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The Proto-Oncogene MET in Liver Differentiation and Liver Cancer

by

Aaron Daniel Tward

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Ap[redacted]

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by

Aaron Daniel Tward

For Ira

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The Proto-Oncoogene *MET* in Liver Differentiation and Liver Cancer

by

Aaron Daniel Tward

Clifford Lowell M.D. Ph.D., Chair

ABSTRACT

The proto-oncogene *MET* encodes a receptor tyrosine kinase Met with diverse roles in development and cancer. I have utilized mouse models and samples from human tumors to explore the role of *MET* in liver differentiation and liver cancer. Overexpression of human *MET* in the liver during embryogenesis and continuously thereafter induced the sustained proliferation of hepatocyte progenitor cells and prevented their differentiation into mature hepatocytes. Inactivation of the overexpressed *MET* then permitted the progenitor cells to exit the cell cycle, differentiate into mature hepatocytes, and form a normal liver. Overexpression of *MET* in adult mice induced dysplasia that preceded hepatocellular carcinoma. In transgenic lines that expressed *MET* at relatively lower levels in the liver, mice first developed hyperplastic and dysplastic lesions and later hepatocellular carcinomas and adenomas. Activation of the Met kinase coincided with the onset of hyperplasia, and continued to be present in dysplastic foci, hepatocellular carcinomas, and hepatocellular adenomas. The transition to hepatocellular carcinoma coincided with activating mutations of the gene encoding β-catenin. The transition to hepatocellular adenoma coincided with loss of phenylalanine hydroxylase, a target gene of hepatocyte nuclear factor 1α (HNF1α). I confirmed the pathogenic roles of activation of Met and β-catenin or loss of HNF1α by hydrodynamically transfecting alleles of each of these into the liver of wild type mice. Activation of Met and β-catenin in combination induced multifocal hepatocellular carcinoma, whereas activation of Met and inactivation of HNF1α in combination induced multifocal hepatocellular adenoma. In a subset of human hepatocellular carcinomas, activation of the Met kinase strongly correlated with activating mutations in the gene encoding β-catenin. This work suggests approaches for the expansion of hepatic progenitor cells for therapeutic use and for the treatment of human hepatocellular carcinoma, and provides mouse models for the pre-clinical testing of those therapeutics.

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Chapter 1: Liver Development and Liver Cancer

The liver is a multifunctional organ whose proper functioning is crucial to the life of mammals. The major functions of the liver include glucose homeostasis, lipid metabolism, small molecule detoxification, nitrogen metabolism, serum protein secretion, bile production, and first-pass metabolism. All of these functions are in operation in a single cell type: the hepatocyte. In addition to being required for all of these roles, the liver has the ability to regenerate its full mass after damage. Further, even single hepatocytes have the ability to repopulate an entire liver (Sandgren et al. 1991). Hence, the liver represents an excellent model system in which to study differentiation, morphogenesis, and growth control.

Liver development

The development of the liver proceeds through a number of stereotyped stages that have been studied in a variety of model organisms. Specification is the earliest stage of development of the liver. During specification, a number of cells in the monolayer of cells of the ventral foregut endoderm begin to express liver-specific genes (Zhao and Duncan 2005). These specified cells are known as hepatoblasts. Studies using explants of mouse embryonic endoderm have demonstrated that signals from the adjacent cardiogenic mesoderm are required for specification of the liver (Gualdi et al. 1996). Among those paracrine signals required for specification of the liver are members of the fibroblast growth factor (FGF) (Jung et al. 1999), and bone morphogenetic protein (BMP) (Rossi et al. 2001) families. The FoxA forkhead box transcription factors, which are produced by the endoderm, are required for specification of the liver (Lee et al. 2005). In culture, embryonic endoderm from *foxa1*$^{-/-}$*foxa2*$^{-/-}$ mice failed to induce liver-specific genes after treatment with FGF2. Hence, the FoxA transcription factors appear to function in endodermal cells in a cell-autonomous manner downstream of FGFs to specify the liver.

After specification, these hepatoblasts then break down the adjacent basement membrane and begin to proliferate and migrate into the surrounding septum transversum mesenchyme (Zhao and Duncan 2005). A growing number of proteins have been implicated in these processes. The transcription factor Hex is required for the expansion of the nascent hepatoblasts (Bort et al. 2004). The ligand and receptor pair hepatoctye growth factor (HGF) and Met are necessary for the expansion of the hepatoblast population at later stages (E10-E15) of liver development (Schmidt et al. 1995; Maina et al. 2001), as is the multi-functional protein β-catenin (Monga et al. 2003). The transcription factor prospero-related homoebox 1 (Prox1) is required for the migration of the nascent hepatoblasts into the nascent liver lobes in a cell-autonomous manner (Sosa-Pineda et al. 2000). Despite the complete failure of hepatoblast migration in *prox1*$^{-/-}$ embryos, liver lobes still formed, implying that other mesenchymal components are likely to play a predominant role in the formation of liver lobes.

The endothelium is one such mesenchymal component that is required for liver lobe formation and migration of hepatoblasts into the septum transversum mesenchyme, as hepatic buds from *flk1*$^{-/-}$ embryos failed to expand (Matsumoto et al. 2001).

Hepatoblasts ultimately differentiate toward either the hepatocyte or biliary lineage (Zhao and Duncan 2005). The molecules involved in the process by which lineage determination occurs are still largely unknown. Notch signaling appears to be important in the differentiation of bile duct cells, as defects in Notch signaling caused a paucity of intrahepatic bile ducts in both humans (Li et al. 1997; Oda et al. 1997) and mice (McCright et al. 2002). Animals with a null allele of the gene encoding hepatocyte nuclear factor 6 (HNF6) showed a premature differentiation of intrahepatic bile ducts at the expense of hepatocytes, as well as a failure of the bile ducts to localize exclusively to portal zones (Clotman et al. 2002). The action of HNF6 may be mediated through hepatocyte nuclear factor 1β (HNF1β), as animals with a null allele of HNF6 had decreased expression of HNF1β in the liver, and animals with a null allele of HNF1β have a similar phenotype to HNF6 null animals (Coffinier et al. 2002).

HGF may promote commitment to the hepatocyte lineage from a more primitive bipotential stem cell. Treatment with HGF of sorted hepatic stem cells that do not express albumin induced the expression of albumin (Suzuki et al. 2003). The induction of albumin in the hepatic stem cells by HGF was dependent on the action of CAAT enhancer binding proteins (C/EBPs), as transfection with a dominant negative allele of C/EBP prevented the upregulation of albumin by HGF. Although HGF appeared to induce commitment to the hepatocyte lineage, treatment of sorted hepatic stem cells with HGF alone did not induce markers of mature hepatocytes. However, the treatment of sorted hepatic stem cells with the combination of HGF and oncostatin M (OSM) was sufficient to induce markers of mature hepatocytes. This finding is consistent with the finding that mice deficient for the receptor for OSM (gp130) showed defects in the maturation of hepatocytes (Kamiya et al. 1999).

Once cells are committed to the hepatocyte lineage, the production of liver-specific proteins is ultimately regulated at the level of transcription (Derman et al. 1981). As a result, a great deal of attention has been paid to identifying and characterizing the constellation of transcription factors that are responsible for expressing liver-specific genes. Studies of knockout mice have implicated HNF1α, HNF1β, C/EBPα, HNF4α, and the FoxA transcription factors in the expression of different subsets of mature liver-specific genes (Zhao and Duncan 2005).

In summary, although we are beginning to assemble knowledge of a collection of molecules that are involved in the development of the liver, a great deal remains unknown. In particular, gaining an understanding of how the different factors work in concert to pattern a mature liver may yield important principles for liver development and development in general. A better understanding of liver development may permit tissue engineering to progress to a stage where the availability of livers for transplant is no longer limiting.

Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is one of the most common and deadliest cancers, with an annual worldwide incidence and mortality of over 500,000 cases per year (Parkin et al. 2005). The primary causes of HCC are infection with either hepatitis B or C virus, chronic alcohol ingestion, or exposure to the fungal toxin aflatoxin B1, although any disease process that induces liver cirrhosis predisposes to HCC (Bruix et al. 2004). Because of endemic hepatitis B virus infection, HCC is more frequent in Asia and sub-Saharan Africa. The incidence of HCC is increasing in North America as a result of hepatits C infection (Parkin et al. 2005). The current treatments for HCC are limited to surgical resection, liver transplantation, and radiofrequency or ethanol ablation (Bruix et al. 2004). In patients where the HCC was diagnosed at an early stage, these treatments provide up to 50% survival five years after treatment.

There are two hypotheses, which are not mutually exclusive, that attempt to explain how infection with the hepatitis viruses contributes to HCC. The first hypothesis holds that hepatitis causes HCC indirectly by causing immune mediated damage to the liver. This damage culminates in multiple cycles of cell death and hepatocyte proliferation and creates a permissive environment for mutations to arise. A number of lines of evidence support this model. Infection of cultured hepatocytes with either hepatitis B or C virus induces no cytopathic effect (Ganem and Prince 2004). Any agent that damages the liver and leads to cirrhosis has a similar ability to induce HCC (Bruix et al. 2004). Also, HCC rarely arises on a background that is not first cirrhotic (Llovet et al. 2003).

The alternative hypothesis holds that the hepatitis viruses play a more direct role in the pathogenesis of HCC. In support of this hypothesis, proteins encoded by both hepatitis B and C viruses have been shown to modulate signaling pathways known to be important in cancer, and some (but not all) transgenic mouse lines overexpressing these proteins have a propensity for tumor formation (Brechot 2004). Although the normal life cycle of hepatitis viruses does not include integration of the viral genome, in HCC nodules there is an increased frequency of integration. Infection with either virus also induces cytokine production, which has been shown to affect hepatocyte proliferation (Rehermann and Nascimbeni 2005).

HCC is believed to ultimately arise from perturbations in tumor suppressor genes and proto-oncogenes that confer a selective advantage upon tumor cells. However, only a limited number of these alterations have been identified and validated. One frequent alteration is loss-of-function mutation of the tumor suppressor gene p53 (Thorgeirsson and Grisham 2002). Aflatoxin B1 is believed to exert at least part of its HCC promoting activity by preferentially mutating serine 249 of p53 (Aguilar et al. 1993). Mutation and loss of heterozygousity (LOH) of the insulin-like growth factor receptor 2 (IGF2R) is also a frequent event in HCC (De Souza et al. 1995). The IGF2R functions by internalizing and degrading soluble insulin-like growth factor 2 (IGF2), so loss-of-function mutations in the gene encoding IGF2R are predicted to cause increased IGF signaling (Nakae et al. 2001). Other genes reported to frequently harbor mutations in HCC include *THRA, THRB, RB1, PTEN,* and *E2F4* (Thorgeirsson and Grisham 2002).

One pathway that is frequently activated by mutation in HCCs is the Wnt signaling pathway (Giles et al. 2003). Mutations in one of the genes encoding β-catenin, Axin, or Axin2 have been found in up to 40% of all HCCs. The mutation of the gene encoding β-catenin is likely to be a late event in HCC, as mutation of β-catenin is detected in HCC nodules, but not in precancerous dysplastic nodules in humans (Park et al. 2005). In its uninduced state, β-catenin exists in two different cellular compartments. The first compartment is adjacent to the membrane, where β-catenin forms part of the functional link between cadherin proteins and the actin cytoskeleton. The second compartment is in the cytoplasm, where β-catenin exists in a complex with the kinases GSK3β and CKIε and the scaffolding proteins APC and Axin. All components of this complex are necessary for the phosphorylation of β-catenin on four critical residues near the N-terminus by the two kinases in the complex. When β-catenin is phosphorylated on all four residues, it can interact with the E3 ubiquitin ligase β-TcRP, which polyubiquinates β-catenin. This polyubiquinated β-catenin is then degraded by the proteasome. Addition of the extracellular ligand Wnt, mutation of β-catenin in the region necessary for interaction with β-TcRP, or inactivating mutation of Axin or APC prevents the proteasomal degradation of β-catenin. In the absence of rapid degradation, cytoplasmic levels of β-catenin increase. β-catenin then shuttles to the nucleus, where it can interact with members of the Tcf family of DNA binding proteins. In the absence of β-catenin, Tcf family members are bound to DNA and recruit transcriptional corepressors of the groucho family of proteins. In the presence of β-catenin, the groucho proteins are displaced from the Tcf proteins, and transcriptional activators are recruited to the sites where the Tcf proteins are bound (Giles et al. 2003). Although in cell lines from other tissues the proliferative effects of activated β-catenin have been shown to require the upregulation of the target genes encoding c-Myc, cyclinD1, and PPARδ, it is unclear whether any of these targets are mediators of the effects of β-catenin in the liver (Cadoret et al. 2001).

Another perturbation implicated in HCC is overexpression (Tavian et al. 2000) or mutation (Park et al. 1999) of Met. Overexpression of a wild type allele of human Met in transgenic mice was sufficient to induce HCC (Wang et al. 2001). Met contains a multi-functional docking site at its C-terminus that recruits multiple signaling molecules, including Gab1, Grb2, PI3K, and Src (Ponzetto et al. 1994; Birchmeier et al. 2003). The Gab1 signaling adapter is crucial for physiological responses to Met signaling, as *Gab1*$^{-/-}$ mice showed a similar phenotype to *Met*$^{-/-}$ mice (Sachs et al. 2000). Enzymatically active Met can, in turn, activate a variety of downstream effectors, including Src, PI3K, STAT3, Ras, PLCγ, SHP2, Crk, and Cdc42 (Birchmeier et al. 2003).

Although different constellations of these effectors have been implicated in a variety of cell biological responses to Met signaling, the crucial effectors that mediate the ability of Met to induce and sustain HCC are unknown. One possible effector of Met that has been implicated in DNA synthesis induced by HGF in cultured hepatocytes is the epidermal growth factor receptor (EGFR) pathway (Scheving et al. 2002). Treatment of rat hepatocytes with HGF induced expression of the EGFR ligand TGFα. The addition of antisense oligonucleotides against TGFα, a blocking antibody to TGFα, or a small molecule EGFR kinase inhibitor blocked HGF induced DNA synthesis in cultured rat hepatocytes. The relevance of these findings *in vivo* remains to be determined.

Multiple lines of evidence support the hypothesis that Met is activated in HCC by a ligand independent mechanism. Despite the overexpression of Met in human HCC, expression of its ligand HGF is downregulated (Tavian et al. 2000). Overexpression of HGF suppressed tumorigenesis in mouse models of HCC (Shiota et al. 1995; Santoni-Rugiu et al. 1996; Thorgeirsson and Santoni-Rugiu 1996), whereas overexpression of Met rapidly induced HCC (Wang et al. 2001). Further, although treatment of primary hepatocytes with HGF induces DNA synthesis, treatment of a variety of HCC cell lines with HGF suppresses proliferation and induces apoptosis (Shiota et al. 1992). The ability of HGF to suppress hepatic tumorigenesis may be related to the ability of HGF to induce apoptosis by liberating the Fas death receptor from complexes formed between Met and Fas (Wang et al. 2002).

The progression to HCC appears to occur through a variety of pathways (Thorgeirsson and Grisham 2002). As such, there remain many new genetic and epigenetic events to be discovered. With a more complete set of these events in hand, we should have the ability to combine our knowledge of tumor biology, developmental biology, and cell signaling to better understand how multiple events can work in concert to initiate and sustain HCC. Such knowledge will hopefully result in new therapeutics for the treatment of this largely intractable disease.

REFERENCES

Aguilar, F., Hussain, S.P., and Cerutti, P. 1993. Aflatoxin B1 induces the transversion of G-->T in codon 249 of the p53 tumor suppressor gene in human hepatocytes. *Proc Natl Acad Sci U S A* **90**(18): 8586-8590.

Birchmeier, C., Birchmeier, W., Gherardi, E., and Vande Woude, G.F. 2003. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* **4**(12): 915-925.

Bort, R., Martinez-Barbera, J.P., Beddington, R.S., and Zaret, K.S. 2004. Hex homeobox gene-dependent tissue positioning is required for organogenesis of the ventral pancreas. *Development* **131**(4): 797-806.

Brechot, C. 2004. Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. *Gastroenterology* **127**(5 Suppl 1): S56-61.

Bruix, J., Boix, L., Sala, M., and Llovet, J.M. 2004. Focus on hepatocellular carcinoma. *Cancer Cell* **5**(3): 215-219.

Cadoret, A., Ovejero, C., Saadi-Kheddouci, S., Souil, E., Fabre, M., Romagnolo, B., Kahn, A., and Perret, C. 2001. Hepatomegaly in transgenic mice expressing an oncogenic form of beta-catenin. *Cancer Res* **61**(8): 3245-3249.

Clotman, F., Lannoy, V.J., Reber, M., Cereghini, S., Cassiman, D., Jacquemin, P., Roskams, T., Rousseau, G.G., and Lemaigre, F.P. 2002. The onecut transcription factor HNF6 is required for normal development of the biliary tract. *Development* **129**(8): 1819-1828.

Coffinier, C., Gresh, L., Fiette, L., Tronche, F., Schutz, G., Babinet, C., Pontoglio, M., Yaniv, M., and Barra, J. 2002. Bile system morphogenesis defects and liver dysfunction upon targeted deletion of HNF1beta. *Development* **129**(8): 1829-1838.

