Published in final edited form as:

Hepatology. 2016 November ; 64(5): 1711–1724. doi:10.1002/hep.28721.

Combined systemic elimination of MET and EGFR signaling completely abolishes liver regeneration and leads to liver decompensation

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Abstract

Receptor tyrosine kinases MET and EGFR are critically involved in initiation of liver regeneration. Other cytokines and signaling molecules also participate in the early part of the process. Regeneration employs effective redundancy schemes to compensate for the missing signals. Elimination of any single extracellular signaling pathway only delays but does not abolish the process. Our present study, however, shows that combined systemic elimination of MET and EGFR signaling (METKO+EGFRi mice) abolishes liver regeneration, prevents restoration of liver mass and leads to liver decompensation. METKO or simply EGFRi mice had distinct and signaling-specific alterations in Ser/Thr phosphorylation of mTOR, AKT, ERK1/2, PTEN, AMPK α etc. In the combined MET and EGFR signaling elimination of METKO+EGFRi mice, however, alterations dependent on either MET or EGFR combined to create shutdown of many programs vital to hepatocytes. These included decrease in expression of enzymes related to fatty acid metabolism, urea cycle, cell replication, and mitochondrial functions and increase in expression of glycolysis enzymes. There was however increase in expression of genes of plasma proteins. Hepatocyte average volume decreased to 35% of control with proportional decrease in dimensions of the hepatic lobules. Mice died at 15–18 days after hepatectomy with ascites, increased plasma ammonia and very small livers.

Conclusion—The study shows that MET and EGFR separately control many non-overlapping signaling endpoints, allowing for compensation when only one of the signals is blocked. The combined elimination of the signals however is not tolerated. The results provide critical new information on interactive MET and EGFR signaling and the contribution of their combined absence to regeneration arrest and liver decompensation.

Liver regeneration after partial hepatectomy (PHx) is the standard model for studying liver regeneration in the last five decades. Signals associated with liver regeneration include receptor tyrosine kinases MET and EGFR, and their associated ligands (HGF, EGF, TGF α , amphiregulin and HB-EGF). Other signals also studied include IL6, TNF α , Wnt/beta catenin, Hedgehog ligands, bile acids, leptin, etc. (1). Currently, there are no known extracellular signals related to cell proliferation which, when inhibited alone or in combination, result in complete and sustained inhibition of liver regeneration. The effects of inhibition of such signals on liver regeneration have been tested with IL6, (2), bile acids (3),

TNF α (4), Wnt/beta catenin (5), Hedgehog (6), FGF15 (7), etc. In all such situations, there is typically a delay in regeneration; within the first five days, however, the liver weight reaches levels of control hepatectomized animals with eventually full restoration of liver weight. Short-term elimination of either HGF/MET or EGFR signaling inhibited regeneration for two days (8, 9). Systemic elimination of the *Hgf* gene resulted in partial suppression of liver regeneration only after the pre-existing stores of HGF protein had been eliminated from hepatic extracellular matrix following two regenerative cycles induced by chemical injury with CCl₄ (10).

Previous studies with systemic elimination of EGFR gave contradictory results, with most studies suggesting little effect beyond day 5 of regeneration (11, 12). Another study focused on conditional systemic elimination of Met but concentrated on signaling changes only in the first 5 days, without any information on the impact of loss of MET on liver regeneration (13). A similar study examined the effects of elimination of MET signaling only in hepatocytes; liver became more sensitive to Fas activation and there was delayed healing after CCl₄ administration, but no effects on liver regeneration after PHx were reported (14). Systemic elimination of EGFR had mild effects on regeneration but combination with hepatocyte-specific elimination of MET was associated with impaired wound healing following CCl₄ injury in the double KO mice. The study was limited to 5 days and there was no information as to healing of the centrilobular damage beyond that time point (15). In a more recent study, expression of a hepatocyte-specific dominant negative EGFR resulted in delay in liver regeneration. The process proceeded to completion however due to compensatory increase of MET (16). Our current study reports for the first time a successful complete suppression of liver regeneration after PHx by systemic elimination of both MET and EGFR signaling.

