Genomic Progression in Mouse Models for Liver Tumors

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The principal cause of human liver cancer is infection with hepatitis viruses B and C, but tumor progression is fueled by ensuing perturbations that confer gain of function on proto-oncogenes or loss of function on tumor suppressor genes. Frequent among these perturbations is overexpression of the proto-oncogene MET. We have modeled the pathogenesis of liver tumors by expressing conditional transgenes of MET in the hepatocytes of inbred mice. The response to the MET transgene varied with both the magnitude and timing of its expression but included hyperplasia of hepatic progenitor cells, as well as benign and malignant tumors that display both phenotypic and genotypic resemblances to human counterparts. The results reveal MET to be a crucial switch in the development of the liver; dramatize how different cellular compartments within a developmental lineage can give rise to distinctive tumor cell lines; delineate rules of tumor progression; provide evidence that the experimental tumors in mice are authentic models for human tumors; and support a role for MET in the genesis of human liver tumors. The models should be useful in elucidating the mechanisms of tumorigenesis and in the preclinical testing of new therapeutics.

Neoplastic tumors arise from a stepwise sequence of events known as tumor progression (Nowell 1976; Kinzler and Vogelstein 1996). Each step in the sequence is thought to represent a discrete genetic or epigenetic aberration, typically affecting the structure or expression of either a proto-oncogene or tumor suppressor gene. This scheme engenders a number of questions. What causes each of the steps in tumor progression? How does each step contribute to tumorigenesis? Are there discrete and specific genotypic pathways to each form of neoplasm? Must the steps in these pathways occur in a particular chronological sequence? How constrained is the tumorigenic pathway, once an initiating event has occurred? Which of the steps in any given pathway might be suitable targets for therapeutic intervention? We cannot answer these questions solely by the study of human tumors. Instead, we need to reconstruct the events of tumorigenesis in a prospective manner. The most desirable means to this end would be the development of animal models that replicate tumorigenesis as it occurs in humans. For a host of reasons, the animal of choice is the laboratory mouse. There is a venerable tradition of denigrating mouse models for cancer, and with good cause, but now the tide has turned. Recent years have seen the development of mouse models that are based on the faithful reconstruction of genetic lesions found in human cancer. The result has been models that offer both biological and molecular authenticity. Here we illustrate this advance by describing the development and use of mouse models for benign and malignant tumors of the liver, the genesis of which resembles that of hepatocellular tumors in humans. Cancer of the liver is among the most common and grievous of human malignancies (Parkin et al. 2005). Although relatively infrequent in developed nations, at least 600,000 new cases occur worldwide every year. The average survival time is 6 months. The only therapeutic recourse of note is surgical resection and liver transplantation.

The principal cause of human liver cancer is infection with either hepatitis B virus or hepatitis C virus, although dietary toxins and chronic alcoholism have also been implicated (Llovet et al. 2003). Downstream of these causes, however, lie perturbations that confer gain of function on proto-oncogenes or loss of function on tumor suppressor genes (Thorgersson and Grisham 2002). Frequent among these perturbations is overexpression of the proto-oncogene MET (Tavian et al. 2000), which encodes a receptor protein-tyrosine kinase (Met), activated by a ligand known as either hepatocyte growth factor or scatter factor (Trusolino and Comoglio 2002). We have modeled the pathogenesis of liver tumors by expressing conditional transgenes of MET in the liver cells of inbred mice. The response to the MET transgene varied with both the magnitude and timing of its expression, but included arrested development of the liver, as well as benign and malignant liver tumors that display both phenotypic and genotypic resemblances to human counterparts. Our results provide insight into the genotypic underpinnings of tumor progression. The models should be useful in studying the mechanisms of tumorigenesis and in the preclinical testing of new therapeutics.

PATHOGENIC EFFECTS OF MET VARY WITH THE MAGNITUDE AND DEVELOPMENTAL TIMING OF GENE EXPRESSION

To replicate the overexpression of MET found frequently in human liver tumors, we targeted the expression of a transgene representing human MET to liver cells by using the transcriptional control element for the liver-enriched activator protein gene (Wang et al. 2001). Four in-
expression of a MET on the protein without reducing its abundance (Ishikawa et al. 2001 and data not shown). However, the liver occurs throughout embryogenesis and into adult life and timing of expression (Fig. 1).