De Souza, A.T., Hankins, G.R., Washington, M.K., Orton, T.C., and Jirtle, R.L. 1995. M6P/IGF2R gene is mutated in human hepatocellular carcinomas with loss of heterozygosity. *Nat Genet* **11**(4): 447-449.

Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray, M., and Darnell, J.E., Jr. 1981. Transcriptional control in the production of liver-specific mRNAs. *Cell* **23**(3): 731-739.

Ganem, D. and Prince, A.M. 2004. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* **350**(11): 1118-1129.

Giles, R.H., van Es, J.H., and Clevers, H. 2003. Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta* **1653**(1): 1-24.

Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J.R., and Zaret, K.S. 1996. Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev* **10**(13): 1670-1682.

Jung, J., Zheng, M., Goldfarb, M., and Zaret, K.S. 1999. Initiation of mammalian liver development from endoderm by fibroblast growth factors. *Science* **284**(5422): 1998-2003.

Kamiya, A., Kinoshita, T., Ito, Y., Matsui, T., Morikawa, Y., Senba, E., Nakashima, K., Taga, T., Yoshida, K., Kishimoto, T., and Miyajima, A. 1999. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *Embo J* **18**(8): 2127-2136.

Lee, C.S., Friedman, J.R., Fulmer, J.T., and Kaestner, K.H. 2005. The initiation of liver development is dependent on Foxa transcription factors. *Nature* **435**(7044): 944-947.

Li, L., Krantz, I.D., Deng, Y., Genin, A., Banta, A.B., Collins, C.C., Qi, M., Trask, B.J., Kuo, W.L., Cochran, J., Costa, T., Pierpont, M.E., Rand, E.B., Piccoli, D.A., Hood, L., and Spinner, N.B. 1997. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* **16**(3): 243-251.

Llovet, J.M., Burroughs, A., and Bruix, J. 2003. Hepatocellular carcinoma. *Lancet* **362**(9399): 1907-1917.

Maina, F., Pante, G., Helmbacher, F., Andres, R., Porthin, A., Davies, A.M., Ponzetto, C., and Klein, R. 2001. Coupling Met to specific pathways results in distinct developmental outcomes. *Mol Cell* **7**(6): 1293-1306.

Matsumoto, K., Yoshitomi, H., Rossant, J., and Zaret, K.S. 2001. Liver organogenesis promoted by endothelial cells prior to vascular function. *Science* **294**(5542): 559-563.

McCright, B., Lozier, J., and Gridley, T. 2002. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development* **129**(4): 1075-1082.

Monga, S.P., Monga, H.K., Tan, X., Mule, K., Pediaditakis, P., and Michalopoulos, G.K. 2003. Beta-catenin antisense studies in embryonic liver cultures: role in proliferation, apoptosis, and lineage specification. *Gastroenterology* **124**(1): 202-216.

Nakae, J., Kido, Y., and Accili, D. 2001. Distinct and overlapping functions of insulin and IGF-I receptors. *Endocr Rev* **22**(6): 818-835.

Oda, T., Elkahloun, A.G., Pike, B.L., Okajima, K., Krantz, I.D., Genin, A., Piccoli, D.A., Meltzer, P.S., Spinner, N.B., Collins, F.S., and Chandrasekharappa, S.C. 1997. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet* **16**(3): 235-242.

Park, J.Y., Park, W.S., Nam, S.W., Kim, S.Y., Lee, S.H., Yoo, N.J., Lee, J.Y., and Park, C.K. 2005. Mutations of beta-catenin and AXIN I genes are a late event in human hepatocellular carcinogenesis. *Liver Int* **25**(1): 70-76.

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. 1999. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. *Cancer Res* **59**(2): 307-310.

Parkin, D.M., Bray, F., Ferlay, J., and Pisani, P. 2005. Global cancer statistics, 2002. *CA Cancer J Clin* **55**(2): 74-108.

Ponzetto, C., Bardelli, A., Zhen, Z., Maina, F., dalla Zonca, P., Giordano, S., Graziani, A., Panayotou, G., and Comoglio, P.M. 1994. A multifunctional docking site mediates signaling and transformation by the hepatocyte growth factor/scatter factor receptor family. *Cell* **77**(2): 261-271.

Rehermann, B. and Nascimbeni, M. 2005. Immunology of hepatitis B virus and hepatitis C virus infection. *Nat Rev Immunol* **5**(3): 215-229.

Rossi, J.M., Dunn, N.R., Hogan, B.L., and Zaret, K.S. 2001. Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev* **15**(15): 1998-2009.

Sachs, M., Brohmann, H., Zechner, D., Muller, T., Hulsken, J., Walther, I., Schaeper, U., Birchmeier, C., and Birchmeier, W. 2000. Essential role of Gab1 for signaling by the c-Met receptor in vivo. *J Cell Biol* **150**(6): 1375-1384.

Sandgren, E.P., Palmiter, R.D., Heckel, J.L., Daugherty, C.C., Brinster, R.L., and Degen, J.L. 1991. Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. *Cell* **66**(2): 245-256.

Santoni-Rugiu, E., Preisegger, K.H., Kiss, A., Audolfsson, T., Shiota, G., Schmidt, E.V., and Thorgeirsson, S.S. 1996. Inhibition of neoplastic development in the liver by hepatocyte growth factor in a transgenic mouse model. *Proc Natl Acad Sci U S A* **93**(18): 9577-9582.

Scheving, L.A., Stevenson, M.C., Taylormoore, J.M., Traxler, P., and Russell, W.E. 2002. Integral role of the EGF receptor in HGF-mediated hepatocyte proliferation. *Biochem Biophys Res Commun* **290**(1): 197-203.

Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. 1995. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373**(6516): 699-702.

Shiota, G., Kawasaki, H., Nakamura, T., and Schmidt, E.V. 1995. Characterization of double transgenic mice expressing hepatocye growth factor and transforming growth factor alpha. *Res Commun Mol Pathol Pharmacol* **90**(1): 17-24.

Shiota, G., Rhoads, D.B., Wang, T.C., Nakamura, T., and Schmidt, E.V. 1992. Hepatocyte growth factor inhibits growth of hepatocellular carcinoma cells. *Proc Natl Acad Sci U S A* **89**(1): 373-377.

Sosa-Pineda, B., Wigle, J.T., and Oliver, G. 2000. Hepatocyte migration during liver development requires Prox1. *Nat Genet* **25**(3): 254-255.

Suzuki, A., Iwama, A., Miyashita, H., Nakauchi, H., and Taniguchi, H. 2003. Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. *Development* **130**(11): 2513-2524.

Tavian, D., De Petro, G., Benetti, A., Portolani, N., Giulini, S.M., and Barlati, S. 2000. u-PA and c-MET mRNA expression is co-ordinately enhanced while hepatocyte growth factor mRNA is down-regulated in human hepatocellular carcinoma. *Int J Cancer* **87**(5): 644-649.

Thorgeirsson, S.S. and Grisham, J.W. 2002. Molecular pathogenesis of human hepatocellular carcinoma. *Nat Genet* **31**(4): 339-346.

Thorgeirsson, S.S. and Santoni-Rugiu, E. 1996. Transgenic mouse models in carcinogenesis: interaction of c-myc with transforming growth factor alpha and hepatocyte growth factor in hepatocarcinogenesis. *Br J Clin Pharmacol* **42**(1): 43-52.

Wang, R., Ferrell, L.D., Faouzi, S., Maher, J.J., and Bishop, J.M. 2001. Activation of the Met receptor by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice. *J Cell Biol* **153**(5): 1023-1034.

Wang, R., Kobayashi, R., and Bishop, J.M. 1996. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. *Proc Natl Acad Sci U S A* **93**(16): 8425-8430.

Wang, X., DeFrances, M.C., Dai, Y., Pediaditakis, P., Johnson, C., Bell, A., Michalopoulos, G.K., and Zarnegar, R. 2002. A mechanism of cell survival: sequestration of Fas by the HGF receptor Met. *Mol Cell* **9**(2): 411-421.

Zhao, R. and Duncan, S.A. 2005. Embryonic development of the liver. *Hepatology* **41**(5): 956-967.

Chapter 2: The Proto-Oncogene *MET* Restricts the Differentiation of Hepatocytes

ABSTRACT

The signals that govern the transition from hepatocyte progenitors to mature hepatocytes are largely unknown. The proto-oncogene *MET* is required for expansion of hepatocyte progenitors *in vivo* (Maina et al. 2001). Here we show that transgenic overexpression of *MET* in mice blocks the differentiation of hepatocyte progenitors and induces their proliferation. Inactivation of the *MET* transgene at one month of age relieves the block to differentiation and allows construction of a normal liver. After inactivation of the *MET* transgene, these animals survive into adulthood, but eventually succumb to carcinomas composed of cells of both the hepatocyte and bile duct lineages. Despite the ability of *MET* to impede the differentiation of hepatocyte progenitors, activation of *MET* in adult animals is not sufficient to revert mature hepatocytes to progenitors. Rather, these animals develop hepatic dysplasia and ultimately hepatocellular carcinoma (HCC). We conclude that *MET* may serve two distinct roles in liver development: maintaining progenitor proliferation, and restricting progenitor differentiation.

INTRODUCTION

The mature liver is composed of two major types of epithelial cells: the hepatocyte and the cholangiocyte. During development, both of these cell types arise from a common hepatic progenitor, or hepatoblast (Lemaigre and Zaret 2004). There likely exist multiple steps of differentiation in both of these lineages, and the point at which a differentiating hepatoblast might be irreversibly committed to either lineage is unclear. We will refer to any cell of the hepatocyte lineage prior to the mature hepatocyte as a hepatocyte progenitor. Previous studies have defined some of the signals that play a role in the decision to become either a hepatocyte or cholangiocyte, but the signals that are necessary to permit the differentiation of mature hepatocytes from hepatocyte progenitors are less well defined.

The receptor tyrosine kinase Met and its ligand hepatocyte growth factor (HGF) are candidate regulators of hepatocyte progenitor cell expansion and differentiation (Suzuki et al. 2003). At embryonic day 14.5, both *Met*$^{-/-}$ and *Hgf*$^{-/-}$ embryos have small livers and widened sinusoids. However, the *Met*$^{-/-}$ embryos still have hepatoblasts present (Schmidt et al. 1995; Maina et al. 2001). Hence, Met signaling is necessary for expansion of the hepatocyte progenitor pool, but not necessary for specification of the liver.

Deletion of *Met* in the liver of postnatal mice yields little phenotype in the absence of hepatic injury (Borowiak et al. 2004; Huh et al. 2004). However, after partial hepatectomy or chemical liver damage, mice with *Met*$^{-/-}$ livers have severe defects in liver regeneration. Hence, Met signaling in adult mice is unnecessary to the liver until the proliferation of liver cells with properties of progenitor cells is required. *In vitro*, HGF is sufficient to induce proliferation of isolated liver stem cells (Suzuki et al. 2003). Taken together, these studies indicate that Met signaling is required for hepatic progenitor expansion during both development and regeneration.

We have previously generated strains of mice that express a transgene of human *MET* in hepatocytes (Wang et al. 2001). Two of these strains (Lines 1 and 2) are born with enlarged livers and die within three to eight weeks after birth. We now report that the hepatomegaly in these mice is due to hyperplasia of hepatic precursor cells and a failure of these cells to differentiate into mature hepatocytes. Death is apparently due to the absence of mature liver function. Inactivation of the *MET* transgene at one month of age relieves the block to differentiation and allows construction of a normal adult liver. The animals survive into adulthood, but eventually succumb to hepatic carcinomas composed of both biliary and hepatocytic elements.

We conclude that Met may play two roles in hepatic development. First, the enzymatic activity of Met sustains the proliferation of hepatocyte progenitors. Second, the enzymatic activity of Met may prohibit differentiation of the progenitors and, unless inactivated, can prevent the formation of the mature liver. Our results also demonstrate that a normal liver can arise in the adult from progenitor cells, in the absence of preexisting mature hepatocytes or tissue architecture. The transgenic mice described here represent a source of abundant hepatic precursor cells that should be useful in further study of hepatocyte differentiation, tissue engineering, and tumorigenesis from tissue progenitor cells.

RESULTS

Activity of murine Met *during hepatic development*

In accord with previous reports (Ishikawa et al. 2001; Spijkers et al. 2001), expression of Met protein was readily detectable during embryogenesis, after birth, and into adulthood (Figure 2.1A, bottom row). In contrast, the kinase activity of Met was readily detectable during embryogenesis, but not after birth or in adulthood, as measured by the surrogate means of testing for phosphorylation of the protein (Figure 2.1A, top row). Thus, the enzymatic activity of Met in the liver is apparently repressed by controls that act directly on the protein, without decreasing its abundance.

The decline in Met kinase activity was concurrent with maturation of the liver, reflected in the disappearance of alpha-fetoprotein (AFP), a marker for primitive progenitor cells, and a rise in glutamine synthetase (GS) and

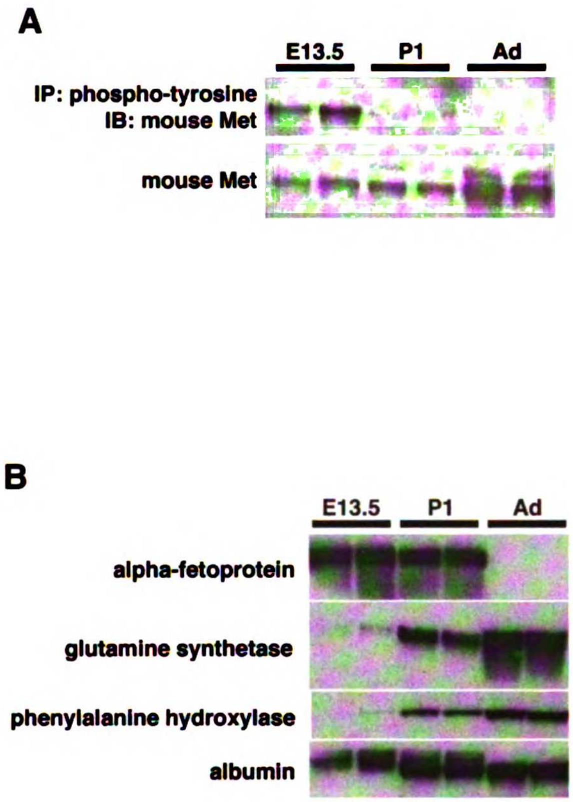
A
E13.5
P1
Ad
IP: phospho-tyrosine
IB: mouse Met
mouse Met
B
E13.5
P1
Ad
alpha-fetoprotein
glutamine synthetase
phenylalanine hydroxylase
albumin

Figure 2.1: Expression and kinase activity of Met during normal development.
(A) Top row, Immunopreciptiation with an anti-phospho-tyrosine antibody was performed on whole tissue lysates from livers of LAP-tTA mice at the stages of development indicated. Western Blotting was then performed with an antibody against mouse Met. Bottom row, Western Blotting was performed on whole tissue lysates from livers of LAP-tTA mice at the stages of development indicated with an antibody against mouse Met. (B) Western Blotting was performed with antibodies against the indicated antigens on whole tissue lysates from livers of LAP-tTA mice at the stages of development indicated. E13.5, embryonic day 13.5; P1, postnatal day 1; Ad, adult. The duplicate lanes represent separate animals.

phenylalanine hydroxylase (PAH), markers for mature hepatocytes (Figure 2.1B). These findings prompted the suspicion that inactivation of the Met kinase might be required for hepatic differentiation. In the following work, we provide support for that suspicion by the study of mice bearing a transgene of human *MET*.

Strains of mice expressing a transgene of human MET

We have previously reported the development of mice that express a transgene of human *MET* specifically in hepatocytes (Wang et al. 2001). Expression of the transgene can be inactivated by administration of doxycycline. Four independent strains of mice were obtained. Two strains expressed the transgene at relatively high levels (previously designated as Lines 1 and 2), whereas two expressed it at much lower levels (previously designated as Lines 3 and 4) (Figure 2.2A).