Materials and Methods (See also Supplemental Files)

Generation of the MET^{ff/ff}: TamCre^{+/-} mice

MET^{ff/ff} mice with a targeted deletion for exon 16 were a kind gift of Dr. Snorri Thorgeirsson (ref PMC384772, mice now available at The Jackson Laboratory as 016974, *Metm1Sst*). The MET^{ff/ff} animals were crossbred with a tamoxifen inducible Cre recombinase (*Gt (ROSA) 26Sortm1 (cre/ERT2) Tyj*, (The Jackson Laboratory) to produce MET^{ff}: Tamcre^{+/-} mice. The ensuing MET^{ff/ff}: Tamcre^{+/-} were crossbred and then carried as a stable line. To remove exon 16 from MET, male MET^{ff/ff}: Tamcre^{+/-} mice were injected IP for five consecutive days with tamoxifen (100 μ l of a 10mg/ml solution in corn oil). Controls consisted of siblings injected with vehicle corn oil only. The activating domain encoded by exon 16 of *c-met* gene was systemically eliminated (METKO mice) in male Met^{ff/ff}: ERCre^{+/-} mice by administration of tamoxifen. The deletion was confirmed by designing three primer pairs, E15, E16 and E21 that amplified exon 15, exon 16 and exon 21 respectively (Fig. S1A). Hepatocytes from mice treated with Tamoxifen did not respond to HGF in primary culture (Fig. S1B). Partial hepatectomies were carried at least 5 days after the last dose of Tamoxifen. No effect of Tamoxifen on hepatocyte proliferation was noted under these conditions (Fig. S2).

Partial Hepatectomy

All procedures performed on mice were approved under the University of Pittsburgh IACUC protocol 14053713 and conducted according to National Institute of Health guidelines, and carried out as described. At defined time points, the animals were sacrificed according to AAALAC guidelines.

Canertinib Diet

The pan-ErbB EGFR inhibitor Canertinib (CI-1033) is a water-soluble, irreversible, type 2 kinase inhibitor, primarily of EGFR (IC_{50} 0.8 nM), with weaker effects on ErbB2/HER-2 (IC_{50} 19 nM), ERBB3 (14 nM) and ErbB4 (IC_{50} 7 nM). In EGFR, Canertinib binds to the ATP-binding site and alkylates cysteine 773 (17). Binding of Canertinib to either EGFR or ErbB2 inhibits the tyrosine kinase activity and also directs the receptor into intracellular trafficking routes for degradation (18). Canertinib was added to the mouse diet with the aim of daily administering 80 mg/kg body weight (19). Animals were fed Prolab® Isopro® RMH 3000. 5P75*. 5P76 (LabDiet, St. Louis, MO). Canertinib, Dihydrochloride Salt (LC Laboratories Woburn, MA) was added at 480 mg/Kg assuming that mice weighing 30 gm consumed 5 gm of food/day. In terms of EGFR inhibition, mice were injected with 80 mg/kg Canertinib in saline for two days prior to PHx and then placed on Canertinib diet thereafter for the duration of the experiment.

Immunohistochemistry

Immunohistochemistry was carried out as described earlier (50). The primary antibodies used are listed in Table S5. Secondary antibodies were donkey anti-rabbit and donkey anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA), used at 1:50,000 dilution. Biotinylated secondary antibodies used for immunohistochemistry were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). They were used at 1:500 dilution.

Protein Analyses

Tissue Homogenization and Western Blot Analyses was carried out as previously described (11).

Pathway-Specific Rt-qPCR Arrays

Pathway-specific expression profiling of genes associated with Oxidative Stress; Cat# 065Z and mouse LncRNA PCR Array cat # LAMM 001Z (QIAGEN Inc., Valencia, CA) was performed as previously described (11). Liver tissues from mice (n=4) were pooled from each group.

Microarrays

The GeneChip® Mouse Genome 430 2.0 Array Set 430 (Affymetrix, Santa Clara, CA) was used for microarray analysis. Liver tissues from mice (n=4) were pooled from each group for RNA isolation. C RNA, c DNA synthesis, and Affymetrix chip hybridization was done as previously described (50). MIAME-Compliant Microarray Data was submitted to GEO database. The accession number is GSE77867.

Gene Tight Clustering

mRNA microarray data were collected and quantile-normalized for groups Control Day 0 (CTRD0), Control Day 14 (CTRD14), METKO Day 14 (METD14), EGFR inhibitor treated Day 14 (CAND14) and combined METKO+EGFR inhibited Day 14 (BOTH14). Each sample was further normalized by log-ratio (base 2) against the CTRD0 sample. Tight clustering method (36) was applied to the remaining genes to identify 5 clusters of expression patterns. In the heatmap, each row block represents one gene cluster and the four columns stand for log₂ (CTRD14/CTRD0), log₂ (METD14/CTRD0), log₂ (CAND14/CTRD0) and log₂ (BOTH14/CTRD0) respectively.