If instead, the same transgene was inactivated 1 month after birth (Fig. 1, second protocol), the hyperplastic livers were remodeled into mature tissue, and survival of the animals was prolonged (Fig. 2 and data not shown). Within several months, however, the mice developed fatal hepatic carcinomas composed of both biliary and hepatic lineages (Fig. 2). The mixed nature of the tumors suggested that tumorigenesis was initiated from a relatively primitive, bipotential cell. The tumors displayed a marker of hepatic progenitor cells, PAH and GS are markers for mature hepatocytes—AFP for the former, PAH and GS for the latter (data not shown). Similar tumors arose, albeit more slowly, even if activation of the MET transgene was delayed until after weaning of the mice (data not shown).

Figure 1. Protocols for the induction of liver tumors with transgenic MET. The strains of transgenic mice used here have been described previously (Wang et al. 2001). Expression of the MET transgene could be repressed by administration of doxycycline (OFF) or permitted by withholding doxycycline (ON). Two strains of mice expressed the transgene at relatively high levels (Lines 1 and 2 in Wang et al. 2001); two others expressed it at relatively low levels (Lines 3 and 4 in Wang et al. 2001). The first three protocols diagrammed in the figure employed Line 2, the fourth protocol employed Line 3. Doxycycline was administered (circles) or withheld (squares) at the indicated time points. Solid black indicates activity of Met kinase, solid white indicates absence of such activity despite the presence of Met protein.

dependent strains of transgenic mice were developed, in each of which expression of the MET transgene could be inactivated by administration of doxycycline (Wang et al. 2001). Two strains expressed the transgene at relatively high levels (previously designated lines 1 and 2), whereas two expressed it at lower levels (previously designated lines 3 and 4) (for details, see Wang et al. 2001). The phenotypic response was dependent on both the magnitude and timing of expression (Fig. 1).

Physiological expression of MET in the normal mouse liver occurs throughout embryogenesis and into adult life (Ishikawa et al. 2001 and data not shown). However, the kinase activity of the gene product subsides soon after birth, apparently in response to controls that act directly on the protein without reducing its abundance (Ishikawa et al. 2001 and data not shown). In contrast, hepatic expression of a MET transgene at a high level in utero and continuing after birth produced sustained activity of the kinase (data not shown), and this in turn led to death within 3–8 postnatal weeks (Fig. 1, first protocol). Prior to death, the animals had greatly enlarged, hyperplastic livers composed of immature precursor cells (Fig. 2 and data not shown). Death was apparently caused by the absence of mature liver function.

If instead, the same transgene was inactivated 1 month after birth (Fig. 1, second protocol), the hyperplastic livers were remodeled into mature tissue, and survival of the animals was prolonged (Fig. 2 and data not shown). Within several months, however, the mice developed fatal hepatic carcinomas composed of both biliary and hepatic lineages (Fig. 2). The mixed nature of the tumors suggests that tumorigenesis was initiated from a relatively primitive, bipotential cell. The tumors displayed a marker of precursor cells (AFP, PAH) and a marker of mature hepatocytes (phenylalanine hydroxylase, PAH). A second marker of mature cells, glutamine synthetase (GS), was absent. The extensive proliferation of progenitor cells and mature hepatocytes (phenyalanine hydroxylase, PAH) and glutamine synthetase (GS). AFP is a marker for hepatic precursor cells, PAH and GS are markers for mature hepatocytes. The tumors displayed a marker of precursor cells (AFP).
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mor progression occurring in response to a distinctive genotypic change (see below, Conclusions).

GENOTYPIC PROGRESSION IN THE GENESIS OF LIVER TUMORS

The morphological progression to HCC elicited by relatively low expression of transgenic MET had biochemical and genomic counterparts. Phosphorylation of the Met protein was used as a surrogate marker for activation of the protein-tyrosine kinase. Despite abundant expression of the transgenic protein (data not shown), no kinase activity could be detected in normal liver of the adult mice (Fig. 3), so it appears that the transgenic protein was susceptible to posttranslational control of its enzymatic activity, reminiscent of that observed for endogenous Met in normal mice (see above). Met kinase activity became apparent in cells of hyperplastic foci, intensified in dysplastic cells, and was abundant in end-stage malignancies (Fig. 3).