Animals of Lines 3 and 4 were normal at birth, but eventually developed HCC and died by one year of age (Wang et al. 2001). Throughout postnatal life in the absence of doxycycline, the *MET* transgene was expressed at lower levels than in Lines 1 and 2, but Met kinase activity was not detectable in normal hepatocytes (Figure 2.2B). Very different results were obtained with Lines 1 and 2. All of the results that follow are from the analysis of Line 2, but similar results were obtained with Line 1. If doxycycline was not administered at any point (designated as *MET* ON/ON), animals from these lines were smaller than control

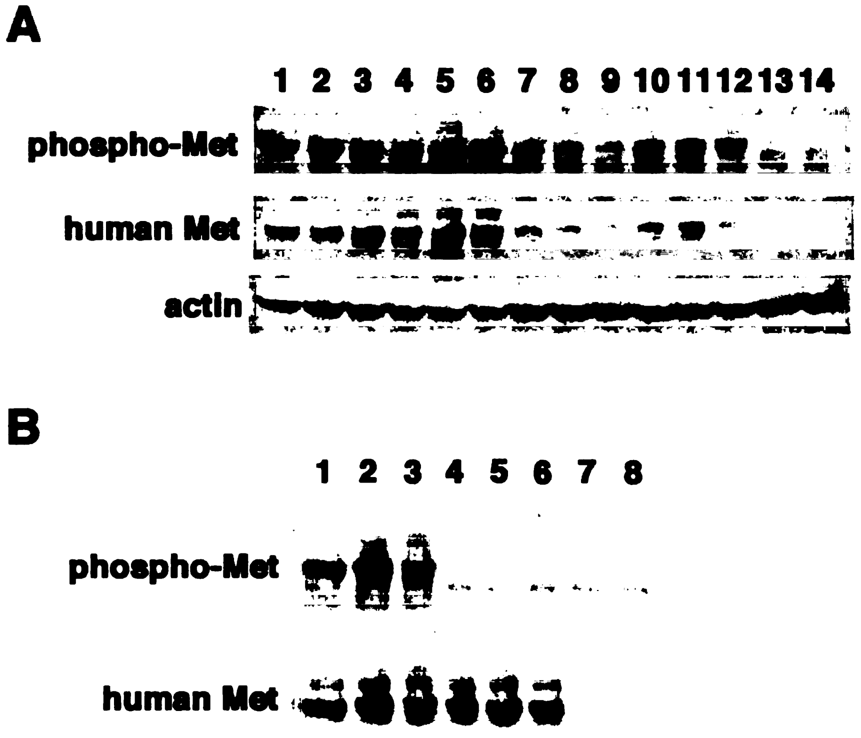

Figure 2.2: Level of Met expression in *MET* transgenic mice.
(A) Western Blot analysis with antibodies against the antigens indicated was performed on whole tissue lysates from livers of newborn mice maintained in the absence of doxycycline. The triplicate lanes represent separate animals. Lanes 1-3, Line 2 LAP-tTA/TRE-MET; lanes 4-6, Line 1 LAP-tTA/TRE-MET; lanes 7-9, Line 4 LAP-tTA/TRE-MET; lanes 10-12, Line 3 LAP-tTA/TRE-MET; lanes 13-14, LAP-tTA control mice. (B) Western Blot analysis with antibodies against the antigenss indicated was performed on whole tissue lysates from livers of 10 day old mice maintained in the absence of doxycycline. The triplicate lanes represent separate animals. Lanes 1-3, Line 1 LAP-tTA/TRE-MET; lanes 4-6 Line 3 LAP-tTA/TRE-MET; lanes 7-8 LAP-tTA littermates.

littermates (Figure 2.3A and Table 2.1), had enlarged livers (Figure 2.3B and Table 2.1) and died within three to eight weeks after birth (Figure 2.3C; Wang et al. 2001). The transgenic Met protein was expressed at high levels in these animals and had readily detectable protein-tyrosine kinase activity (Figure 2.4). Expression of the transgene was limited to hepatocytes, when analyzed with immunohistochemistry (data not shown). As anticipated, administration of doxycycline quickly repressed expression of the transgene (Figure 2.4).

Met can impede hepatocyte differentiation

We explored the genesis of the hepatomegaly in animals of Line 2 mice that had received no doxycycline during gestation or postnatal life. The enlarged livers were histologically hyperplastic (Figure 2.5B) and were composed of cells that appeared smaller, rounder and more basophilic than normal hepatocytes (Figure 2.5A and 2.5B). In accord with the hyperplasia, more cells than normal expressed a marker for proliferation, Ki67 (Figure 2.5I and 2.5J). Apoptosis was also increased (Table 2.1), but failed to prevent hepatomegaly as a result of the hyperplasia.

The morphology of the hyperplastic cells was similar to that of hepatocyte progenitors, so we performed analyses for molecular markers of hepatocyte differentiation. The cells expressed AFP, a marker for progenitors (Figure 2.4, 2.5E, and 2.5F), whereas expression of markers for mature hepatocytes, GS and PAH, was greatly reduced (Figure 2.4). We conclude that sustained

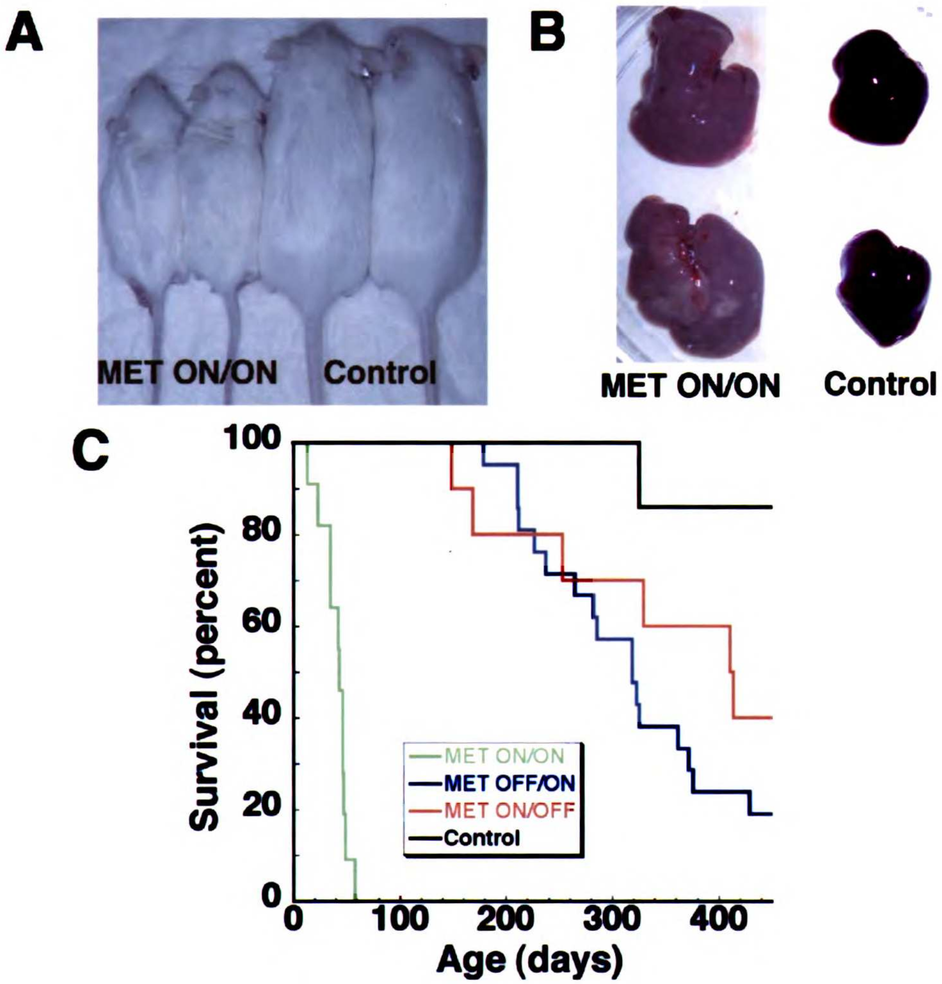

Figure 2.3: Body size, liver size, and survival of *MET* transgenic animals.
(A) Animals at one month of age. *MET* ON/ON, LINE 2 LAP-tTA/TRE-MET mice with the *MET* transgene expressed continuously throughout gestation and after birth; Control, LAP-tTA littermates. (B) Livers from the animals shown in A. (C) Survival of transgenic animals. *MET* ON/ON, Line 2 LAP-tTA/TRE-MET with *MET* transgene expressed continuously throughout gestation and after birth; *MET* ON/OFF, Line 2 LAP-tTA/TRE-MET with *MET* transgene expressed throughout gestation and for one month after birth; *MET* OFF/ON, LAP-tTA/TRE-MET Line 2 with *MET* transgene expressed only after weaning; Control, LAP-tTA littermates.

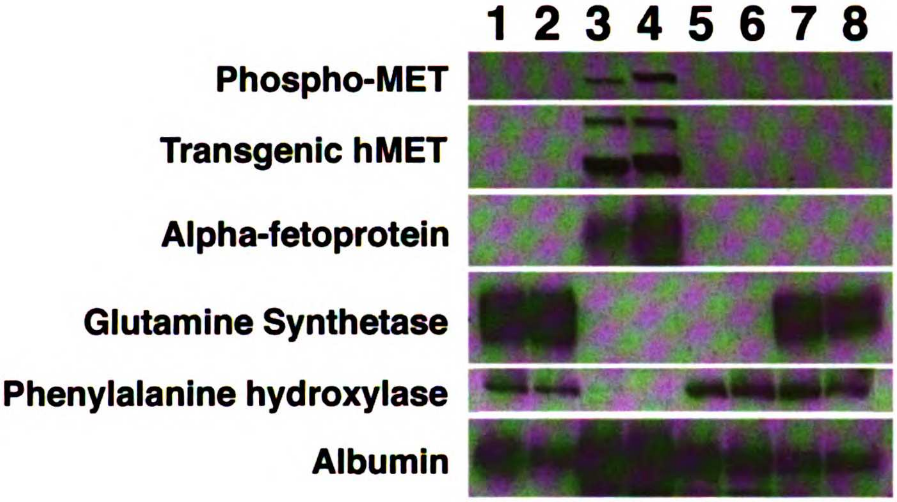
1 2 3 4 5 6 7 8
Phospho-MET
Transgenic hMET
Alpha-fetoprotein
Glutamine Synthetase
Phenylalanine hydroxylase
Albumin

Figure 2.4: Reversible block of differentiation by Met.
Western Blotting was performed on whole tissue lysates of livers using antibodies against the indicated antigens. The duplicate lanes represent separate animals. Mouse strains are as described in Figure 2.3. Lanes 1-2, LAP-tTA mice at one month of age; lanes 3-4, Line 2 *MET* ON/ON mice at one month of age with the *MET* transgene expressed continuously throughout gestation and after birth; lanes 5-6, Line 2 *MET* ON/OFF mice at 1 month of age with the *MET* transgene expressed continuously throughout gestation and after birth for 1 month, then inactivated for 3 days; lanes 7-8, Line 2 *MET* ON/OFF mice at 2 months of age with the *MET* transgene expressed continuously throughout gestation and after birth for 1 month, then inactivated for 25 days. The upper band in the human Met lane is the immature 170 kDa Met precursor, whereas the lower band is the mature 140 kDa chain of Met.

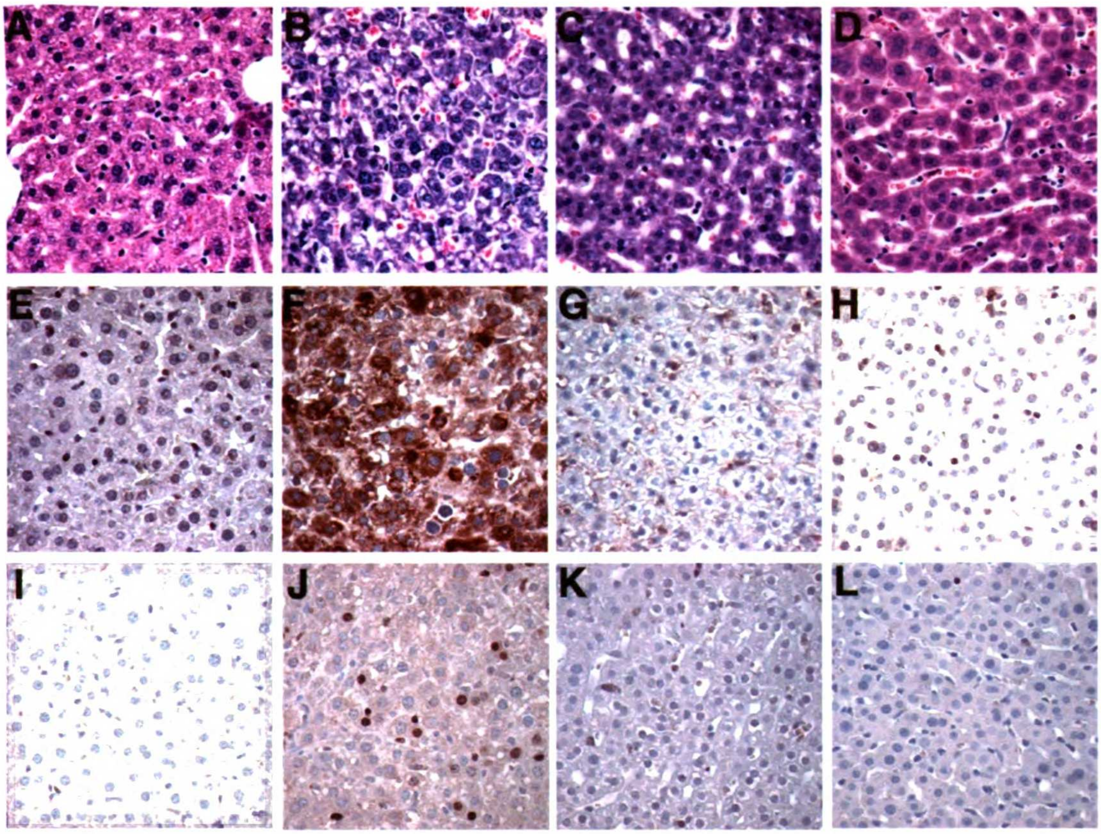

Figure 2.5: Reversible block of differentiation by Met.
Mouse strains are as described in Figure 2.3. Sections from fixed and paraffin embedded livers were subjected to staining with H+E (A-D), or immunohistochemistry with antibodies against either alpha-fetoprotein(E-H) or Ki67 (I-L). Sections are shown of LAP-tTA mice at one month of age (A, E, I), Line 2 *MET* ON/ON mice at one month of age with the *MET* transgene expressed continuously throughout gestation and after birth (B, F, J), Line 2 *MET* ON/OFF mice at 1 month of age with the *MET* transgene expressed continuously throughout gestation and after birth for 1 month, then inactivated for 3 days (C, G, K), and Line 2 *MET* ON/OFF mice at 2 months of age with the *MET* transgene expressed continuously throughout gestation and after birth for 1 month, then inactivated for 25 days (D, H, L).

overexpression of the *MET* transgene throughout late gestation and into postnatal life prevented the differentiation of progenitor cells into mature hepatocytes, and that continuing proliferation of the progenitors gave rise to the enlarged, hyperplastic livers. This in turn supports the view that the physiological inactivation of Met kinase activity late in normal gestation may be essential for hepatocyte differentiation.

Reversing the block to differentiation

Because overexpression of *MET* caused a block of differentiation, we predicted that cessation of overexpression should release the block. To test this prediction, we administered doxycycline to Line 2 mice at one month of age (designated as *MET* ON/OFF). Inactivation of transgenic Met expression in this way prevented the premature death seen in the *MET* ON/ON regimen (Figure 2.3B). Three days after inactivating the *MET* transgene, the liver weight of *MET* ON/OFF animals decreased (Table 2.1), cellular proliferation decreased (Figure 2.5J and 2.5K), apoptosis increased (Table 2.1), and the state of differentiation of hepatocytes appeared intermediate between hepatic progenitors and mature hepatocytes as assessed by both histology (Figure 2.5C) and marker expression (Figure 2.4). 25 days after inactivating the *MET* transgene in *MET* ON/OFF animals, body and liver weight, apoptosis (Table 2.1), proliferation (Figure 2.5L), histological appearance of hepatocytes (Figure 2.5D), and marker expression

	Body weight[a] (grams)	Liver weight[b] (grams)	Hepatic Index[c] (percent)	Apoptotic Index[d] (number per high power field)
Control	24.8±1.4	1.38±.03	5.57±0.25	2.8±0.8
MET ON/ON	15.7±1.0	3.11±.22	19.79±0.92	11.4±2.7
MET ON/OFF 3 days	14.7±1.1	2.06±0.19	14.01±0.67	17.0±4.6
MET ON/OFF 25 days	21.7±0.6	1.58±0.05	7.32±0.20	6.0±2.5

Table 2.1: Reversion of liver enlargement.
[a] Whole animals and [b] livers were weighed from animals as listed in Fig. 2.3. [c] The hepatic index is the liver weight to body weight ratio, expressed as a percentage. [d] Sections from livers of mice were subjected to the TUNEL assay to reveal apoptotic cells. The apoptotic index is the average number of apoptotic cells per high power field. Ten randomly selected high power fields per section were analyzed. Numbers are listed as mean ± SEM. Four animals per group were used.

(Figure 2.4) were similar to those in livers of controls. We conclude that inactivation of the *MET* transgene permitted the persistent hepatic progenitors to differentiate into mature hepatocytes and construct a normal liver.

Tumorigenesis following inactivation of the MET *transgene*

Although the *MET* ON/OFF animals were rescued from the very early death observed with *MET* ON/ON mice, they nevertheless died prematurely around one year of age (Figure 2.3C). The apparent cause of death was liver tumors, which contained cells of both the hepatocyte and bile duct lineages (Figure 2.6A), and expressed AFP and PAH, but did not express GS (data not shown). The mixed nature of the tumors suggests a bipotential cell of origin.