Gene Ontology analysis

Functional annotation of gene list in Cluster 3 was carried out using PANTHER and visualized as pie charts. Gene Ontology enrichment analysis of genes in cluster 3 into functional biological groups using PANTHER Overrepresentation Test (release 04-30-2015) to determine GO terms that were over-represented in Cluster 3 genes list (GO Ontology database Released 2015-08-06) compared to the *Mus musculus* reference list. p-values are corrected by Bonferroni for multiple hypothesis testing (20).

Statistical Analyses

Unpaired Student's *t*-test was performed for evaluation of significant differences between the control and different treatment groups by using the Minitab computer program (Minitab, Inc., State College, PA). Data are expressed as mean $\pm$ SEM. For real-time PCR *P* values were determined by student's *t*-test with *P* < 0.05 being significant.

Results

Deletion of exon 16 resulted in MET with no kinase activity

HGF binding to MET results in receptor dimerization and autophosphorylation at Tyr 1230, 1234 and Tyr 1235 in the activation loop resulting in activation of MET (20). Biological activity of Met depends on phosphorylation of tyrosine 1349 and 1356 (21). Levels of Tyr1349 reflect MET activation (22). Activated MET translocates to the nucleus to initiate calcium signaling (23). Complete absence of phosphoMet (Tyr 1234/1235) and PhosphoMet (Tyr1349), a docking site for extensive downstream signaling, was seen in MetKO mice at all time points compared to Control and EGFRi mice (Fig. 2A). Expression of recombinant MET was also reduced in METKO+EGFRi mice at all time points. A compensatory increase in total MET and Phospho MET Tyr1234/1235 levels was seen in EGFRi mice (9, 16).

Canertinib mediated inhibition of tyrosine kinase activity of EGFR

Treatment with Canertinib eliminated both Tyr 1068 and Tyr 845 of EGFR, consistent with the described effects on EGFR internalization and degradation (17). Total EGFR and PhosphoEGFR Tyr 1068 and PhosphoEGFR Tyr 845 increased in Met KO mice, probably reflecting compensatory changes. Of interest, PhosphoERBB2 did appear in the METKO +EGFRi mice, despite administration of Canertinib. This is consistent with literature suggesting that ERBB2 can be activated by the IGF1 receptor (24). PhosphoERBB2 and

Phospho ERBB3 are decreased in EGFRi (especially in Days 7 and 14) but were not entirely absent. Regardless of any ERBB2 and ERBB3 contribution, however, the combined METKO and Canertinib treatment did completely arrest liver regeneration.

Abolished liver regeneration in METKO+EGFRi mice

METKO or EGFRi mice had a mild decrease in eventual restoration of liver weight at Day 14 after PHx compared to control mice. METKO+EGFRi mice however, had complete suppression of regeneration (Fig. 1 A and B). Control mice were Met^{ff/ff}: ERCre^{+/+} to whom only corn oil (control vehicle, no Tamoxifen) was administered. EGFRi mice had a small decrease of hepatocyte proliferation at Day 2 after PHx, with similar small decreases at Days 7 and 14 (Fig. 1A). The liver-to-body-weight ratio at Days 7 and 14 was modestly affected compared to control mice (Fig. 1B). There were stronger negative effects on hepatocyte proliferation at Day 2 in METKO mice, but proliferation was sustained at higher levels than the other categories at Days 7 and 14. The liver-to-body-weight ratio for Days 7 and 14 in METKO mice showed a decrease to slightly less than that observed in the EGFRi mice (Fig. 1B). Dramatic suppression of both hepatocyte proliferation and liver to body weight ratio was seen, however, in METKO+EGFRi mice. There was a pronounced drop in hepatocyte proliferation at Days 2 and 7 with a complete absence of hepatocyte proliferation (0% of cells) at Day 14 (Fig. 1A). Complete lack of proliferation at Day 14 (0% of cells) was also seen in cholangiocytes (Fig. S3). The effects on liver-to-body-weight ratio were equally significant. In METKO+EGFRi mice there was no increase at all of the liver remnant (or liver to body weight ratio) after PHx at Days 7 or 14 (Fig. 1B). Glutamine synthetase immunohistochemistry identifying the central veins documented a decrease in size of hepatic lobules (Fig. 1C). Noted is the comparable size of the lumen of the central veins in both sections, whereas the distance between the central veins is markedly decreased in the METKO+EGFRi livers. There was no evidence of increased hepatocyte apoptosis by TUNEL assays (data not shown). Assessment of hepatocyte size by photomicrography demonstrated that hepatocytes in METKO+EGFRi mice were on average only 35% of the volume of control mice (Fig. 1D, E). This provides an explanation for the decreased lobular dimensions shown in Fig. 1C. Despite the absence of proliferation of hepatocytes and cholangiocytes, there was no evidence of proliferation of any type of liver progenitor cells. CDKN1A (p21) causes arrest of hepatocyte proliferation (25, 26). There is increase of nuclear p21 in METKO+EGFRi hepatocytes (Fig. 1F). We terminated our studies at Day 14 after PHx because mice were ill, developed ascites and had to be sacrificed. There were no evident histologic changes in any other organs or effects on proliferation of cells of intestine, lungs or kidneys in METKO+EGFRi mice (Fig. S4).