It seemed likely that additional genetic events contributed to tumorigenesis in the model. For hints of what these might be, we turned to the example of human HCC. Among the more frequent genetic anomalies in such tumors is the mutational activation of $\beta$-catenin, a multifunctional protein that mediates signaling to the transcriptional apparatus through the Wnt pathway (Giles et al. 2003). In the absence of Wnt signaling, $\beta$-catenin is located in the cytoplasm and at the plasma membrane. Cytoplasmic $\beta$-catenin is situated in a protein complex that facilitates phosphorylation and consequent destruction of the protein. When activated by signaling from Wnt, however, degradation of $\beta$-catenin is inhibited and much of the protein moves to the nucleus, where it then serves as a transcription factor. Thus, nuclear localization can be used as a surrogate marker for activation of $\beta$-catenin in the Wnt pathway.
Immunostaining for β-catenin revealed intense concentration of the protein at the periphery of normal liver cells, and the cells of both hyperplastic and dysplastic foci (Fig. 3). In contrast, the protein was abundant in the nuclei of most cells in the full-blown HCC (Fig. 3). The activation of β-catenin in malignant cells was attributable to mutations found in the β-catenin gene (Fig. 5). The mutations were analogous to those found in human HCC and included both point mutations and small deletions. These changes preclude the phosphorylations that elicit the degradation of β-catenin (Giles et al. 2003) and thus serve to constitutively activate the protein.

Multiple nodules of HCC developed in most affected livers. Individual nodules always contained a single mutant allele of the β-catenin gene (CTNNB1), indicating that the nodule was probably clonal in origin (data not shown). However, the mutations varied from one nodule to another, signaling independent origins of each nodule. The consistency with which the mutations were found in tumors suggests that they confer some selective advantage during clonal evolution of the emerging tumor cells and provides strong circumstantial evidence for a role in tumorigenesis.

The adenomas in transgenic animals displayed activation of the Met kinase, but not of β-catenin (data not shown). Sequencing of the β-catenin gene in adenomas also failed to uncover any mutant alleles (data not shown). In search for other possible genetic lesions, we turned again to the example of human tumors. Hepatic adenomas of humans occur in both congenital and sporadic forms (Zucman-Rossi 2004). In the congenital form, tumor susceptibility is apparently transmitted by a heterozygous deficiency in the HNF-1α gene (TCF1) (Bacq et al. 2003), and homozygous deficiencies of HNF-1α are frequent in sporadic adenomas (Bluteau et al. 2002). We have failed to find any genomic damage to HNF-1α in the adenomas of the mouse model (data not shown), but the signaling pathway to which HNF-1α contributes appears to be deficient, as manifested by an absence of PAH (Fig. 4), production of which is dependent on signaling from HNF-1α (Pontoglio et al. 1997).

**RECONSTRUCTION OF TUMORIGENESIS BY HYDRODYNAMIC TRANSFECTION**

Knowing that abnormalities of both Met and β-catenin apparently contributed to the genesis of HCC in the transgenic mice, we sought to explore their individual roles in tumor progression. To this end, we used hydrodynamic transfection to introduce and express ectopic genes in the livers of adult mice (Liu et al. 1999; Zhang et al. 1999; Yant et al. 2000). We used a wild-type allele of MET, as in the transgenic model, and a mutant allele of the β-catenin gene that encodes a constitutively active protein (Barth et al. 1997). The results are summarized in Figure 6. Introduction of MET alone gave rise to microscopic foci of slightly dysplastic cells (Fig. 4), whereas transfection of the β-catenin gene had no apparent effect (data not shown). When the two genes were introduced and expressed together, the recipient animals developed HCC within a month or so (Figs. 4 and 6). Moreover, sequential introduction of the two genes at an interval of one month produced tumors, and the sequence of introduction had no effect on the outcome (data not shown). Thus, cooperation between MET and the β-catenin gene in the genesis of HCC appears not to be strictly dependent on the chronological sequence with which the two genes go awry.

We were also able to reconstruct the genesis of hepatic adenomas by hydrodynamic transfection. To do this, we created a dominant negative allele of HNF-1α found in human hepatic adenomas (Vaxillaire et al. 1999), which can mimic the effect of a genetic deficiency in the endogenous gene. Transfection of the inhibitory allele of HNF-1α produced numerous individual cells that were...
deficient in PAH (Fig. 4), but no tumors (Fig. 6). When MET and the dominant negative HNF-1α were introduced and expressed together, however, they rapidly gave rise to adenomas (Figs. 4 and 6). The tumors displayed histological morphology and differentiation markers identical to those of the adenomas found in transgenic mice (Fig. 4).