Overexpression of MET *in adult mice*

Because *MET* overexpression was sufficient to block the differentiation of hepatic progenitors to mature hepatocytes, we wondered whether *MET* overexpression might cause reversion of mature hepatocytes to progenitors. To address this question we allowed development to proceed normally, then induced *MET* overexpression in Line 2 mice at three weeks of age (designated as *MET* OFF/ON). We confirmed that the conditionally expressed Met was enzymatically active 3 months after induction in the *MET* OFF/ON livers (Figure 2.7A, top row). Instead of the small round basophilic hepatocytes in the *MET* ON/ON livers (Figure 2.5B), *MET* OFF/ON hepatocytes were dysplastic, with variegated cell

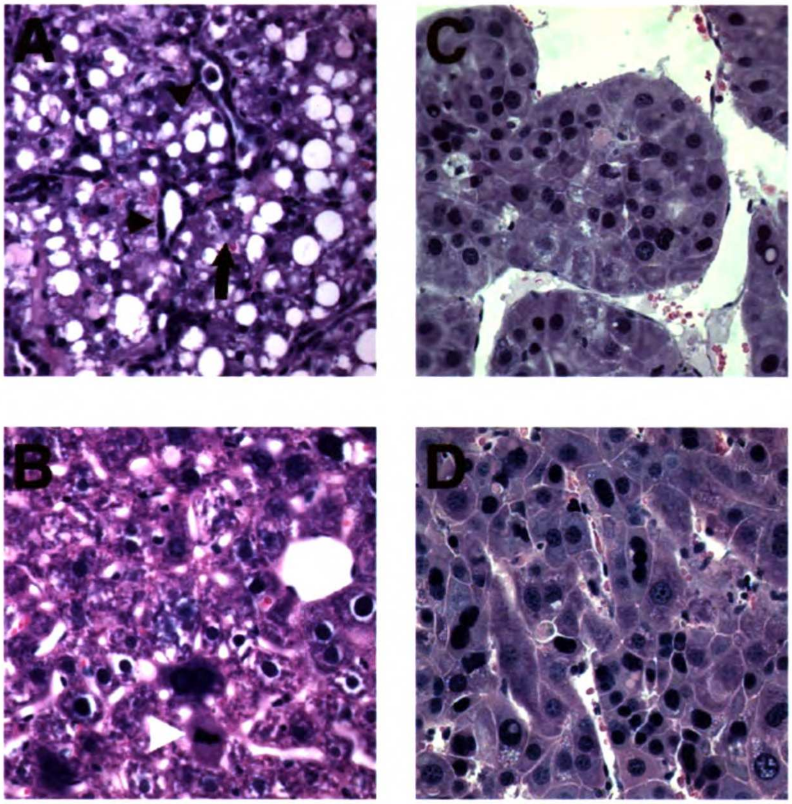

Figure 2.6: Histology of hepatic tumors in *MET* trangenic animals. Strains are designated as in Figure 2.3. All images are of H+E stained sections. (A) Tumor from a Line 2 *MET* ON/OFF animal more than one year after inactivation of the *MET* transgene. Biliary elements are marked with black arrowheads and hepatocyte elements are marked with black arrows. (B) Liver from Line 2 *MET* OFF/ON animal 3 months following induction of Met. The white arrowhead denotes a mitotic figure. (C-D) Tumors from two separate Line 2 *MET* OFF/ON animals at one year of age.

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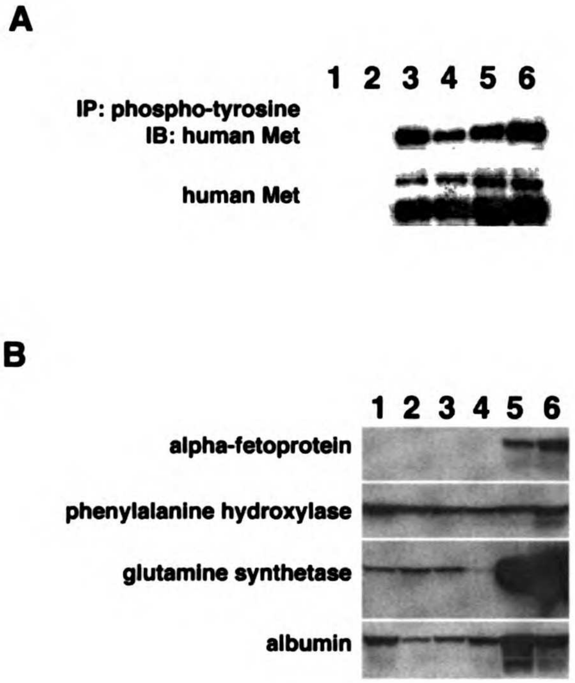
A
1 2 3 4 5 6
IP: phospho-tyrosine
IB: human Met
human Met
B
1 2 3 4 5 6
alpha-fetoprotein
phenylalanine hydroxylase
glutamine synthetase
albumin

Figure 2.7: Differentiation markers in livers of *MET* OFF/ON animals. Strains are designated as in Figure 2.3. (A) Top row, Immunopreciptiation with an anti-phospho-tyrosine antibody was performed on whole tissue lysates from livers of LAP-tTA mice (lanes 1-2) or Line 2 *MET* OFF/ON mice (lines 3-6). Western Blotting was then performed with an antibody against human Met. Bottom row, Western Blotting was performed on whole tissue lysates from livers of the mice listed above with an antibody against mouse Met. (B) Western Blotting was performed with antibodies against the indicated antigens using whole tissue lysates from livers of LAP-tTA (lanes 1-2) mice or Line 2 *MET* OFF/ON mice (lanes 3-6). Lanes 1-2, lysates of livers from LAP-tTA littermates at four months of age. Lanes 3-4, lysates of livers from animals of the Line 2 *MET* OFF/ON protocol three months after induction of the *MET* transgene. Lanes 5-6, lysates of HCCs from animals of the Line 2 *MET* OFF/ON protocol more than one year after induction of the *MET* transgene. The duplicate lanes represent separate animals.

size and abnormal nuclear size and shape in the livers of animals 3 months after induction of *MET* (Figure 2.6B). These *MET* OFF/ON livers retained the molecular markers of mature hepatocytes, expressing PAH and GS but not AFP (Figure 2.7B). We conclude that Met overexpression is sufficient to block differentiation of progenitors, but cannot revert mature hepatocytes to a progenitor state. Rather, overexpression of Met in hepatocytes gives rise to histological abnormalities that are harbingers of eventual tumorigenesis.

As we previously reported, the *MET* OFF/ON mice begin to develop hepatocellular carcinoma at approximately 6 months of age (Wang et al. 2001). In contrast to *MET* ON/OFF tumors, *MET* OFF/ON tumors contain cells that are predominantly hepatocyte like, rarely contain bile ducts, and closely resemble human hepatocellular carcinoma (HCC), with trabecular (Figure 2.6C) and sheet-like (Figure 2.6D) architectures. Cells of these tumors expressed both the progenitor marker AFP and the mature markers GS and PAH (Figure 2.7B).

DISCUSSION

We have used a conditional transgene of *MET* to explore the role of the Met kinase in hepatic development and tumorigenesis. The results indicate that Met may function as a switch in the differentiation of hepatocytes. Physiological inactivation of Met occurs late in embryogenesis. By overexpressing Met and sustaining the activity of Met beyond that time, we were able to demonstrate that the activity of Met allowed ongoing proliferation of undifferentiated hepatocyte precursor cells *in vivo*. Sustained activity of Met eventually gave rise to hepatic tumors, the nature of which varied with the developmental context within which Met was abnormally active.

Three distinctive causes of death from sustained activity of Met

We used three different protocols that subjected mice to sustained activity of transgenic Met. In the first (*MET* ON/ON), Met was active throughout embryogenesis and continued so until the animals died between 3 and 8 weeks after birth. The animals had enlarged, hyperplastic livers composed of immature precursors of hepatocytes. We presume but have not documented that death of the animals was caused by diminished liver function. These results demonstrate that the Met kinase can block differentiation, and that the homeostatic mechanisms that normally govern the size of the liver are unable to override the effect of Met, perhaps because they are not effective against hepatic precursor cells.

In the second protocol (*MET* ON/OFF), the transgene of *MET* was inactivated one month after birth. The hyperplastic livers were remodeled into mature, functioning tissue, prolonging survival of the animals. Within one year, however, the mice had developed fatal tumors composed of both biliary and hepatocytic lineages. The mixed nature of the tumors suggests that tumorigenesis initiated with a relatively primitive, bipotential precursor cell. We suggest that the prolonged proliferation of progenitor cells in these mice allowed the accretion of genetic damage that gave rise to tumorigenesis.

In the third protocol (*MET* OFF/ON), the *MET* transgene was held inactive until three weeks after birth, then activated by the withdrawal of doxycycline. Over the ensuing months, there was no evidence that hepatocytes reverted to the progenitor form found in *MET* ON/ON and *MET* ON/OFF mice. Instead, the livers first became morphologically dysplastic, then developed neoplastic tumors resembling the hepatocellular carcinomas of humans (HCC). The neoplastic cells displayed molecular markers of hepatic progenitor cells and mature hepatocytes. We suggest that the tumors originated from relatively mature, unipotential progenitor cells, perhaps even mature hepatocytes themselves. The genesis of these tumors may approximate what happens in the genesis of human HCC, during which MET is often anomalously overexpressed in the context of the adult liver (Ueki et al. 1997; Tavian et al. 2000). The difference between the tumors that arise in *MET* ON/OFF mice and *MET* OFF/ON mice dramatizes how activating the same oncogene at distinct points within a developmental lineage can alter the ultimate identity of the resulting tumor.

We have previously reported the induction of HCC by Met in an ON/ON protocol with Lines 3 and 4 of the LAP-tTA/TRE-MET mice (Wang et al. 2001). The livers of these mice have relatively low levels of Met kinase activity throughout embryogenesis. The kinase activity disappears by 10 days after birth, and returns only in the context of hyperplastic foci that precede the development

of carcinoma (Figure 2.2B; and Chapter 3). The disappearance of kinase activity after birth may explain why differentiation is not impeded in Lines 3 and 4. In contrast, the livers of Lines 1 and 2 display a high level of Met kinase activity throughout embryogenesis and after birth, and it is this activity that apparently blocks differentiation in the hepatocyte lineage. The initiation of tumorigenesis that occurs in the *MET* ON/ON protocol with Lines 3 and 4 may be similar to that in the *MET* OFF/ON protocol with Lines 1 and 2. In both instances, Met kinase is silent soon after birth. In the *MET* OFF/ON protocol with Lines 1 and 2, the kinase appears by virtue of an experimental manipulation. In the *MET* ON/ON protocol with Lines 3 and 4, the kinase activity reappears spontaneously in abnormal cells, for reasons that have yet to be ascertained (Chapter 3). But in either instance, the appearance of Met kinase activity leads eventually to HCC.

Hepatocytes can differentiate in the context of the adult animal

The adult liver can regenerate following partial hepatectomy or chemical damage. Thus, it is apparent that the adult remains capable of providing whatever external factors are required for differentiation and tissue morphogenesis, operating in conjunction with surviving hepatocytes and tissue architecture. We have now shown that a normal liver can also arise in the adult from progenitor cells without apparent benefit of preexisting mature hepatocytes or tissue architecture. This finding has promising implications for tissue engineering as a source of organ grafts. It may not be necessary to create mature liver tissue in vitro prior to transplantation. Instead, it may be possible to seed progenitor cells on an artificial scaffold and then allow differentiation and morphogenesis to occur in vivo following implantation. The abundant hepatic progenitors available from the *MET* ON/ON mice described here may be valuable reagents for pursuing this possibility experimentally.

The Met kinase as a switch in hepatic development

Previous work has demonstrated that the Met kinase is required for the proliferation of hepatic progenitor cells, but not for the original lineage specification of the progenitors (Maina et al. 2001). It appears that Met may have no function in the adult liver, unless a requirement for regeneration arises (Borowiak et al. 2004; Huh et al. 2004). Indeed, the work reported here and previously (Wang et al. 2001) indicates that the anomalous expression and activation of Met can both impede hepatic differentiation and be tumorigenic.

Met may function as a crucial switch in the development of the liver. Its activity during embryogenesis supports the proliferation of hepatocyte progenitor cells (Maina et al. 2001; and the present report), and its perinatal inactivation may be required to permit these cells to differentiate. The abundance of Met enzymatic activity in murine liver is apparently controlled by direct action on the protein rather than by altering its abundance. Control of this sort would facilitate a prompt proliferative response to any need for regeneration of liver tissue.

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 activity might itself trigger differentiation. The *MET* ON/ON protocol with the transgenic mice employed here could provide an abundant source of progenitor cells for the experimental study of hepatic differentiation, tumorigenesis, and tissue engineering.

MATERIALS AND METHODS

Mice

Transgenic mice that express the tet-transactivator in a liver specific fashion (LAP-tTA) were mated with transgenic mice that express wild type *MET* under the control of the tetracycline response element (TRE-MET) to generate double transgenic mice (LAP-tTA/TRE-MET) as we previously reported (Wang et al. 2001). All mice were on the FVB/N background. Age matched LAP-tTA littermates were used as controls. Doxycycline was administered either in the food (200 mg/kg) or in the water (2 mg/mL) for suppression of transgene expression. Genotyping was performed by PCR as described (Wang et al. 2001).

Histology

Animals were euthenized and their livers removed and rinsed in PBS. One piece was snap frozen in liquid nitrogen for preparation of lysates and other pieces fixed overnight in freshly prepared cold 4% paraformaldehyde. Fixed tissue samples were then washed three times in PBS and stored in 70% ethanol until they were embedded in paraffin. Five micron sections were placed on slides and stained with hematoxylin and eosin.

Immunohistochemistry

Paraffin was removed from unstained slides with xylenes. The slides were then rehydrated through a series of washes with incrementally decreasing percentages of ethanol. Antigen retrieval was done in 10 mM sodium citrate buffer pH 6.0 by placing in a microwave on high for 10 minutes followed by 20 minute cool down at room temperature. Samples were then subjected to 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity. Blocking was done with the Avidin-Biotin Blocking Kit in combination with either goat serum or the Mouse On Mouse Peroxidase Kit (Vector labs, Burlingame California). Primary antibody binding was done for either 30 minutes at room temperature or overnight at 4°. Detection was performed with the ABC-Elite Peroxidase Kit (Vector labs), using the DAB Substrate Kit (Vector labs). Counterstaining was done by a 5 second dip in hematoxylin Gill 3 (Sigma). Antibodies and dilutions were as follows: anti-Phospho-Met (Tyr1234/1235) Antibody, 1:25 (Cell Signaling Technology); anti-human Met, 1:500 (Zymed); anti-alpha-fetoprotein, 1:1000 (Dako); anti-glutamine synthetase, 1:500 (BD Bioscience); anti-phenylalanine hydroxylase, 1:500 (BD Bioscience). Apoptosis was detected with the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Serologicals Corporation). Apoptosis was quantitated by counting the number of apoptotic hepatocytes per 10 random high power fields by a pathologist who did not know the identity of the samples.

Immunoprecipitation

For each sample, 10 μL of anti-phospho-tyrosine antibody (4G10) was added to 1 mg of protein in 200 μL of lysis buffer and placed on a rocker overnight at 4°. 12 μL of protein G beads were added to each sample, which was placed on a rocker at 4° for 1 hour. The beads were washed three times with 1 mL of lysis buffer, then boiled in 50 μL of SDS sample buffer. 20 μL was then loaded per lane and subjected to Western Blotting as described below.

Western Blots

Lysates were made by taking a sample of frozen liver tissue and placing it in a tissue grinder with lysis buffer. The lysis buffer was composed of 1% NP40, 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 183 mg/mL $NaVO_4$, and 100 mM NaF and contained a cocktail of protease inhibitors consisting of leupeptin, aprotinin, and Pefabloc (Roche). After homogenization, insoluble debris was removed by centrifugation. Samples from

the lysates were then subjected to a BCA protein assay (Pierce). Samples of 50 micrograms of protein each were subjected to SDS-PAGE. Proteins were transferred to PVDF membranes and blocked with 5% milk. Primary antibody binding was done for either one hour at room temperature or overnight at 4°. Detection was performed by ECL (Amersham). Antibodies and dilutions were as follows: : anti-Phospho-Met (Tyr1234/1235) Antibody, 1:1000 (Cell Signaling Technology); anti-human Met, 1:2000 (Santa Cruz Biotech); anti-mouse Met, 1:1,000 (Santa Cruz Biotech); anti-glutamine synthetase, 1:5000 (BD Biosciences); anti-phenylalanine hydroxylase, 1:5000 (BD biosciences); anti-albumin, 1:10,000 (Dako); and anti-alpha fetoprotein,1:5000 (Dako).

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REFERENCES

Borowiak, M., Garratt, A.N., Wustefeld, T., Strehle, M., Trautwein, C., and Birchmeier, C. 2004. Met provides essential signals for liver regeneration. *Proc Natl Acad Sci U S A* **101**(29): 10608-10613.

Huh, C.G., Factor, V.M., Sanchez, A., Uchida, K., Conner, E.A., and Thorgeirsson, S.S. 2004. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proc Natl Acad Sci U S A* **101**(13): 4477-4482.

Ishikawa, K.S., Masui, T., Ishikawa, K., and Shiojiri, N. 2001. Immunolocalization of hepatocyte growth factor and its receptor (c-Met) during mouse liver development. *Histochem Cell Biol* **116**(5): 453-462.

Lemaigre, F. and Zaret, K.S. 2004. Liver development update: new embryo models, cell lineage control, and morphogenesis. *Curr Opin Genet Dev* **14**(5): 582-590.

Maina, F., Pante, G., Helmbacher, F., Andres, R., Porthin, A., Davies, A.M., Ponzetto, C., and Klein, R. 2001. Coupling Met to specific pathways results in distinct developmental outcomes. *Mol Cell* **7**(6): 1293-1306.

Schmidt, C., Bladt, F., Goedecke, S., Brinkmann, V., Zschiesche, W., Sharpe, M., Gherardi, E., and Birchmeier, C. 1995. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* **373**(6516): 699-702.