Effect of combined inhibition on key downstream signaling pathways

mTORC-AKT-Pi3k pathway (Figure 2B)—Inhibition of mTORC suppresses liver regeneration (27). mTORC exists in two distinct complexes, mTORC1 and mTORC2. mTOR is the catalytic component of both complexes. Expression of Phospho-mTOR by immunoblot was performed by using antibodies against phospho-Ser2481, (activated mTORC2 complex), and phospho-Ser2448, (activated mTORC1 complex) (28). Phospho mTOR (Ser2448) is decreased in most regeneration days in EGFRi mice, whereas phospho-mTOR (Ser2481) is primarily decreased in the METKO mice. As a result, both Phospho

mTOR proteins are decreased in the combined METKO+EGFRi mice (Days 2, 7, 14). Rictor, interacting with mTOR is also decreased in METKO+EGFRi. Recent studies have shown that AKT activity is essential for full liver regeneration (7), acting primarily through control and inhibition of Foxo1 (29). The reduced levels of Rictor-mTORC complex 2 affected AKT activation. AKT becomes fully activated only after phosphorylation at Ser473 by the mTORC 2 complex (30). Phosphorylation by PDK1 at the Thr308 residue located in the activation domain is also necessary for activation of AKT. Levels of both Ser473-pAKT and Thr308-pAKT are decreased in METKO+EGFRi mice. An increase in the active (non-phosphorylated) form of PTEN in EGFRi and METKO+EGFRi mice shows the effect to be primarily dependent on EGFR and also suggests down-regulation of the PI3K/AKT pathways, of key importance for regulation of cell metabolism and cell proliferation (31). In summary, in METKO+EGFRi mice there was complete suppression of activation of both mTOR and Pi3K-AKT pathways.

ERK1/2 activation and Cyclin D1 are decreased in METKO+EGFRi mice (Fig. 2C). Activation of MET and EGFR leads to differential regulation of pathways associated with cell proliferation, like p44/42 MAPK (Erk1/2), Cyclin D1, cellular energy sensors like AMPK and Acetyl-CoA Carboxylase (ACC) regulating fatty acid biosynthesis. METKO+EGFRi mice had lower levels of Phospho-Erk1/2 (Thr202/Tyr204) at all time points compared to control mice. (Fig. 2C). Following PHx, levels of Cyclin D1 normally increase (32). Cyclin D1 levels however were reduced in the METKO+EGFRi. Higher levels of Cyclin D1 were seen at time 0 and day 2 in the EGFRi mice but decreased at day 7 and day 14.

Activation of AMPK and reduced expression of enzymes associated with fatty acid biosynthesis in METKO+EGFRi mice—AMPK is an important regulator of cell energy status functioning as a major sensor of ATP levels (33). When ATP/ADP ratios are affected due to reduced ATP levels, AMPK is activated by phosphorylation at Thr172 by LKB (34). Activated AMPK inhibits (via phosphorylation) several pathways in order for cells to conserve energy. A typical effect is inhibition of ACC involved in synthesis of fatty acids, an ATP consuming process (35). Figure 2C, shows activation (Thr172 phosphorylation) of AMPK in EGFRi and METKO+EGFRi mice. No such effect is seen in METKO mice. Activated AMPK inhibits ACC by phosphorylation at Ser79. This is shown by phospho-ACC immunoblot in EGFRi mice but not in Control and METKO, documenting the dominant role of EGFR (and not MET) in regulation of this activity. A recent study in mice expressing dominant negative EGFR in hepatocytes found suppression of fatty acid synthesis (16). The EGFR (but not MET) dependent activation of AMPK (Fig. 2C) is consistent with the findings in hepatocyte-specific EGFR-dominant mice by Lopez-Luque et al. (16). There was also dramatic decrease in mRNA expression of key enzymes involved in fatty acid synthesis (Fig. 4A), consistent with a decrease in expression of SREBP1 in METKO+ EGFRi mice to 1/3 of that of Control (data not shown). Phosphorylation of AMPK α is noted already at Day 2 after PHx in the EGFRi mice but not in the METKO mice (Fig. 2C). In view of the known effects of AMPK α in downregulation of fatty acid synthesis and lipid metabolism, the results in this figure correlate with Fig. 7 of Supplement. Oil Red-O stain for triglycerides at Day 2 after PHx (Figure S7) shows massive lipid accumulation in