Previous work demonstrated that the targeted expression of transgenic normal MET in liver cells gave rise to a primitive tumor known as hepatoblastoma (Shachaf et al. 2004). The same tumor occurred when hydrodynamic transfection was used to express a normal allele of human MET in the liver of adult mice (Fig. 6). The tumors arose rapidly, without any readily apparent intermediate stage, and contained neither detectable Met kinase activity nor mutations in the β-catenin gene (data not shown).

### REGRESSION OF HCC AFTER INACTIVATION OF THE INITIATING TRANSGENE

The prevailing scheme of tumor progression raises the question of which steps in that progression remain essential for maintenance of the eventual malignancy and, thus, might be suitable targets for therapeutic intervention. Recent efforts to address this question with mouse models have shown that many forms of malignancy initiated by transgenes will regress when the responsible transgene is inactivated (data not shown). We conclude that the relapses were driven by a new and different genetic event that bypassed the requirement for Met kinase and presumably cooperated with the activity of β-catenin in tumorigenesis.

### CONCLUSIONS

We have developed mouse models for both benign and malignant liver tumors. The results support a role for MET in the genesis of liver tumors by complementing previous circumstantial evidence from studies of human tumors; provide insight into the rules of tumor progression; dramatize how different cellular compartments within a developmental lineage can give rise to distinctive tumors in response to the same tumorigenic influence, presumably by generating distinctive tumor stem cells; and provide evidence that nonmalignant tumors in mice are authentic models for human tumors.

We initiated tumorigenesis by overexpression of a normal allele of the MET proto-oncogene. Although this anomaly is common in human HCC, it apparently lies downstream of an external cause, such as chronic infection with either hepatitis B or C virus. It is not presently clear how viral infection might lead to the genetic anomaly required for tumorigenesis. One hypothesis holds that ongoing death and regeneration of liver tissue in response to chronic infection sets the stage for the occurrence and fixation of genetic damage (Thorgeirsson and Grisham 2002). This view is consistent with the lengthy time that separates the onset of infection from the appearance of malignant tumors. In contrast, tumorigenesis in the mouse models is relatively quick, with tumors arising after a few months at the most. At least three factors may contribute to this difference.

First, by introducing the overexpression of MET into numerous, if not all, liver cells, we have both bypassed earlier events that would otherwise be necessary for tumorigenesis and increased the likelihood of subsequent events.
events by creating a vast number of potential tumor stem cells. It is also possible that sustained activity of MET might destabilize the genome, much as described for MYC (Mai et al. 1996; Felson and Bishop 1999). Second, the rapidity of tumorigenesis in the models is in accord with the natural history of cancer in mice, which develop spontaneous malignancies much more quickly than do humans (Campisi 2003). This difference may reflect inferior defenses against permanent genetic damage in rodents (Ames et al. 1993). Third, cells in the dysplastic foci induced by MET were proliferating (data not shown), providing a permissive environment for the occurrence and fixation of mutations.

Although overexpression of MET and mutational activation of β-catenin are common in human HCC, neither is universal. In contrast, the mouse HCC described here contained both abundant Met kinase and activating mutations of β-catenin, almost without exception. The former was by experimental design; the latter must have arisen from spontaneous mutagenesis followed by selection during the course of tumor progression. We suggest that in constructing the mouse model, we have imposed one of several possible genetic pathways to HCC.

In pursuit of this idea, we have surveyed more than 50 human HCCs for the presence of enzymatically active Met and activating mutations of β-catenin. The results indicate that the two were frequently congruent, and in particular, robust Met kinase activity was virtually never present in the absence of a β-catenin mutation (A.D. Tward et al., unpublished). These correlations have not previously been appreciated. Thus, the findings in the mouse model have led us to a subset of human tumors in which the course of tumorigenesis was apparently akin to that in the model. The remainder of human HCC would arise from at least one additional pathway that has not been replicated by our mouse model. A further inference would be that activation of Met kinase constrains the subsequent pathway of tumorigenesis.

We have not detected Met kinase activity in the normal cells of adult transgenic livers (Fig. 3 and data not shown). Activity became apparent only with the appearance of hyperplastic foci and then persisted throughout the course of tumorigenesis. We presume that only occasional liver cells express the MET transgene above the threshold required to initiate tumorigenesis, and that such cells give rise to the hyperplastic foci.

The tumors in our mouse models all arose from overexpression of the MET proto-oncogene, but the specific outcome of tumorigenesis was determined by which genetic anomalies occur subsequently. We dramatized this conclusion by using hydrodynamic transfection to introduce various genetic anomalies into the mouse liver in vivo. The results demonstrated that MET and β-catenin cooperate to produce HCC, whereas MET and a deficiency of HNF-1α cooperate to produce benign adenomas. We have found no evidence that the abnormalities of β-catenin and HNF-1α ever coincide in tumors, and we cannot presently say whether either of these preempts the other, should they occur coincidentally in the same cell, but the issue is amenable to study by hydrodynamic transfection.