Spijkers, J.A., van den Hoff, M.J., Hakvoort, T.B., Vermeulen, J.L., Tesink-Taekema, S., and Lamers, W.H. 2001. Foetal rise in hepatic enzymes follows decline in c-met and hepatocyte growth factor expression. *J Hepatol* **34**(5): 699-710.

Suzuki, A., Iwama, A., Miyashita, H., Nakauchi, H., and Taniguchi, H. 2003. Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. *Development* **130**(11): 2513-2524.

Tavian, D., De Petro, G., Benetti, A., Portolani, N., Giulini, S.M., and Barlati, S. 2000. u-PA and c-MET mRNA expression is co-ordinately enhanced while hepatocyte growth factor mRNA is down-regulated in human hepatocellular carcinoma. *Int J Cancer* **87**(5): 644-649.

Ueki, T., Fujimoto, J., Suzuki, T., Yamamoto, H., and Okamoto, E. 1997. Expression of hepatocyte growth factor and its receptor c-met proto-oncogene in hepatocellular carcinoma. *Hepatology* **25**(4): 862-866.

Wang, R., Ferrell, L.D., Faouzi, S., Maher, J.J., and Bishop, J.M. 2001. Activation of the Met receptor by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice. *J Cell Biol* **153**(5): 1023-1034.

Chapter 3: Distinct Secondary Mutations Determine Tumor Identity in the Liver

ABSTRACT

The combinations of genetic and epigenetic events that give rise to hepatocellular carcinoma and hepatocellular adenoma are largely unknown. Using transgenic mouse models and hydrodynamic transfection of mice, we report that the proto-oncogene *MET,* which encodes the receptor tyrosine kinase Met, can cooperate with activation of β-catenin to give rise to hepatocellular carcinoma. In contrast, activation of *MET* in combination with inactivation of hepatocyte nuclear factor 1 alpha gave rise to benign hepatocellular adenoma. Despite activating mutations in the β-catenin gene, inactivation of transgenic Met induces tumor regression of HCC, although relapse eventually occurs. The cooperation of *MET* and β-catenin may be a general mechanism of hepatocarcinogenesis as Met and β-catenin are frequently activated together in human hepatocellular carcinoma. We conclude that distinct secondary mutations can give rise to distinct tumor types in mice, and demonstrate the utility of hydrodynamic transfection for studying liver tumorigenesis.

INTRODUCTION

Cancer is believed to arise from a stepwise progression of genetic and epigenetic alterations in proto-oncogenes and tumor suppressor genes (Kinzler and Vogelstein, 1996). It is well established that in different tissues the same initiating oncogene can give rise to distinct tumor types. It is also established that targeting different initiating oncogenes to the same tissue may give rise to similar or different tumor types. It is currently unclear, however, whether an initiating oncogene can give rise to distinct tumor types in the same tissue. Furthermore, if a given initiating oncogene does induce distinct tumor types in a tissue, what role do secondary mutations play in determining the identity of the resulting tumors?

We have addressed these questions in the setting of tumorigenesis in the liver. Liver cancer is one of the deadliest human malignancies, with an annual worldwide incidence of 600,000 cases and a mean survival time of six months from time of diagnosis (Parkin et al., 2005). Partial hepatectomy, liver transplantation, and ethanol or radiofrequency ablation are the major treatments tfor liver cancer; there is currently no chemotherapy that shows survival benefit (Llovet, 2005).

The major causes of liver cancer are infection with Hepatitis B or C Virus, chronic alcoholism, aflatoxin exposure, or presence of any other condition that predisposes to cirrhosis. These causes are believed to produce liver cancer by inducing repeated rounds of hepatocyte death and hepatocyte proliferation. This process creates a permissive environment for genetic or epigenetic changes to occur that confer gain of function on proto-oncogenes or loss of function on tumor suppressor genes (Thorgeirsson and Grisham 2002). Many such changes have been reported, but the combinations of these changes that operate in concert to produce human hepatocellular carcinoma (HCC) remain largely uncharacterized.

Frequent among the changes known to occur in human HCC are overexpression or mutation of the receptor tyrosine kinase Met (Tavian et al. 2000; Park et al. 1999), and activation of the Wnt signaling pathway by mutation of the genes encoding β-catenin (*CTNNB1),* Axin (*AXIN1),* or Axin 2 (*AXIN2)* (Giles et al., 2003). We have previously confirmed that *MET* overexpression can initiate and maintain HCC *in vivo*, thereby strengthening the argument that Met plays a pathogenic role in human HCC (Wang et al., 2001). It has also been shown that activation of β-catenin by loss of APC (Colnot et al. 2004) or inducible mutation of the endogenous β-catenin gene in combination with mutation of the Ha-Ras gene (Harada et al., 2004) is sufficient to initiate HCC in mice. However, activation of the Met and β-catenin pathways in concert has not been implicated in human HCC.

Hepatocellular adenoma (HCA) is a relatively rare benign tumor of the liver found most frequently in women with a history of oral contraceptive use. In contrast to HCC, only one genetic change has been clearly implicated in HCA: inactivating mutations in the gene encoding the transcription factor hepatocyte nuclear factor 1α (HNF1α) (*TCF1/Tcf1*) (Zucman-Rossi, 2004). Biallelic inactivating mutations of *TCF1* are found in 50% of all HCA, and some families with heterozygous germline mutations in *TCF1* develop an adenomatosis syndrome, in which individuals develop 10 or more HCAs that exhibit a loss of heterozygosity for *TCF1*. Although *Tcf1*$^{-/-}$ mice develop hepatomegaly and die around weaning, they have not been reported to show evidence of neoplasia (Pontoglio et al., 1996). Hence, other events in addition to loss of *TCF1* are likely to be necessary for development of HCA.

Here, we report that despite a common initiating mutation, distinct secondary mutations yield distinct tumor identities. We show that mice that overexpress *MET* in the liver developed HCA in addition to HCC. After activation of the Met kinase in the transgenic animals, HCC development is correlated with activation of β-catenin, whereas HCA development is correlated with loss of HNF1α pathway function. To confirm the causative role of these events, we used hydrodynamic transfection to express exogenous genes in the liver. We show that hydrodynamic transfection of *MET* and a mutant allele of β-catenin rapidly induces multifocal HCC, and transfection of *MET* and a dominant negative allele of HNF1α rapidly induces multifocal HCA. We also show that tumor cells did not durably differentiate into normal hepatocytes after inactivation of the *MET* transgene. We conclude that although the identity of the initiating oncogene partly constrains the pathway of tumorigenesis, distinct secondary mutations can determine the ultimate identity of the tumors that arise.

RESULTS

Distinct tumors in MET *transgenic mice*

We have previously generated strains of mice that overexpress a wild type allele of human *MET* specifically in hepatocytes, under the control of doxycycline (Wang et al., 2001). Four independent *MET* transgenic lines were generated, designated Lines 1, 2, 3, and 4. Newborn mice from Lines 1 and 2 expressed Met at a higher level than did Lines 3 and 4 (Figure 2.2A). At ten days of age, mice from Lines 1 and 2 maintained phosphorylation of Met, which we used as a surrogate for Met kinase activity (Naldini et al., 1991), whereas mice from Lines 3 and 4 had lost Met phosphorylation (Figure 2.2B). Mice from Lines 1 and 2 died around a month of age as a result of a block of differentiation in hepatocytes when maintained in the absence of doxycycline throughout embryogenesis and after birth (Wang et al., 2001; this work, Chapter 2). In contrast, hepatocytes of mice from Lines 3 and 4 did not show a block of differentiation. Rather, mice from these lines developed hyperplastic foci, which progressed to dysplastic foci (Figure 3.1B and 3.1C). These mice then started developing tumors at three months of age (Wang et al., 2001; and data not shown).

Two distinct types of tumors developed in Line 3 and 4 *MET* transgenic mice: HCC and HCA, often coexisting in the same liver. Typically there were between one and five independent tumor nodules of either type in any given liver.

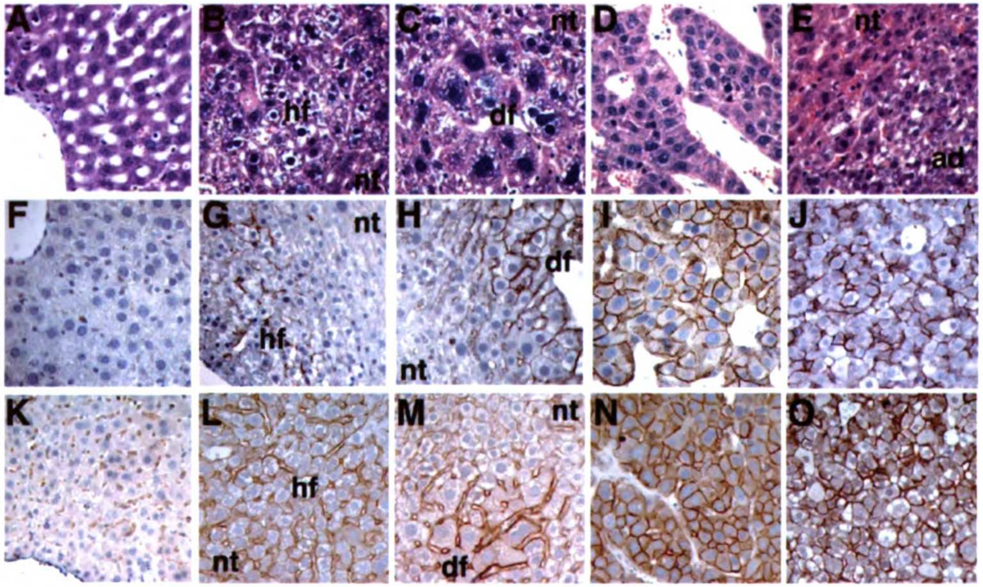

Figure 3.1: Activation of Met in pre-neoplastic and neoplastic lesions from *MET* transgenic mice.
Doxycyline was withheld for the life of LAP-tTA transgenic animals (A, F, and K), or Line 3 LAP-tTA/TRE-MET animals at one month of age (B, G, and L), two months of age (C, H, and M), or greater than three months of age (D, E, I, J, N, and O). Livers were sectioned and analyzed by microscopy after H+E staining (A-E), immunohistochemistry with an antibody against phosphorylated Met as a surrogate for enzymatic activity (F-J), or immunohistochemistry with an anitbody against human Met (K-O). Analyses were performed on control livers (A. F, and K), hyperplastic foci (B, G, and L), dysplastic foci (C, H, and M), HCCs (D, I, and N) or HCAs (E, J, and O). hf denotes hyperplastic focus, df denotes dysplastic focus. nt denotes normal tissue. and ad denotes HCA.

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The HCCs were composed predominantly of hepatocytic cells, had cell plates greater than three cells thick, had a paucity of bile duct cells, and lacked lobular architecture (Wang et al., 2001; and Figure 3.1D). In addition, the HCCs expressed the fetal marker alpha-fetoprotein (AFP) (Figure 3.2S). The HCAs were also composed of hepatocytic cells, had a paucity of bile duct cells, and lacked lobular architecture (Figure 3.1E). Unlike in the HCCs, however, the cell plates in the HCAs were one to two cells thick (Figure 3.1E), and the HCAs did not express the fetal marker AFP (Figure 3.2T). We did not observe HCCs growing within HCAs, or vice versa. HCAs tended to arise later and typically in smaller numbers than the HCCs (data not shown). We conclude that the HCC and HCA tumors probably arose independently of one another.

Distinct pathways of tumorigenesis in MET *transgenic mice*

To determine whether activation of Met is responsible for the generation of hyperplastic and dysplastic foci, we performed immunohistochemistry with an antibody directed against phosphorylated Met. Although Met was expressed in all hepatocytes in the livers of Line 3 *MET* transgenic animals (Figure 3.1L and 3.1M), phosphorylated Met was detected only in hyperplastic foci (Figure 3.1G), dysplastic foci (Figure 3.1H), HCCs (Figure 3.1I), and HCAs (Figure 3.1J). Furthermore, hyperplastic foci were not observed in Line 3 *MET* transgenic animals maintained in the absence of doxycycline for 6 months then placed on doxycycline for 6 months. We conclude that activation of Met correlated with and

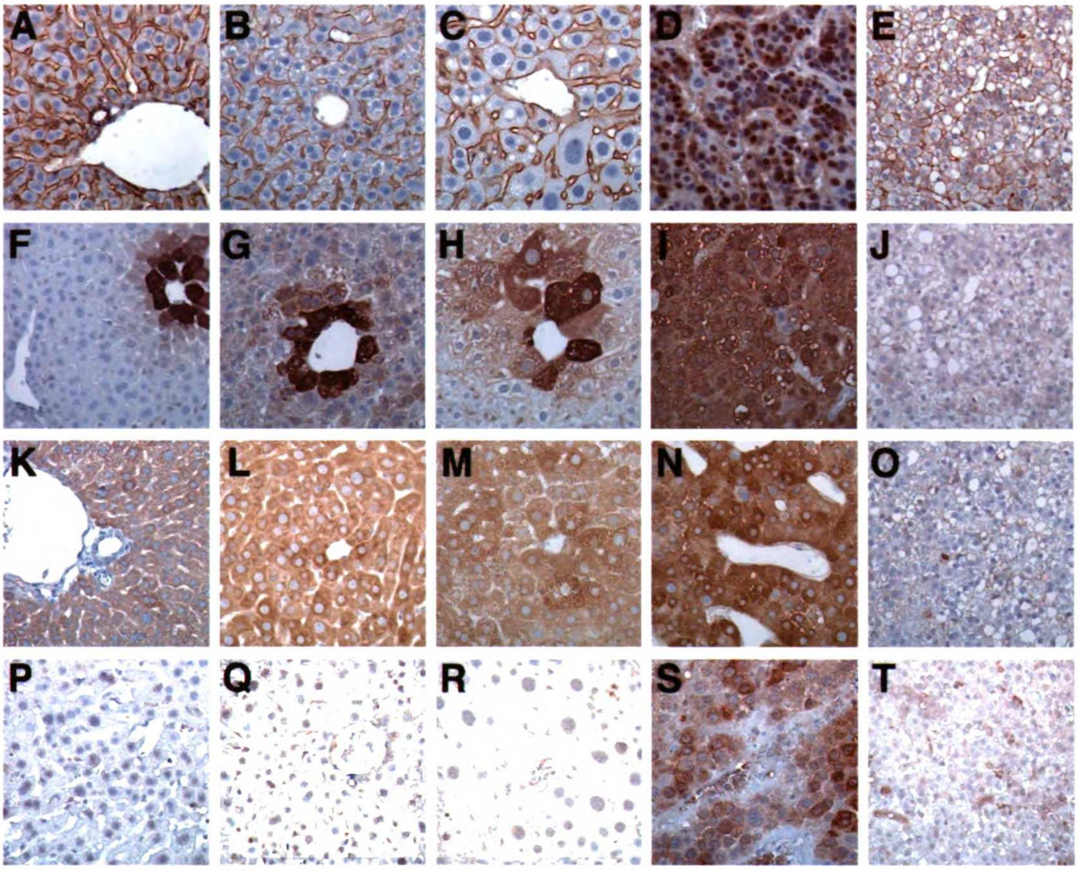

Figure 3.2: Markers of progression in pre-neoplastic and neoplastic lesions from *MET* transgenic mice.
Doxycyline was withheld for the life of LAP-tTA transgenic animals (A, F, K, and P), or Line 3 LAP-tTA/TRE-MET animals at one month of age (B, G, L, and Q), two months of age (C, H, M, and R), or greater than three months of age (D, E, I, J, N, O, S, and T). Livers were sectioned and analyzed by microscopy after immunhistochemistry with antibodies against β-catenin (A-E), glutamine synthetase (F-J), phenylalanine hydroxylase (K-O), or alpha-fetoprotein (P-T). Analyses were performed on control livers (A. F, K, and P), hyperplastic foci (B, G, L, and Q), dysplastic foci (C, H, M, and R), HCCs (D, I, N, and S) or HCAs (E, J, O, and T).

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may be responsible for the induction of hyperplasia in Line 3 *MET* transgenic livers.

We next wished to determine if activation of β-catenin was involved in the progression from hyperplastic or dysplastic focus to HCC. We looked for evidence of activation of β-catenin by two separate assays: nuclear accumulation of β-catenin (Giles et al., 2003), and expression of the β-catenin target glutamine synthetase (GS) (Cadoret et al., 2002). Nuclear β-catenin was detected in sections from HCC nodules (Figure 3.2D), whereas only membrane staining was present in sections from normal liver, hyperplastic foci, dysplastic foci, and HCAs (Figure 3.2A-3.2C and 3.2E). In agreement with the nuclear β-catenin staining, GS was expressed in HCC nodules, but absent from HCA nodules (Figure 3.2I-3.2J). As in the normal liver, GS was expressed in hyperplastic and dysplastic foci by hepatocytes immediately adjacent to the central vein, but not beyond (Figure 3.2F-3.2H). We conclude that β-catenin is activated in the progression of HCC, but not HCA in Line 3 and 4 *MET* transgenic mice.