hepatocytes of control mice, complete absence in EGFRi mice and some residual accumulation in METKO mice. The suppression of triglyceride accumulation in METKO mice is less than the severe suppression of EGFRi group, and the results in Fig. 2 suggest that this effect on triglyceride accumulation seen in METKO is not dependent on AMPK α phosphorylation.

There was no evident change in any of the mouse categories in Yes-associated protein (Yap) or beta-catenin at the mRNA, protein expression level or nuclear localization by immunohistochemistry (data not shown).

Dependence on both EGFR and MET signaling of expression of the genes in one of the five tight gene clusters

We attempted to identify the genes which were not affected by single receptor signaling deletion but rather by the elimination of both EGFR and MET signals. To that end, we performed gene expression analysis of microarray data at Day 14 of regeneration (see Methods) and the results revealed five tight Gene Clusters (36). A heat map analysis of each cluster in the four different treatment categories is shown in Fig. 3A. Each row block represents one Gene Cluster. Each column shows expression data as $\log_2(\text{expression value of Category at Day 14} / \text{expression value of Control at Day 0})$. We focused on Cluster 3, (marked by arrow) in which Control, EGFRi and METKO mice had high expression of most genes, whereas METKO+EGFRi mice had low expression. Single elimination of either MET or EGFR did not affect expression of most of these genes in Cluster 3. Their expression dramatically decreased only after elimination of both MET and EGFR (Fig. 3A, arrow indicating color change from red to green for all genes in the cluster). This cluster fulfilled the criteria for the genes that we were trying to identify. The adjacent pie charts show the variety of the processes to which the genes present in Cluster 3 contribute (Figure 3B). There is dramatic suppression of the expression of all of these genes in METKO+EGFRi mice at Day 14. As shown in Fig. 3C, several of these genes (e.g. members of the CYP family, fatty acid binding protein 1 (FABP1) and FKBP4) are associated with metabolic and synthetic functions in normal liver.

Gene Ontogeny Enrichment analysis of genes in Cluster 3 indicates a predominance of genes in critical biosynthetic pathways

GO enrichment analysis of genes in Cluster 3, controlled by METKO+EGFRi, was carried out (Supplemental Materials and Methods). GO biological process “enrichment” analysis of genes in cluster 3 is shown in Supplemental Tables S1 and S2. Genes associated with aromatic amino acid catabolism (GO: 0009074), glutathione metabolism (GO: 0006749), amino acid catabolism (GO: 0009063), fatty acid beta-oxidation (GO: 0006635) and catabolism (GO: 0009062) were found to be enriched more than fivefold (and thus most likely to be affected) at day 14 PHx in METKO+EGFRi mice. GO cellular component analysis revealed a more than fivefold enriched dependence in oxidoreductase complex (GO: 1990204), peroxisome (GO: 0005777) and microbody (GO: 0042579) cytoplasmic organelles.

Dysregulation of oxidative stress pathways in METKO+EGFRi mice

Deletion of MET signaling is associated with disruption of redox homeostasis (37). q RT-PCR analysis of genes associated with the oxidative stress response in METKO+EGFRi mice at Days 0, 2, 7 and 14 post PHx was carried out. The results are shown in Figure 4. Significant downregulation was observed in genes that protect against oxidative stress, such as *Cat*, *Ccs*, *Gclc*, *GCLM*. Genes that promote oxidative stress (*Txnip*) were upregulated, suggestive of a compromised response against oxidative stress. (For enzymatic function details and references, see Supplemental Table S3).

