector used in the present work expresses in a variety of cell types (data not shown), including progenitor cells that give rise to mammary gland outgrowth (as in Fig. 1). Furthermore, repopulation of the mammary gland after transplantation of MEC requires progenitor cell expansion (15, 30), so mature outgrowths that overexpress *MET* presumably arose from progenitor cells overexpressing *MET*.

***MET*-Induced Neoplastic Lesions Display Characteristics of Mammary Progenitor Cells.** Luminal epithelial cells and myoepithelial cells within the mammary gland are derived from a common progenitor (36, 37). Most mouse mammary tumors display a loss of cytokeratin 14-positive myoepithelial cells as the luminal epithelium expands. However, we found that tumors resulting from overexpression of *MET* and *MYC* in our system contained both luminal and myoepithelial cells, suggesting that the tumors might arise from an expanded population of bipotential mammary progenitor cells.

Expansion of mammary progenitor cells in this model is further supported by our finding that NPN initiated by overexpression of *MET* alone consisted of CK6-positive cells. An abundance of CK6-positive cells is thought to reflect expansion of a progenitor cell population (10). It has previously been found that expression of *MET* increases 5-fold during differentiation of human breast progenitor cells *in vitro* (37). Although *MET* expression may be regulated during differentiation of breast epithelium, the outcome of aberrant expression of *MET* in progenitor cells has not directly been tested.

Similar to our results, hyperplasias and tumors induced by Wnt-1, β -catenin, or Myc are heterogeneous in nature and express both CK6 and Sca-1. On the other hand, hyperplasias and tumors driven by Neu, H-Ras, or the polyomavirus middle T antigen do not express CK6 or Sca-1 and are much more homogenous in nature (10). Because of the presence of early developmental markers and the coexistence of multiple epithelial cell lineages, the former group of tumors is thought to arise from expansion of a transformed progenitor cell population, whereas the latter group might arise from transformation of a more differentiated cell type. Thus far, tumors suspected to arise from a putative progenitor cell population all express oncogenes that lead to activation of components of the Wnt signaling pathway (10). Our data remain consistent with this: β -catenin was found in the cytoplasm and nuclei of cells within *MET*-induced NPN, indicating that it is active (Fig. 8, which is published as supporting information on the PNAS web site). Tumors arising in WAP-HGF transgenic animals also displayed activation of β -catenin (32), and *MET* has been implicated in β -catenin activation in other cell types as well (38, 39).

It is important to note that not all hyperplasias or tumors arising from the retroviral transduction/transplantation system express CK6. For example, we have found that tumors arising from expression of the Polyomavirus middle T antigen were CK6-negative whether the oncogene was expressed by retroviral transduction/transplantation (Fig. 9, which is published as sup-

porting information on the PNAS web site) or from the MMTV promoter (10). In contrast, hyperplasias resulting from overexpression of *MYC* were CK6-positive in both MMTV-*MYC* transgenic mice (10) and in outgrowths resulting from retroviral transduction of *MYC* (Fig. 9). Based on these results, it is likely that expression of an oncogene in progenitor cells (as with the retroviral transduction/transplant system) is not sufficient for generation of tumors that consist of an expanded progenitor cell population. Instead, transformation and expansion of progenitor cells may also require the function of particular oncogenes.

There is a clear association between CK6 expression and the Sca-1⁺ mammary progenitor cell population: CK6⁺ cells are present in the Sca-1⁺ cell population and absent in the Sca-1[−] cell population (10). CK6 expression might be a better marker of mammary progenitor cells, because it is more restrictive than Sca-1 expression (10). Still, it should be noted that CK6⁺ cells have not yet been proven to be bona fide progenitor cells that are capable of giving rise to multiple cell types in the mammary gland. This will require either identification of new progenitor cell markers in the mammary gland, or a method to sort viable cells based on expression of intracellular markers such as CK6 without permeating the cell membrane.

MET and Metastasis. Met activity has been implicated in cell migration and invasion, and there is a strong clinical association between *MET* overexpression and poor prognosis due to metastasis. We did not detect metastasis from *MET/MYC*-induced mouse mammary tumors (unpublished data), although our experimental system is capable of giving rise to metastatic tumors initiated by other protooncogenes (unpublished data). Our data suggest that, at least in mice, *MET* overexpression is not sufficient for tumor metastasis. We have not ruled out that *MET* plays a role in this process, perhaps in cooperation with other factors.

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