To determine the mechanism of β-catenin activation in HCCs from Line 3 and 4 *MET* transgenic mice, we analyzed the mouse β-catenin gene (*Ctnnb1*) for evidence of mutation by sequencing genomic DNA. Almost all (20/21) HCC nodules that we analyzed harbored a heterozygous activating mutation in *Ctnnb1* (Table 3.1). In contrast, no HCA nodule that we analyzed (0/12) harbored a

Table 3.1: *Catnb1* mutations in HCCs from *MET* transgenic mice.

[a]DNA was extracted from the tissues indicated then subjected to PCR analysis with primers specific for exon two of the *Catnb1* gene. [b]All mutations were validated by sequencing at least two independent PCR products per sample with both forward and reverse primers. In addition, when possible, PCR products from samples with mutations were subjected to differential restriction enzyme digestion to validate the presence of mutations.

Strain	Sample[a]	Sample ID	Mutation[b]	codon
FVB/N	Tail		none	
FVB/N	Liver		none	
Line 3	Non-tumor	10683 NT	none	
Line 3	Non-tumor	10682 NT	none	
Line 3	Non-tumor	10679 NT	none	
Line 4	Non-tumor	487 NT	none	
Line 3	HCC	10683 T1	S37C	TCT->TGT
Line 3	HCC	10682 T1	Del I35->G38	
Line 3	HCC	10679 T1	Del E9->Q76	
Line 3	HCC	10679 T3	G34E	GGA->GAA
Line 3	HCC	437 T1	S33F	TCT->TTT
Line 3	HCC	479 T1	S33Y	TCT->TAT
Line 3	HCC	393 T1	Del I35-> G38	
Line 3	HCC	393 T2	G34R	GGA->AGA
Line 3	HCC	476 T1	S33F	TCT->TTT
Line 3	HCC	345 T1	D32N	GAT->AAT
Line 3	HCC	345 T2	none	
Line 3	HCC	475 T1	S33F	TCT->TTT
Line 3	HCC	10687 T1	G34E	GGA->GAA
Line 3	HCC	10687 T2	G34R	GGA->AGA
Line 3	HCC	10686 T1	G34E	GGA->GAA
Line 3	HCC	10686 T2	S37C	TCT->TGT
Line 3	HCC	10685 T1	S33F	TCT->TTT
Line 3	HCC	10685 T2	S37F	TCT->TTT
Line 4	HCC	165 T2	S37Y	TCT->TAT
Line 4	HCC	165 T3	S33Y	TCT->TAT
Line 4	HCC	160 T1	S33A	TCT->GCT
Line 4	HCC	430 T4	splice site	G->A
Line 4	HCA	487 T1	none	
Line 4	HCA	487 T2	none	
Line 4	HCA	487 T3	none	
Line 4	HCA	487 T4	none	
Line 4	HCA	165 T1	none	
Line 4	HCA	160 T2	none	
Line 4	HCA	160 T3	none	
Line 4	HCA	160 T4	none	
Line 4	HCA	160 T5	none	
Line 4	HCA	430 T1	none	
Line 4	HCA	430 T2	none	
Line 4	HCA	430 T5	none	
Line 4	HCA	430 T6	none	

mutation in *Ctnnb1* (Table 3.1). In the instances where we analyzed multiple nodules from the same liver, separate HCC nodules harbored different *Ctnnb1* mutations, indicating that each nodule represents an independent clone (Table 3.1). We conclude that β-catenin is activated by mutation in the progression of HCC.

Because the HCAs did not show activation of β-catenin, we explored the possibility that loss of HNF1α was involved in the progression of HCA. The HNF1α target phenylalanine hydroxylase (PAH) (Pontoglio et al., 1997) was not expressed in HCA nodules (Figure 3.2O), but was expressed in normal liver, hyperplastic and dysplastic foci, and in HCC nodules (Figure 3.2K-3.2N). However, Western Blot, quantitative RT-PCR, and sequencing analysis revealed no alterations in HNF1α protein levels, mRNA levels, or DNA sequence, respectively, in HCAs relative to normal liver (data not shown). Sequencing analysis of the genes encoding the two dimerization cofactors of HNF1α (DCoH1 and DCoH2) also revealed no mutations. Although the identities of the mutations involved in the progression of the HCAs is unknown, we suggest that these mutations may ultimately affect the transactivation function of HNF1α , and do not involve β-catenin.

Distinct pathways of tumorigenesis in mice after hydrodynamic transfection

Our results with the Line 3 and 4 *MET* transgenic mice suggested a forked pathway whereby activation of the Met kinase initiates tumorigenesis, and progression to HCC or HCA is favored by activation of β-catenin or loss of HNF1α function, respectively. To confirm this model, we employed hydrodynamic transfection to deliver exogenous genes to the liver (Liu et al., 1999; Zhang et al., 1999). Because we required integration of the exogenously delivered genes, we used a transposon system composed of two plasmids: the first was an expression vector expressing a hyperactive allele of the Sleeping Beauty transposase, and the second was an expression vector in which the cassette containing the gene of interest was flanked by inverted repeats that permitted integration by the Sleeping Beauty transposase (Yant et al., 2000; Yant et al. 2004). Using this system we can routinely stably transfect 5-20% of hepatocytes (Yant et al. 2000; and data not shown).

In an effort to recapitulate the pathogenesis of HCCs and HCAs using hydrodynamic transfection, we generated transposons containing genes with epitope tages that encode GFP, a wild type allele of human *MET*, a constitutively active allele of β-catenin similar to those found in HCCs in humans and mice (ΔN90-β-catenin), and a dominant negative allele of HNF1α containing only the N-terminal 290 amino acids (DNHNF1α) (Vaxillaire et al., 1999) similar to those found in the germline of families with familial adenomatosis (Reznik et al., 2004).

First, we verified that the alleles that we generated were expressed and functional after hydrodynamic transfection. We detected expression of the transfected alleles by immunohistochemistry with antibodies against their respective epitope tags (Figure 3.3A – 3.3D). We inferred functionality of the

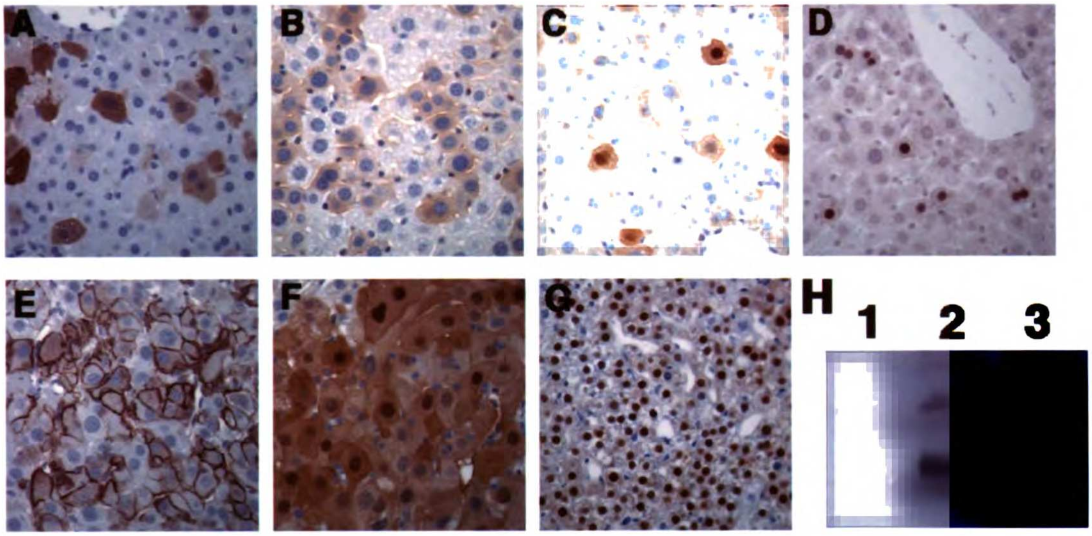

Figure 3.3: Expression of hydrodynamically transfected genes.
(A-G) Sections from livers of FVB/N mice hydrodynamically transfected at 6-8 weeks of age were subjected to immunohistochemistry with antibodies against the V5 tag (A, B, D, and G), the myc tag (C and F), or phospho-Met (E). Mice were hydrodynamically transfected with the listed alleles, then euthenized after the indicated time. (A) GFP with a C-terminal V5 tag; animal euthenized 24 hours after hydrodynamic transfection. (B) Met with a C-terminal V5 tag; animal euthenized one month after hydrodynamic transfection. (C) ΔN90-β-catenin with a N-terminal myc tag; animal euthenized one month after hydrodynamic transfection. (D) DNHNF1α with a C-terminal V5 tag; animal euthenized 11 months after hydrodynamic transfection. (E-F) Met with a C-terminal V5 tag and ΔN90-β-catenin with a N-terminal myc tag; animal euthenized three months after hydrodynamic transfection. An HCC nodule is shown. (G) Met with a C-terminal V5 tag and DNHNF1α with a C-terminal V5 tag; animal euthenized three months after hydrodynamic transfection. An HCA nodule is shown. (H) Lysates of the normal liver (lane 1) or tumor (lanes 2-3) were subjected to immunoprecipitation with an anti-phospho-tyrosine antibody (4G10), followed by Western Blotting with an antibody against human Met. Lane 1, liver from one year old FVB/N control mouse; lane 2, HCA nodule from an animal hydrodynamically transfected with *MET* and the DNHNF1α allele, then euthenized three months later; lane 3, HCC nodule from an animal hydrodynamically transfected with *MET* and the ΔN90-β-catenin allele, then euthenized three months later.

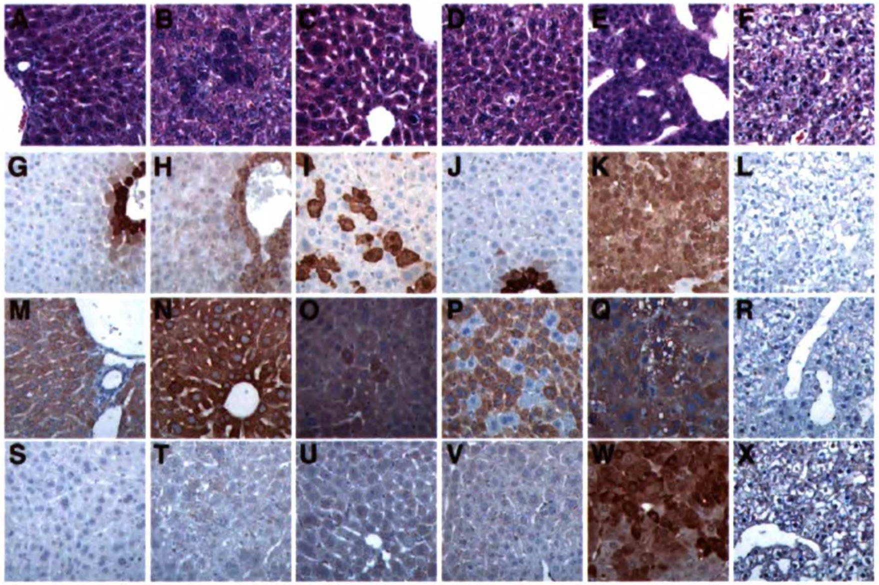

Figure 3.4: Tumorigenesis after hydrodynamic transfection.
Hydrodynamic transfections were performed on six to eight week old FVB/N mice. Mice were euthenized one to three months later. Mice were hydrodynamically transfected with a plasmid containing the gene for the sleeping beauty transposase and plasmids containing the genes for GFP with a C-terminal V5 tag (A, G, M, and S), wild type human Met with a C-terminal V5 tag (B, H, N, and T), an activated mutant of β-catenin that lacks the 90 N-terminal amino acids with an N-terminal myc tag, ΔN90-β-catenin (C, I, O, and U), a dominant negative allele of HNF1α that has only the N-terminal 290 amino acids and a C-terminal V5 tag, DNHNF1α (D, J, P, and V), both Met and ΔN90-β-catenin (E, K, Q, and W), or both Met and DNHNF1α (F, L, R, and X). Livers were sectioned and analyzed by microscopy after H+E staining (A-F), or immunohistochemistry with antibodies against glutamine synthetase (G-L), phenylalanine hydroxylase (M-R), or alpha-fetoprotein (S-X). Images depict non-tumor bearing liver (A-D, G-J, M-P, and S-V), HCC nodules (E, K, Q, and W), or HCA nodules (F, L, R, and X).

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MET allele by the induction of dysplasia (Figure 3.4B) as well as upregulation of E-cadherin (data not shown) after hydrodynamic transfection of *MET*. Hydrodynamic transfection of ΔN90-β-catenin induced the β-catenin target GS in hepatocytes not adjacent to the central vein (Figure 3.4I). Hydrodynamic transfection of the DNHNF1α allele gave rise to scattered hepatocytes with a foamy appearance (Figure 3.4D), and was able to inhibit expression of the HNF1α target PAH (Figure 3.4P). Within three months none of the animals transfected with the genes for GFP, Met, ΔN90-β-catenin, or DNHNF1α alone showed any evidence of tumors, although a small number of animals succumbed to tumors after that point (Figure 3.5), presumably after enough time had elapsed for a cooperating mutation to occur. We conclude that the alleles we generated are functional, but are not sufficient to rapidly induce tumors alone.

In contrast to hydrodynamic transfection with each allele alone, the combination of *MET* and ΔN90-β-catenin rapidly gave rise to multi-focal HCC in 74% (20/27) of animals (Figures 3.4E and 3.5). The HCC that arose was multifocal, with more than 50 nodules per liver. The presence of HCC was apparent within one month of transfection. Unlike after transfection of each allele independently, 21% (7/34) of animals hydrodynamically transfected with *MET* in combination with ΔN90-β-catenin died within three days after hydrodynamic transfection (Figure 3.5). In the HCC nodules, we could detect ΔN90-β-catenin by immunohistochemistry and phosphorylated Met by immunohistochemistry and Western Blotting (Figure 3.3E, 3.3F, and 3.3H). These animals died with

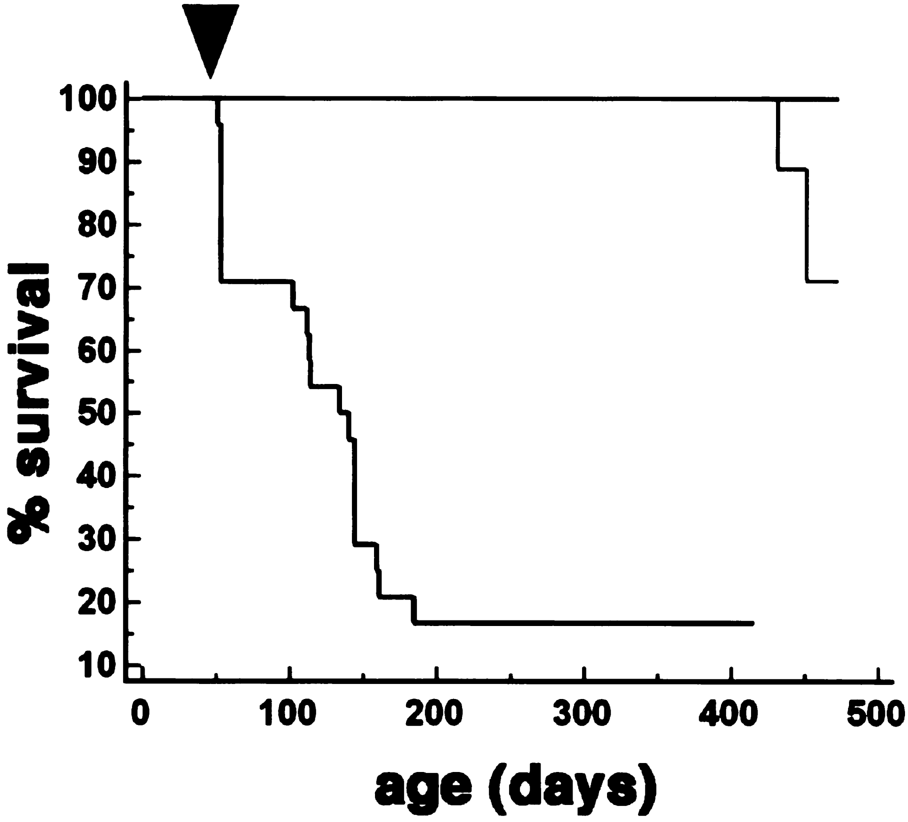

Figure 3.5: Survival of mice after hydrodynamic transfection.
Six to eight week old FVB/N mice were hydrodynamically transfected with the indicated constructs and observed. Black line, *MET* and ΔN90-β-catenin (N=24); Red line, *MET* (N=9); Blue line, ΔN90-β-catenin (N=15), DNHNF1α (N=8), and *MET* and DNHNF1α (N=8). The black arrowhead indicates the point of hydrodynamic transfection.

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massive liver tumors around three months after hydrodynamic transfection (Figure 3.5; and data not shown). Like the HCCs from the *MET* transgenic mice, the tumors induced by hydrodynamic transfection with *MET* and the ΔN90-β-catenin allele expressed GS, PAH, and AFP (Figure 3.4K, 3.4Q, and 3.4W). Livers from these animals showed no evidence of HCA. Sequential hydrodynamic transfection with *MET* followed by hydrodynamic transfection with the ΔN90-β-catenin allele three weeks later, or the reverse, yielded similar results to injection of the two genes in combination (data not shown). We conclude that activated Met and β-catenin could cooperate to rapidly induce multifocal HCC, irrespective of the order in which the events occurred.