LncRNA profiling in METKO+EGFRi mice indicates differential regulation of LncRNAs regulating proliferation, transcription and translation

Rt-qPCR analysis of long non-coding RNAs in METKO+EGFRi mice at day 14 showed changes in expression of specific LncRNA genes associated with cell proliferation and cell cycle (Supplemental Table S4). Significant upregulation in expression of LncRNAs that function as tumor suppressors and block proliferation (*Gas5*, and *Meg3*) was seen in MET+EGFRi mice. LncRNAs that regulate transcription (*Neat 1* and *Malat 1*) were downregulated. Notable is the increase in lncRNA-p21, which functions as a global translation blocker and regulates cell cycle progression. (For quantitative details and references, see Supplemental Table S4).

Expression of genes associated with fatty acid synthesis, urea cycle, mitochondrial functions, glycolysis and plasma protein synthesis in Day14 METKO+EGFRi mice

There was suppression (less than 10% of control) in METKO+EGFRi mice of many enzymes associated with fatty acid synthesis (Fig. 5A). There was decrease in mitochondrial enzymes of the urea cycle (fig. 4D schematic) OTC (ornithine transcarbamylase) (Fig. 5B) and CPS1 (Carbamoyl synthetase) (Fig. 5C). (Plasma ammonia values in Control and METKO+EGFRi mice at Day 14 were 5.52 and 41.3 µg/ml respectively). Decrease in expression of multiple genes associated with mitochondria and Krebs cycle is paralleled with an increase in enzymes of the glycolytic pathway (Fig. 5E). Fig. 5F shows increased expression in METKO+EGFRi livers at Day 14 of genes of plasma proteins (serum amyloid A2, transferrin, hemopexin, several lipoproteins, etc.). In 17 of these genes, however, the expression is stronger in the EGFRi category, reflecting a more direct involvement of EGFR mediated pathways in regulation of synthesis of plasma proteins.

Decreased expression of genes regulating cell proliferation, DNA replication and metabolism in METKO+EGFRi mice

Fig. S4 illustrates downregulation of genes MCM2, MMC7, MCM5 associated with general cell proliferation in METKO+EGFRi mice at Days 2 and 14 of regeneration (Fig. S4A). *Gadd45b* has been associated with inhibitory effects in hepatocyte cell cycle related to regeneration but facilitates cell growth in hyperplasia induced by xenobiotic compounds (38). There is strong increase in expression of *Gadd45b* at Day 14 in the METKO+EGFRi mice (Fig. S4B).

DISCUSSION

Liver has a high propensity to regenerate and liver failure due to serious injury is considered de facto as failure of regeneration. This study demonstrates that complete suppression of regeneration can lead to severe decompensation of most key metabolic and differentiated hepatocyte functions, as seen in liver failure. It should be noted however, that typically, liver failure results from massive exposure of the liver to injurious stimuli (chemicals, viruses, etc.) resulting in massive hepatocyte death. This is not the case in our study. Hepatocyte death at Day 14 in any of the categories is minimal. The loss of key hepatic functions (urea cycle, lipid synthesis etc.) is not due to insufficient number of hepatocytes caused by massive cell death. It is due to selective shut-down of key components of hepatocyte function due to elimination of extracellular signals of MET and EGFR. Eventually, the mice die with their hepatocytes still alive and their livers small but intact, and simply turned off. The development of ascites and high plasma ammonia levels in METKO+EGFRi mice attests to the impaired liver function. Ascites may be due to the decrease in liver and lobule size (Fig. 1C), resulting in decrease of the cross-section of hepatic sinusoid surface and thus causing portal hypertension. The lack of proliferation of cholangiocytes and absence of progenitor cells in METKO+EGFRi mice despite the drop in liver weight and lack of hepatocyte proliferation shows that MET and EGFR signaling are fundamental for proliferation of all hepatic epithelial cells, including progenitor cells.

Our results demonstrate the complementarity of the two receptors in support of crucial pathways. EGFR seems to be more involved in lipid biosynthesis and plasma protein secretion (Figure 2: AMPK α) whereas MET is more involved in maintaining the redox balance of the cell (Figure 4). The combined effect of elimination of signaling of both receptors should be taken into account in therapeutic considerations.