The determining nature of genetic lesions in tumorigenesis is further illustrated by the fact that targeted expression of MYC in liver cells, using the same control element as in the present work, gave rise to hepatoblastomas, rather than either HCC or adenomas (Shachaf et al. 2004 and data not shown). We were able to recapitulate that finding by using hydrodynamic transfection to express a normal allele of human MYC in the liver of adult mice. The transected mice developed hepatoblastomas.

The reconstitution of tumorigenesis in the transgenic mouse models places overexpression of Met upstream of the other genetic events required for the genesis of either HCC or adenomas. We have not conducted an exhaustive search for such cooperating events, focusing instead on known candidates deduced from data obtained with human tumors. However, the rapidity with which mouse tumors arise after simultaneous transfection of MET and mutants of either β-catenin or HNF-1α suggests that, in the mouse at least, relatively few additional spontaneous events may be required. Our results with hydrodynamic transfection also raise the possibility that HCC will arise irrespective of the order in which overexpression of Met and mutation of β-catenin occur, but more extensive experimentation will be required to address this issue in a definitive manner.

A major objective of modeling tumorigenesis in the mouse is to identify individual steps in tumor progression that are suitable targets for therapeutic intervention. Our work with the mouse model for HCC illustrates this prospect. Inactivation of MET, even in the most advanced tumors, led to prompt and universal tumor regression. Thus, MET not only initiated tumorigenesis, but also served as a tumor maintenance function. The efficiency and relative durability of regression were presumably due to the permanent removal of Met kinase from the tumor cells. The mouse tumors do recur, but only after extended periods of time, and only after the advent of some event that can bypass the need for MET and, perhaps, exploit the presence of residual cells with mutant β-catenin.

These results highlight Met as a possible target in the treatment of HCC. They also emphasize the existence of mutations that bypass any requirement for the target could give rise to drug resistance during the course of therapy.

In aggregate, this and many other reports of similar results with mouse models further justify the pursuit of therapies that are targeted at single genetic lesions in human tumors—therapies of the sort represented by the landmark pharmaceutical, Gleevec (Sawyers 2004). Mouse models with the authenticity exemplified by the present work should be useful in evaluating both the suitability of individual targets and the potential efficacy of additional targeted therapies, once they are in hand.

It is now apparent that MET is a crucial switch in the development of the liver (Fig. 8). The function of the gene is required to sustain proliferation of hepatic progenitor cells, but it must be inactivated in order to permit differentiation of those cells (A.D. Tward et al., in prep.). Unphysiologically, the activity of the Met kinase can disturb the differentiation of hepatocytes and engender tumorigenesis from both
bipotential and unipotential cells within the hepatocyte lineage, as dramatized by the work presented here. We cannot discern whether the effects of Met on progenitor proliferation and differentiation are independent of one another. Alternatively, one of the effects might follow the other. For example, the mere withdrawal of progenitor cells from the cell cycle after depletion of Met kinase activity might itself trigger differentiation. Whatever its genesis, the massive hyperplasia of progenitor cells in response to the in utero and postnatal overexpression of \textit{MET} could provide an abundant source of such cells for the experimental study of hepatic differentiation, tumorigenesis, and tissue engineering.

\textbf{ACKNOWLEDGMENTS}

We thank Linda Prentice for assistance with histology, and Luda Urisman for assistance with animal husbandry. The work was supported by funds from the National Cancer Institute (CA009043), the George W. Hooper Research Foundation, and the National Institutes of Health (DK49022 to M.A.K.). ADT was supported by the UCSF Medical Scientist Training Program NIH NIGMS (5T32GM07618).

\textbf{REFERENCES}


\begin{figure}[h]
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\includegraphics[width=\textwidth]{mouse_models_for_hepatitis_tumorogenesis.png}
\caption{Pathways of tumor progression. The diagram is based on results for tumor progression reported in the present paper and elsewhere (Wang et al. 2001; Shachaf et al. 2004) and on results describing the role of Met in hepatic development, which demonstrate that the physiological activity of Met both drives proliferation and blocks differentiation of hepatic precursor cells (Suzuki et al. 2003, and in prep.).}
\end{figure}


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