Hydrodynamic transfection with a combination of *MET* and DNHNF1α gave rise to multifocal HCA in 50% (5/10) of animals (Figure 3.4F). These animals were still alive after 10 months of observation, probably due to the indolent nature of HCA (Figure 3.5). Like the HCAs from the *MET* transgenic mice, the tumors induced by hydrodynamic transfection with *MET* and DNHNF1α did not express GS, PAH, or AFP (Figure 3.4L, 3.4R, and 3,4X). Immunhistochemistry confirmed that the DNHNF1α was expressed in the nuclei of all cells within each HCA nodule (Figure 3.3G). Western Blotting confirmed that phosphorylated Met was present in HCA nodules, albeit at lower levels than in HCC nodules (Figure 3.3H). We conclude that activated Met in combination with inactivation of HNF1α could cooperate to rapidly induce multifocal HCA.

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Activation of Met and β-catenin in human HCC

To explore whether the pathway of tumorigenesis that we delineated in our mouse model also operates in humans, we analyzed human HCC nodules for evidence of Met and β-catenin activation. We assayed β-catenin activation by sequencing the *CTNNB1* locus and by Western Blotting for the β-catenin target GS. We assayed Met activation by immunoprecipitation followed by Western Blotting for phosphorylated Met (Figure 3.6). Of tumors with an activating mutation in *CTNNB1*, 85% (12/14) expressed a high amount of GS, compared with only 7% without a mutation (3/42). The latter population may represent tumors that have activated β-catenin through mutation of other components of the Wnt pathway. Among tumors with easily detectable phosphorylated Met, 60% of tumors (9/15) had an activating mutation in *CTNNB1*. Among tumors with a low or undetectable amount of phosphorylated Met, 12% of tumors (5/41) harbored an activating mutation in *CTNNB1* ($p < .001$ by chi square for the association of *CTNNB1* mutation with presence of phosphorylated Met). Although this subset of HCCs possessed low amounts of phosphorylated Met, high amounts of total Met were detected in 43% of these tumors (18/41). We conclude that in a subset of human HCCs, mutation of *CTNNB1* was a frequent event in combination with activation, but not high expression of Met.

Recurrence after tumor regression

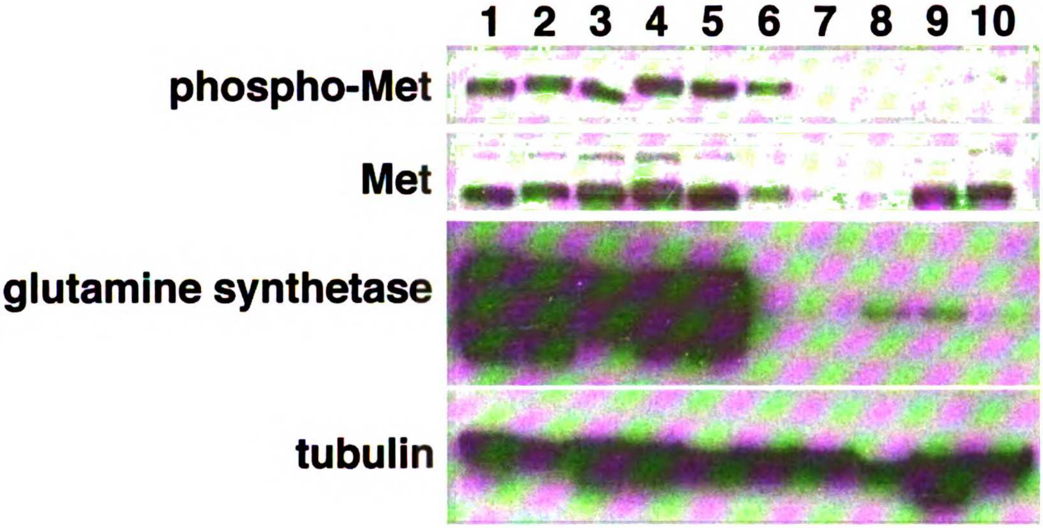

Figure 3.6: Coincidence of Met activity and mutant β-catenin in human HCC. Western Blot analysis was perfomed on lysates from human HCCs with antibodies against the antigens indicated. For the blot marked phospho-Met, immunoprecipitation with an antibody against phosphorylated tyrosine (4G10) was followed by Western Blotting with an antibody against human Met. Whole liver lysates were used for Met, glutamine synthetase, and tubulin. Tubulin is shown to demonstrate equal loading. Lanes 1-5 were loaded with samples that harbored a mutant allele of β-catenin, lanes 6-10 were loaded with samples that harbored a wild type allele of β-catenin.

We previously reported regression of HCC lesions in the *MET* transgenic animals following inactivation of the *MET* transgene by doxycycline (Wang et al, 2001). Tumor regression was accompanied by a cessation of proliferation as well as an induction of apoptosis. Our knowledge that *Catnb1* was mutant enabled us to detect the HCC cells after tumor regression by looking for cells with nuclear accumulation of β-catenin using immunohistochemistry. We maintained Line 3 *MET* transgenic animals in the absence of doxycycline for seven months to permit the development of HCC. We then inactivated *MET* transgene expression by administering doxycycline in the diet and maintained the animals in the presence of doxycycline for six months. After this protocol, we found nests of residual hepatocyte-like cells embedded in scar tissue (Figure 3.7A), which possess nuclear accumulation of β-catenin (Figure 3.7B). However, we never observed normal hepatocytes with nuclear accumulation of β-catenin intercalated in the normal parenchyma (Figure 3.7B). We conclude that after tumor regression, the liver is not reconsituted by tumor cells.

Despite being maintained on doxycycline after developing HCCs, the *MET* transgenic mice began to succumb to recurrent tumors starting within a few months after administration of doxycycline (Figure 3.7E). The recurrent tumors retained the histological appearance of HCC and had nuclear accumulation of β-catenin (Figure 3.7C and 3.7D). Western Blot analysis of lysates from the recurrent tumors demonstrated the absence of phosphorylated Met, as well as no

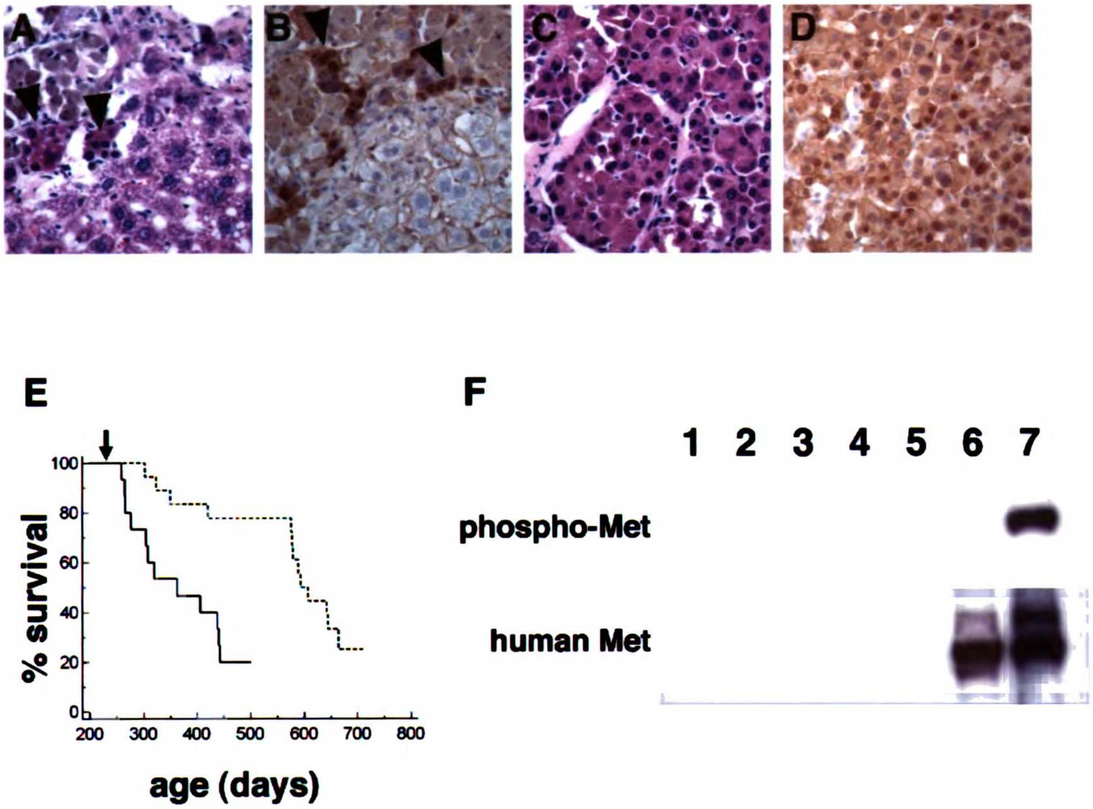

Figure 3.7: Recurrence of HCC after regression.
Eight month old LAP-tTA/TRE-MET Line 3 mice with tumors were placed on doxycycline then followed thereafter (E) or euthenized after 6 months (A-D, F). Livers from euthenized mice were sectioned and analyzed by microscopy after H+E staining (A and C), or immunohistochemistry with an antibody against β-catenin (B and D). A and B depict residual tumor cells (arrowheads) embedded in scar tissue, adjacent to normal parenchyma (lower right). C and D depict cells from a recurrent tumor nodule. Survival of tumor bearing mice was analyzed (E) after administration of doxycycline (black arrow). Animals were given doxycycline in their food (dashed line, N=18), or were given regular food (solid line, N=15) at seven months of age. Western Blot analysis was perfomed on lysates from liver with antibodies against the antigens indicated (F). Lane 1, Non-tumor tissue from a LAP-tTA/TRE-MET Line 3 mouse with recurrent tumor; lanes 2-4, recurrent tumors from LAP-tTA/TRE-MET Line 3 mice maintained in the presence of doxycycline for one year; lane 5, wild-type FVB/N control; lane 6, non-tumor tissue from a LAP-tTA/TRE-MET Line 3 mouse off doxy; lane 7 HCC from a LAP-tTA/TRE-MET Line 3 mouse maintained in the absence of doxycycline.

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reexpression of the *MET* transgene (Figure 3.7F). We conclude that inactivation of the *MET* transgene was sufficient to induce tumor regression, even in the presence of a mutant *Catnb1*. However, the regression that occurred was not durable, and the mice ultimately succumbed to recurrent HCCs.

DISCUSSION

We previously reported that overexpression of human *MET* in the mouse liver induces and maintains HCC (Wang et al. 2001). Here we report that the *MET* transgenic mice developed HCAs and HCCs by distinct pathways of tumorigenesis. In both the HCCs and HCAs, activation of Met was the initiating event. But a subsequent event apparently determined what type of tumor would result (β-catenin activation in HCC, inactivation of HNF1α in HCA). To confirm this hypothesis, we employed hydrodynamic transfection to deliver exogenous oncogenes in the liver. Using hydrodynamic transfection, we recapitulated the pathogenesis of HCCs and HCAs of the *MET* transgenic mice. We then showed that in a subset of human HCCs the activation of Met was strongly correlated with activating mutations of β-catenin. The apparent similarity in genotypic progression in the mouse models of liver tumors descibed above to human liver tumors provides additional evidence of their authenticity as models for human diseases.

A new approach to experimental tumorigenesis in the liver

We have presented here the details of a new approach to experimental tumorigenesis in the liver based upon hydrodynamic transfection. This approach has several advantages over standard transgenic techniques: it permits the expression of genes exclusively in adult animals thus avoiding any artifacts that might arise from aberrant development; it allows for relatively rapid execution and repetition of experiments; and by inserting expression modules at numerous sites throughout the genome, it avoids artifacts that can arise from the chromosomal position of a transgene in the form of either spurious activity or insertional mutatgenesis. Hydrodynamic transfection of cDNA and shRNA librarys should permit rapid screening for novel tumor suppressors and oncogenes.

One limitation of hydrodynamic transfection is that not every animal that received a hydrodynamic transfection maintained long term expression of the transfected genes. This phenomenon can explain the lower than 100% penetrance of tumors in the hydrodynamic transfection protocols with combinations of oncogenes. In support of this suggestion, animals without tumors did not express detectable levels of the transfected genes (data not shown). This effect is likely due to immune mediated clearance as hydrodynamically transfected animals that showed no expression of the transfected genes after one month had numerous large nests of lymphocytes within the liver parenchyma (data not shown).

Activation of Met in preneoplastic and neoplastic lesions

In the *MET* transgenic mice, it is unclear why Met is activated in the preneoplastic and neoplastic lesions, but not in the surrounding tissue that still expresses the transgene. One possibility is that these lesions express higher levels of the transgene. In support of this possibility, cells within the preneoplastic and neoplastic lesions stain more intensely for human Met than the surrounding normal tissue (Figure 3.1). Newborn *MET* transgenic animals from Lines 1 and 2 express Met at a higher level than animals from Lines 3 and 4 (Figure 2.2A), and have Met constitutively activated in all hepatocytes (data not shown). This finding implies a threshold of expression above which Met is constitutively phosphorylated.. We suggest that expression above (Lines 1 and 2) or below (Lines 3 and 4) this threshold at the time of birth is the variable responsible for the differences in phenotype that we see between the two types of transgenic lines. Nonetheless, the mechanism by which Met is activated in these mice remains obscure, although it is apparently dependent on adhesion (Wang et al., 1996). Other possible mechanisms include activation of Met by an unknown ligand, receptor crosstalk with the endogenous Met, or phosphorylation by other intracellular kinases.

Distinct pathways of tumorigenesis

The formation of distinct tumors in the *MET* transgenic animals correlated with the acquisition of distinct cooperating events (activation of β-catenin, or inactivation of HNF1α). However, the evidence that these cooperating events had a causative role in tumorigenesis was circumstantial. Using hydrodynamic transfection of genes that directly induce these cooperating events, we have demonstrated that these events can play a causative role in tumorigenesis.

The exact position within the hepatocyte lineage of the cell of origin for both HCC and HCA is unclear. The HCCs and HCAs may arise from bipotential liver stem cells, committed hepatocyte progenitors, or from the mature hepatocytes themselves. The fact that both tumor types arise rapidly and in great numbers after hydrodynamic transfection argues that this technique transfects the target cell population for both tumors, and that those cells are not rare in adult mouse liver. If in fact the cell of origin of HCAs and HCCs is the same, it is possible that the quantity of enzymatically active Met determines the ultimate outcome. In support of this notion, the amount of phosphorylated Met, although elevated, was much lower in the HCAs than in the HCCs (Figure 3.3H). In addition, HCAs were most frequent in the lowest expressing *MET* transgenic line, Line 4. Regardless, what is clear is that the same protocol of hydrodynamic transfection of distinct combinations of oncogenes was sufficient to determine the ultimate identity of the ensuing tumors.

Cooperation of Met and β-catenin in HCC

One question that arises is why activation of β-catenin is a preferred cooperating mutation after activation of Met in both our mouse model and in a subset of human HCCs We propose that the sequential activation of Met and β-catenin is sufficient to activate an existing progenitor renewal program, but activation of either alone is insufficient to do so. In support of this idea, both Met and β-catenin are activated in circumstances where hepatocyte progenitor expansion occurs, such as during liver development and regeneration (Figure 2.1; Stolz et al., 1999, Monga et al., 2001). In addition, loss-of-function studies of both Met and β-catenin have demonstrated their necessity for proper liver development and regeneration (Huh et al., 2004; Borowiak et al., 2004; Sodhi et al., 2005). We have shown with hydrodynamic transfection with *MET* and ΔN90β-catenin is sufficient to induce the fetal marker AFP, but transfection of either alone is insufficient to do so (Figure 3.4; and data not shown). It may be that following activation of Met, activation of the β-catenin pathway is one of the few events that can sustain this renewal program.

Pathways of tumorigenesis in human HCC

Although that the majority of human HCCs with we analyzed with activated β-catenin also had activated Met, there was a subset of human HCCs with evidence of activation of β-catenin, but not Met. Apparently events other than activation of Met can also cooperate with β-catenin. Analysis of the transgenic HCC that recur with persistent activation of β-catenin, but without reactivation of Met, may provide access to one or more of those events. It is of note that activation of β-catenin strongly correlated with the presence of phosphorylated

Met, but not as strongly with overexpression of Met. Hence, it may be that the presence of active Met as opposed to overexpression of Met may serve as a better marker for tumors where Met is playing a pathogenic role.

Tumor regression

A previous report has suggested that tumors may not regress in the presence of known cooperating mutations that activate oncogenes (D'Cruz et al., 2001). Although other reports have demonstrated tumor regression where subsequent mutations are presumed, in these reports the pathogenic mutations were not identified (Felsher, 2004). In contrast, we found that even in the presence of an activating mutation in *Catnb1*, tumors regressed, although residual cells remained in the scars from the regressed tumors. We suggest performing experiments with the tetracycline-inducible system to determine if combinatorial blockade of Met and β-catenin will prevent recurrence of tumors.

In this work, we found that after inactivation of the *MET* transgene, HCC cells do not reincorporate into the liver. This finding stands in contrast to previous work with *MYC* transgenic animals showing that liver tumors transplanted subcutaneously can reform some mature liver components after inactivation of the *MYC* transgene (Shachaf et al., 2004). The difference in tumor types may explain the discrepancy in our findings. The tumors induced by *MYC* most closely resemble hepatoblastoma, which is believed to be composed of more primitive hepatocytes than those in the HCCs that arise in the *MET* transgenic animals. These cells may have retained the capacity to differentiate into mature liver cells when released from the genetic influence that froze them in a primitive state.