All hepatic cells are constantly exposed to HGF and EGF. HGF, produced by stellate cells, is present in high concentration as inactive (single chain) protein in hepatic extracellular matrix (39). EGF is also in constant supply to hepatocytes through the portal circulation from Brunner's glands of the duodenum (40). Hepatocytes are thus constantly exposed to the "tonic" effect of these two growth factors (41). Based on our findings, we hypothesize that the gene expression cybernetics of normal liver depend on a constant signal from MET and EGFR and that most other signals depend on the foundation of that signaling platform. The results also illustrate the complexity of interactions of apparently diverse pathways regulated separately by MET or EGFR, with many of them becoming inactive when both MET and EGFR signals are eliminated.

Is it possible that the observed results are due to indirect effects on hepatocytes from other cells types? This is not a likely possibility. Complete elimination of macrophages by GdCl₂ had minimal effects on liver regeneration (42). Toxic elimination of cholangiocytes by DAPM also had no effect on hepatocyte growth, in fact it accelerated transformation of hepatocytes to cholangiocytes (43). Sinusoidal endothelial cells do provide HGF to hepatocytes at days 4–7 after PHx but even if this were affected, it would not explain the dramatic decrease in hepatocyte proliferation because HGF supply is irrelevant since MET is already eliminated.

Our results show that, in addition to inhibiting liver regeneration, combined elimination of both MET and EGFR signaling can lead to liver decompensation. This scenario may be clinically relevant. For HGF/MET, data from literature suggest that this scenario is likely to occur in fulminant hepatitis. HGF, present in high concentrations in hepatic extracellular matrix, is released in plasma immediately after PHx as the active heterodimeric form (44) due to programmed remodeling of extracellular matrix induced by urokinase (45). HGF is also released in fulminant hepatitis and high plasma HGF concentrations paradoxically correlate with severity of fulminant hepatitis (46). In contrast to HGF released in blood after hepatectomy, however, HGF released due to massive degradation of extracellular matrix in fulminant hepatitis is present in the monomeric, inactive form (47). Since in fulminant hepatitis most of HGF available in the environment of hepatic cells is inactive, in fulminant hepatic failure all hepatic cells expressing MET are deprived of active HGF. This is de facto similar to the approach in our study; we eliminated HGF/MET signaling from all hepatic cells not by eliminating active HGF (as in fulminant hepatitis) but by eliminating MET.

Clinical scenarios relating EGFR signaling to fulminant hepatitis in humans are not available. Access to EGF from portal circulation is likely to be severely compromised in fulminant hepatitis due to documented widespread damage and occlusion of fenestrae of sinusoidal endothelial cells (48). This could limit availability of all portal components, including EGF, to all hepatic cells.

HGF and EGFR ligands are the only direct mitogens for hepatocytes, causing cell proliferation in a chemically defined serum-free medium and in whole animals (rats and mice) (49). We have also shown that of all growth factors whose receptors exist on hepatocytes, only HGF and EGF were capable of stimulating transformation of hepatocytes to cholangiocytes in an organoid culture (50). The current study complements these previous findings and demonstrates the unique importance that combined EGFR and MET/HGF signaling plays for all aspects of liver function and liver regeneration. A schematic representation of the experimental outline of our study is shown in Figure 6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support for this study was provided by the Grant NIH/NIDDK P01 DK096690 (P.I. David Perlmutter), Cleveland Foundation and the Menten Endowment Foundation.

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Figure 1.

Suppression of cell proliferation and arrest of liver regeneration in METKO+EGFRi mice.

A. Percent of hepatocytes in DNA synthesis (Ki67 positive nuclei) following PHx of mice in the four different treatment categories. The values of the bars reflect the mean and standard error of minimally three separate mice in each treatment group. (The numbers over the bars indicate the absolute values of the mean). The Day 14 value of hepatocyte proliferation in MET-KO+EGFRi mice was 0% and is placed under an arbitrary horizontal bar. **B.**

Comparison of “liver-to-body weight” (LBWR) ratio at days 7 and 14 after PHx with that of normal mice prior to and immediately after PHx. The values of the bars reflect the mean and standard error of minimally three separate mice in each treatment group. (The numbers over the bars indicate the absolute values of the mean). **C.** Glutamine synthetase immunohistochemistry of post-PHx Day 14 of mouse liver in control (upper photo) and MET-KO+EGFRi mice (lower photo) (Magnification: 100X). **(D):** The mean average volume of hepatocytes in the different treatment groups at Day 14 after PHx. (Data derived from E below) **E:** A hematoxylin stain photomicrograph of control (left) and MET-KO

+EGFRi mice (right) at Day 14 after PHx (Magnification 200X). **F**: Immunohistochemistry for CDKN1A (p21) in the nuclei of hepatocytes in Control (upper) and METKO+EGFRi mice (lower) at Day 4 after PHx. (Magnification: 400X). Most of the nuclei in the METKO+EGFRi liver section are positive for p21 (black arrowheads).