MATERIALS AND METHODS

Mice

Transgenic mice that express the tet-transactivator in a liver specific fashion (LAP-tTA) were mated with transgenic mice that express wild type *MET* under the control of the tetracycline response element (TRE-MET) to generate double transgenic mice (LAP-tTA/TRE-MET) (Wang et al., 2001). All mice were on the FVB/N background. LAP-tTA littermates or FVB/N mice were used as controls. Doxycycline was administered in the food (200 mg/kg) for suppression of transgene expression. Genotyping was performed by PCR as described (Wang et al., 2001).

Hydrodynamic transfection

10-50 μg total of the plasmids encoding the Sleeping Beauty transposase and transposons with oncogenes of interest in a ratio of 1:25 were diluted in 2.5 mL of filtered 0.9% NaCl, then injected into the lateral tail vein of six to eight week old FVB/N mice (Charles River). 3 mL syringes (Becton Dickinson) with 26G1/2" needles were used for the injections.

Histology

Animals were euthenized and their livers removed and rinsed in PBS. One piece was snap frozen in liquid nitrogen for preparation of lysates and other pieces fixed overnight in freshly prepared cold 4% paraformaldehyde. Fixed tissue samples were then washed three times in PBS and stored in 70% ethanol until they were embedded in paraffin. Five micron sections were placed on slides and stained with hematoxylin and eosin.

Immunohistochemistry

Paraffin was removed from unstained slides with xylenes. The slides were then rehydrated through a series of washes with incrementally decreasing percentages of ethanol. Antigen retrieval was done in 10 mM sodium citrate buffer pH 6.0 by placing in a microwave on high for 10 minutes followed by 20 minute cool down at room temperature. Samples were then subjected to 3% hydrogen peroxide for 10 minutes to quench endogenous peroxidase activity. Blocking was done with the Avidin-Biotin Blocking Kit (Vector labs) in combination with either goat serum or the Mouse On Mouse Peroxidase Kit (Vector labs). Primary antibody binding was done for either 30 minutes at room temperature or overnight at 4°. Detection was performed with the ABC-Elite Peroxidase Kit (Vector labs), using the DAB Substrate Kit (Vector labs). Counterstaining was done by a 5 second dip in hematoxylin Gill 3 (Sigma).

Antibodies and dilutions were as follows: anti-Phospho-Met (Tyr1234/1235) Antibody, 1:25 (Cell Signalling Technology); anti-human Met, 1:500 (Zymed); anti-alpha-fetoprotein, 1:1000 (Dako); anti-glutamine synthetase, 1:500 (BD Bioscience); anti-phenylalanine hydroxylase, 1:500 (BD Bioscience).

Preparation of lysates

Lysates were made by taking a sample of frozen liver tissue and placing it in a tissue grinder with lysis buffer. The lysis buffer was composed of 1% NP40, 50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EDTA, 183 mg/mL $NaVO_4$, and 100 mM NaF and contained a cocktail of protease inhibitors consisting of leupeptin, aprotinin, and Pefabloc (Roche). After homogenization, insoluble debris was removed by centrifugation. Samples from the lysates were then subjected to a BCA protein assay (Pierce).

Immunoprecipitation

For each sample, 10 μL of anti-phospho-tyrosine antibody (4G10) was added to 1 mg of protein in 200 μL of lysis buffer and placed on a rocker overnight at 4°. 12 μL of protein G beads were added to each sample, which was placed on a rocker at 4° for 1 hour. The beads were washed three times with 1 mL of lysis buffer, then boiled in 50 μL of SDS sample buffer. 20 μL was then loaded per lane and subjected to Western Blotting as described below.

Western Blot analysis

Samples of 50 micrograms of protein each were subjected to SDS-PAGE. Proteins were transfered to PVDF membranes and blocked with 5% milk. Primary antibody binding was done for either 1 hour at room temperature or overnight at 4°. Detection was performed by ECL (Amersham). Antibodies and dilutions were as follows: anti-Phospho-Met (Tyr1234/1235) Antibody, 1:1000 (Cell Signalling Technology); anti-human Met, 1:2000 (Santa Cruz Biotech); anti-glutamine synthetase, 1:5000 (BD Biosciences); anti-tubulin, 1:250 (Abcam).

DNA sequence analysis

Tumor DNA was extracted with a QIAamp Tissue Kit (Qiagen), then subjected to PCR under the following conditions: 94° for 5 minutes; then 35 cycles each of 94° for 30 seconds, 56° for 30 seconds, and 68° for one minute; then a final extension step of 68° for seven minutes. Platinum Pfx polymerase was used for all PCR for sequencing. The sequences of the PCR primers, which were also used as sequencing primers, were: mouse BCAT ex2 F, ctgcccgtcaatatctgaaaa; mouse BCAT ex2 R, tcccatggagctcatactgac; human BCAT ex3 F, caatgggtcatatcacagat; human BCAT ex3 R, agtgacattgctattactctc.

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REFERENCES

Borowiak, M., Garratt, A.N., Wustefeld, T., Strehle, M., Trautwein, C., and Birchmeier, C. 2004. Met provides essential signals for liver regeneration. *Proc Natl Acad Sci U S A* **101**(29): 10608-10613.

Cadoret, A., Ovejero, C., Saadi-Kheddouci, S., Souil, E., Fabre, M., Romagnolo, B., Kahn, A., and Perret, C. 2001. Hepatomegaly in transgenic mice expressing an oncogenic form of beta-catenin. *Cancer Res* **61**(8): 3245-3249.

Colnot, S., Decaens, T., Niwa-Kawakita, M., Godard, C., Hamard, G., Kahn, A., Giovannini, M., and Perret, C. 2004. Liver-targeted disruption of Apc in mice activates beta-catenin signaling and leads to hepatocellular carcinomas. *Proc Natl Acad Sci U S A* **101**(49): 17216-17221.

D'Cruz, C.M., Gunther, E.J., Boxer, R.B., Hartman, J.L., Sintasath, L., Moody, S.E., Cox, J.D., Ha, S.I., Belka, G.K., Golant, A., Cardiff, R.D., and

Chodosh, L.A. 2001. c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. *Nat Med* **7**(2): 235-239.

Felsher, D.W. 2004. Reversibility of oncogene-induced cancer. *Curr Opin Genet Dev* **14**(1): 37-42.

Giles, R.H., van Es, J.H., and Clevers, H. 2003. Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim Biophys Acta* **1653**(1): 1-24.

Harada, N., Oshima, H., Katoh, M., Tamai, Y., Oshima, M., and Taketo, M.M. 2004. Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations. *Cancer Res* **64**(1): 48-54.

Huh, C.G., Factor, V.M., Sanchez, A., Uchida, K., Conner, E.A., and Thorgeirsson, S.S. 2004. Hepatocyte growth factor/c-met signaling pathway is required for efficient liver regeneration and repair. *Proc Natl Acad Sci U S A* **101**(13): 4477-4482.

Kinzler, K.W. and Vogelstein, B. 1996. Lessons from hereditary colorectal cancer. *Cell* **87**(2): 159-170.

Liu, F., Song, Y., and Liu, D. 1999. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* **6**(7): 1258-1266.

Llovet, J.M. 2005. Updated treatment approach to hepatocellular carcinoma. *J Gastroenterol* **40**(3): 225-235.

Monga, S.P., Pediaditakis, P., Mule, K., Stolz, D.B., and Michalopoulos, G.K. 2001. Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration. *Hepatology* **33**(5): 1098-1109.

Naldini, L., Vigna, E., Ferracini, R., Longati, P., Gandino, L., Prat, M., and Comoglio, P.M. 1991. The tyrosine kinase encoded by the MET proto-oncogene is activated by autophosphorylation. *Mol Cell Biol* **11**(4): 1793-1803.

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. 1999. Somatic mutations in the kinase domain of the Met/hepatocyte growth factor receptor gene in childhood hepatocellular carcinomas. *Cancer Res* **59**(2): 307-310.

Parkin, D.M., Bray, F., Ferlay, J., and Pisani, P. 2005. Global cancer statistics, 2002. *CA Cancer J Clin* **55**(2): 74-108.

Pontoglio, M., Barra, J., Hadchouel, M., Doyen, A., Kress, C., Bach, J.P., Babinet, C., and Yaniv, M. 1996. Hepatocyte nuclear factor 1 inactivation results in hepatic dysfunction, phenylketonuria, and renal Fanconi syndrome. *Cell* **84**(4): 575-585.

Pontoglio, M., Faust, D.M., Doyen, A., Yaniv, M., and Weiss, M.C. 1997. Hepatocyte nuclear factor 1alpha gene inactivation impairs chromatin remodeling and demethylation of the phenylalanine hydroxylase gene. *Mol Cell Biol* **17**(9): 4948-4956.

Reznik, Y., Dao, T., Coutant, R., Chiche, L., Jeannot, E., Clauin, S., Rousselot, P., Fabre, M., Oberti, F., Fatome, A., Zucman-Rossi, J., and Bellanne-Chantelot, C. 2004. Hepatocyte nuclear factor-1 alpha gene inactivation: cosegregation between liver adenomatosis and diabetes phenotypes in two maturity-onset diabetes of the young (MODY)3 families. *J Clin Endocrinol Metab* **89**(3): 1476-1480.

Shachaf, C.M., Kopelman, A.M., Arvanitis, C., Karlsson, A., Beer, S., Mandl, S., Bachmann, M.H., Borowsky, A.D., Ruebner, B., Cardiff, R.D., Yang, Q., Bishop, J.M., Contag, C.H., and Felsher, D.W. 2004. MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer. *Nature* **431**(7012): 1112-1117.

Sodhi, D., Micsenyi, A., Bowen, W.C., Monga, D.K., Talavera, J.C., and Monga, S.P. 2005. Morpholino oligonucleotide-triggered beta-catenin knockdown compromises normal liver regeneration. *J Hepatol* **43**(1): 132-141.

Stolz, D.B., Mars, W.M., Petersen, B.E., Kim, T.H., and Michalopoulos, G.K. 1999. Growth factor signal transduction immediately after two-thirds partial hepatectomy in the rat. *Cancer Res* **59**(16): 3954-3960.

Tavian, D., De Petro, G., Benetti, A., Portolani, N., Giulini, S.M., and Barlati, S. 2000. u-PA and c-MET mRNA expression is co-ordinately enhanced while hepatocyte growth factor mRNA is down-regulated in human hepatocellular carcinoma. *Int J Cancer* **87**(5): 644-649.

Vaxillaire, M., Abderrahmani, A., Boutin, P., Bailleul, B., Froguel, P., Yaniv, M., and Pontoglio, M. 1999. Anatomy of a homeoprotein revealed by the analysis of human MODY3 mutations. *J Biol Chem* **274**(50): 35639-35646.

Wang, R., Ferrell, L.D., Faouzi, S., Maher, J.J., and Bishop, J.M. 2001. Activation of the Met receptor by cell attachment induces and sustains hepatocellular carcinomas in transgenic mice. *J Cell Biol* **153**(5): 1023-1034.

Wang, R., Kobayashi, R., and Bishop, J.M. 1996. Cellular adherence elicits ligand-independent activation of the Met cell-surface receptor. *Proc Natl Acad Sci U S A* **93**(16): 8425-8430.

Yant, S.R., Meuse, L., Chiu, W., Ivics, Z., Izsvak, Z., and Kay, M.A. 2000. Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat Genet* **25**(1): 35-41.

Yant, S.R., Park, J., Huang, Y., Mikkelsen, J.G., and Kay, M.A. 2004. Mutational analysis of the N-terminal DNA-binding domain of sleeping beauty transposase: critical residues for DNA binding and hyperactivity in mammalian cells. *Mol Cell Biol* **24**(20): 9239-9247.

Yant, S.R., Wu, X., Huang, Y., Garrison, B., Burgess, S.M., and Kay, M.A. 2005. High-resolution genome-wide mapping of transposon integration in mammals. *Mol Cell Biol* **25**(6): 2085-2094.

Zhang, G., Budker, V., and Wolff, J.A. 1999. High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA. *Hum Gene Ther* **10**(10): 1735-1737.

Zucman-Rossi, J. 2004. Genetic alterations in hepatocellular adenomas: recent findings and new challenges. *J Hepatol* **40**(6): 1036-1039.

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Chapter 4: Conclusions

In this work, I have shown that the receptor tyrosine kinase Met regulates the differentiation and proliferation of cells of the hepatocyte lineage. Cells at different positions within the hepatocyte lineage responded differently to the aberrant activation of Met. The overexpression of Met in fetal hepatocytes induced proliferation and a block of differentiation. The overexpression of Met in adult hepatocytes induced dysplasia and ultimately hepatocellular carcinoma. The response of fetal hepatocytes to the aberrant activation of Met may have been an extension of the physiological ability of Met to induce proliferation and block differentiation, as the overexpression of Met is initiated in the same context in which Met signals during development. Met is not normally activated in adult hepatocytes in the absence of hepatic injury. The non-physiological sustained activation of Met in adult hepatocytes yielded dysplasia and cancer, a phenotype not typically seen in the normal life of an animal. These findings dramatize the critical role that the developmental context plays in the response to growth inducing signals.

The paradigm of cancer as a disorder of inappropriate activation of developmental programs is becoming increasingly apparent in cancers of many tissues including skin (Green and Khavari 2004), colon (Pinto and Clevers 2005), and the hematopoetic system (Passegue et al. 2003). This study now provides further evidence for this paradigm in liver cancer. Both Met and β-catenin are

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tightly regulated during liver development and liver regeneration (Monga et al. 2001; Micsenyi et al. 2004; Michalopoulos and DeFrances 2005). The aberrantly sustained activity of either in isolation was not sufficient to significantly alter the differentiation state or architecture of the liver. However, when these two signals were combined, the hepatocellular carcinomas that arose possessed many properties of hepatocyte progenitors including fetal marker expression, relief of architectural constraints, and proliferation. These findings incorporate the concepts of the multistep nature of cancer and cancer as a disorder of developmental signals. The further analysis of the signals that regulate liver development and liver regeneration may provide important insights into the mechanisms of liver carcinogenesis, and provide new candidate proto-oncogenes and tumor suppressor genes for study.

Recent clinical successes with oncogene-targeted therapies, including Gleevec for chronic myelogenous leukemia and Iressa for non-small cell lung cancer, have provided a validation of the search for mutant proto-oncogenes and tumor suppressor genes in human tumors (Sawyers 2004). However, tumor regression is not proving to be durable, as exemplified by the recurrence of HCCs after inactivation of the *MET* transgene in our mouse model of HCC. The recurrence of tumors that did not have activation of Met implies the presence of other signals that can replace the function of Met in tumor maintenance. These tumors did possess activation of β-catenin, however. Future experiments where both Met and β-catenin are inactivated in established tumors may provide a more durable regression and a proof of principle of the efficacy of combination oncogene-targeted therapy.

I have used hydrodynamic transfection as a tool to validate the pathogenic roles of Met, β-catenin, and HNF1α in liver tumorigenesis. However, hydrodynamic transfection may also be used as a tool for the discovery of novel oncogenes and tumor suppressor genes. cDNA libraries made from human hepatocellular carcinomas can be cloned into transposon vectors and hydrodynamically transfected into mouse livers. If tumors develop, the inserts of the integrated genes can be cloned using PCR. In addition, because the transposon vectors integrate into the genome, each hydrodynamic transfection also functions as an insertional mutagenesis, which may permit identification of novel tumor suppressor genes. Because liver tumor development may be used as a functional readout, the signal to noise ratio of these approaches may be better than approaches to cancer gene identification based on microarray or comparative genomic hybridization.

This work has demonstrated the utility of authentic mouse models for human liver tumors. The analysis of these mouse models provided insights into the rules of tumor progression and tumor regression, suggested specific interactions between oncogenes that occur in human tumors, and generated experimental systems for future mechanistic studies of proto-oncogenes and tumor suppressor genes in liver tumorigenesis. By providing mouse models for pre-clinical drug testing, these studies should facilitate future efforts to develop novel therapeutics for human liver cancer.

REFERENCES

Green, C.L. and Khavari, P.A. 2004. Targets for molecular therapy of skin cancer. *Semin Cancer Biol* **14**(1): 63-69.

Michalopoulos, G.K. and DeFrances, M. 2005. Liver regeneration. *Adv Biochem Eng Biotechnol* **93**: 101-134.

Micsenyi, A., Tan, X., Sneddon, T., Luo, J.H., Michalopoulos, G.K., and Monga, S.P. 2004. Beta-catenin is temporally regulated during normal liver development. *Gastroenterology* **126**(4): 1134-1146.

Monga, S.P., Pediaditakis, P., Mule, K., Stolz, D.B., and Michalopoulos, G.K. 2001. Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration. *Hepatology* **33**(5): 1098-1109.

Passegue, E., Jamieson, C.H., Ailles, L.E., and Weissman, I.L. 2003. Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics? *Proc Natl Acad Sci U S A* **100 Suppl 1**: 11842-11849.

Pinto, D. and Clevers, H. 2005. Wnt, stem cells and cancer in the intestine. *Biol Cell* **97**(3): 185-196.

Sawyers, C. 2004. Targeted cancer therapy. *Nature* **432**(7015): 294-297

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