Figure 2. Western immunoblot analysis of activation status of MET, EGFR and downstream effects on activation of key signaling pathways. For details and interpretation, see Results

A. Western blot analysis of total and activated Met, EGFR ERBB2 and ERBB3. Beta actin was used as loading control. **B.** Western Blot analysis of mTOR-AKT-PTEN Pathway. **C.** Western Blot analysis of Cyclin D1, phospho-Erk1/2, AMPK alpha, Phospho-AMPK α (Thr172), Acetyl-CoA carboxylase and Phospho-Acetyl-CoA Carboxylase (Ser79).

Figure 3.

A: Gene Cluster Analysis of microarray data. Heatmap of gene expression in livers of the different categories at day 14 after PHx and GO analysis of the genes in Cluster 3 (indicated by arrow) in which gene expression decreases only when both MET and EGFR are suppressed. **B:** The complexity of the functions and processes controlled Gene Cluster 3 is illustrated by the adjacent three pie charts illustrating Gene Ontogeny results for the functions performed by the genes which are enriched in that cluster. **C:** The sum of the expression of each gene present in Cluster 3 was obtained by adding the expression values of each of the four categories of treatment. That sum was normalized to 100%. The top 25 genes expressed in the control group of Cluster 3 were analyzed and shown in Fig. 3C. Each treatment category is shown with a specific color. Results show the percent expression of the top 25 most expressed genes in Control treatment at Day 14 after PHx, relative to the other three treatment categories.

Figure 4. Dysregulation of oxidative stress pathways in METKO+EGFRi mice

RT-qPCR analysis of genes associated with the oxidative stress response in METKO+EGFRi mice at Days 0, 2, 7, 14 post hepatectomy. Y-axes indicate fold-change compared to the geometric mean of the six reference genes of the array plate (see Methods). Significant downregulation was observed in genes that protect against oxidative stress, such as *Cat*, *Gcs*, *Gclc*, *GCLM*, *Gstp1*. Genes that promote oxidative stress, such as *Txnip* were upregulated, suggestive of a compromised response against oxidative stress. (For function details and references, see Supplemental Table S3).

Figure 5. Altered gene expression of multiple fundamental enzymes and proteins associated with hepatocyte metabolism and differentiation. **A.** Gene expression of enzymes associated with different aspects of fatty acid synthesis. Expression data at Day 14 for each treatment group are compared to the expression of Day 0. Each expression point is shown by colored-tagged bars and the absolute expression values are shown above the corresponding bar for Control Day 0 (prior to hepatectomy) and Day 14 of regeneration for the METKO+EGFR inhibited mice. All values are suppressed at Day 14 of METKO+EGFR inhibited mice as compared to any other category. Decreased expression of the two mitochondria-localized urea cycle enzymes OTC (**B**) and CPS1 (**C**). Values are compared between Days 0, 2, 7 and 14 after PHx. A schematic (**D**) demonstrates the location of the different enzymes of the urea cycle and their associated products, illustrating the location of both enzymes in the mitochondria. **E.** Log ratio of RNA expression of the ratio of [(expression in METKO+EGFRi)/(Average expression of the other categories)] for genes associated with mitochondrial functions, Krebs

cycle and glycolysis. There is a general shift for down-regulation of expression of mitochondrial and Krebs cycle genes and upregulation of genes associated with glycolysis at Day 14 in the METKO+EGFRi group. **F.** Enhanced expression of plasma proteins produced by hepatocytes at Day 14 in METKO+EGFRi mice (red line bars). For all data in Figure 4F, each bar demonstrates gene expression results obtained by pooling of equal weights of samples from at least four separate mice. The expression value of each bar shown was statistically significant within the array at a p-value of less than 0.05%. For more details and associated references, see text. There is general upregulation of expression of most of the plasma proteins secreted by hepatocytes of METKO+EGFRi group (shown in red), with the exception of 17 genes in the EGFRi group (shown in purple) which surpass the expression of the METKO+EGFRi group.

Figure 6. A schematic diagram of the study and main findings

In standard liver regeneration after 2/3 partial hepatectomy, liver mass is mostly restored within 14–20 days. In the combined absence of MET and EGFR signaling, however, liver regeneration is completely arrested with ensuing liver failure and animal death.