Tumour growth stimulation following partial hepatectomy in mice is associated with increased upregulation of c-Met

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Keywords

Hepatectomy, Liver regeneration, Liver metastases, Growth factors, c-Met.

Abbreviations

CRC, Colorectal Cancer; CLM, Colorectal Liver Metastases; HGF, Hepatocyte Growth Factor; IGF, Insulin Growth Factor; EGF, Epidermal Growth Factor; VEGF, Vascular Endothelial Growth Factor; TGF, Transforming Growth Factor.

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Abstract

Hepatic resection is the preferred option for curative treatment of colorectal liver metastasis (CLM). However, this is associated with significant recurrence rates in both hepatic and extrahepatic sites. The upregulation of growth factors required for liver regeneration after resection is thought to stimulate the growth of micrometastases. The current study describes temporal changes in the expression of hepatocyte growth factor receptor (c-Met), epidermal growth factor receptor (EGFR) and insulin growth factor I receptor (IGF-IR) in an orthotopic mouse model of liver resection and tumour induction. Mice underwent 70% hepatectomy and induction of liver metastases through intrasplenic injection of colorectal cancer cells. Control groups included sham-operated mice and 70% hepatectomy alone. The expression levels of liver and tumour c-Met, EGFR and IGF-IR were quantified by quantitative RT-PCR at different time points. 70% liver resection stimulates tumour growth; increases the expression of c-Met within established tumours and surrounding liver parenchyma; downregulates EGFR expression and increases IGF-IR expression within the liver parenchyma. In conclusion, we demonstrate in our mouse model that major hepatectomy stimulates engraftment and growth of CLM and that this effect is probably due to the upregulation of c-Met as a result of the liver regeneration process. Liver IGF-IR may also contribute to this phenomenon through a paracrine effect on tumour growth. This study provides support for the role of c-Met in the stimulation of tumour growth after resection possibly through the promotion of tumour cell proliferation.

Introduction

The majority of colorectal cancer (CRC) related deaths are associated with liver metastasis, which occurs in up to 70% of patients with CRC [1-3]. Surgical resection of colorectal liver metastases (CLM) offers potential for cure in selected patients. However, 30 - 60% of patients will develop disease recurrence in the liver or at distant sites [4,5]. High tumour recurrence rates following surgical resection for patients with CLM have been linked to the upregulation of growth factors required for liver regeneration [6-9]. The mechanism of this stimulation remains unclear.

Resection of liver tissue initiates the release of a cascade of growth factors that results in proliferation of hepatic cells, leading to the regeneration of the liver to its previous size. Many growth factors and cytokines have been implicated in regulating liver regeneration, including hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factors (TGF), tumour necrosis factor (TNF) and interleukin (IL)-6 [10-14]. Insulin growth factor I (IGF-I) may be another important molecule in this context. Male mice with a liver-specific IGF-IR knockout displayed a strong impairment in hepatocyte proliferation after hepatectomy [15].

Numerous studies have also shown that growth factors such as HGF, vascular endothelial growth factor (VEGF), TGF- β , IGF-I and EGF, and their cognate receptors, stimulate tumour cell proliferation, migration, angiogenesis, resistance to cell death and metastasis in many human cancers and murine models [16-19]. Upregulation of these factors during liver regeneration may thus enhance the metastatic process [4].

Animal studies have confirmed that there is stimulation of tumour growth after liver resection [20,21,9,22,23] We have previously shown that mice which underwent 70%

hepatectomy and were induced with CRC cells immediately after liver resection presented higher percentage of proliferating tumour cells (Ki67 positive cells) [24].

We hypothesized that the upregulation of key growth factor receptors in the liver during liver regeneration stimulate the growth of liver metastases. Candidates for this dual role in liver regeneration and tumour stimulation include: c-Met, EGFR and IGF-IR in conjunction with their associated ligands. The current study describes temporal changes in the expression of these growth factor receptors in a mouse model of liver resection and tumour induction.

Material and methods

Animals

Eight to ten week old male CBA mice (Laboratory Animal services, University of Adelaide, South Australia) were used in all experiments. Mice were maintained in standard cages with access to irradiated food and water *ad libitum*, and exposed to a twelve hour light/dark cycle. All procedures were implemented in accordance with the guidelines of the Austin Health Animal Ethics Committee.

Maintenance of Colon Cancer Cell Line

Primary colon cancer cells were obtained from a mouse colorectal cell line (MoCR)- derived from a dimethylhydrazine induced colon carcinoma in CBA mice, as described previously. Cancer cells were maintained *in vivo* by serial passage in the flanks of male CBA mice. The resultant tumour growth pattern using this cell line reassembles the human cancer growth [24-26].

Experimental Design

Animals were subjected to a 70% liver resection and/or intrasplenic tumour induction. Sham-operated mice were used as a control for liver resection. Sham operations involved access and manipulation of the liver with a cotton swab before the incision was sutured, whereas resected (hepatectomised) animals had 70% of their liver surgically removed. In tumour induced groups, liver metastases were induced through intrasplenic injection of tumour cells before the liver resection or manipulation of the liver (sham operated). Mice used for the normal control did not undergo any surgical procedures or manipulation. This study consisted of four experimental groups: a) liver resection, b) sham-operation, c) liver resection plus tumour induction, and d) sham-operation plus tumour induction, with at least six animals per group. In our model, the presence of micrometastases can be detected around 6 days after tumour induction; angiogenesis is established by day 10, followed by exponential growth of tumours between days 10-16, and a plateau phase from day 18-22 [26]. This study can be divided in two different stages. The early stage consists of time points that cover the process of liver regeneration and appearance of micrometastases: 2, 4, 6, 8 and 10 days after surgery. For these time points, pieces of livers, with or without micrometastases, were snap frozen in liquid nitrogen to extract RNA or fixed in 10% formalin and embedded into paraffin blocks. The late time points; 16, 18 and 21 days after tumour induction, were designed to study established liver metastases and the surrounding liver tissue, separately. For this purpose, macroscopic tumour nodules were carefully dissected from adjacent liver and snap frozen separately. Although extra care was taken, contamination from micrometastases cannot be excluded from samples of the tumour bearing liver. After collection of livers in anaesthetised mice, they were then killed by exsanguinations through division of the abdominal aorta. The difference in tumour growth between groups was confirmed using the liver to body weight ratio as a surrogate for tumour burden [27].

Induction of Colorectal Liver Metastases

Induction of colorectal liver metastases was performed as described previously [25]. Briefly, a transverse mid-line surgical incision was performed in anaesthetized mice and the spleen was exteriorized. An intrasplenic injection of 25000 MoCR cells (0.05 mL of tumour suspension) was given; cells were allowed to circulate for three minutes followed by a splenectomy. According to experimental groups, animals were subjected to sham-operation or liver resection before closure of the wound. All mice were recovered and monitored daily.

70% Partial Hepatectomy (liver resection)

Partial removal of liver lobes was performed as described previously [24,28]. Briefly, a transverse abdominal incision was made to expose all liver lobes in anaesthetized mice. Animals underwent 70% liver resection, where both the left posterior and the median lobes were removed. The abdomen and skin were then closed in layers with running 3/0 silk sutures. Animals were monitored for recovery and daily until the end of the experiment.

Quantitative Assessment of c-Met, EGFR and IGF-1R mRNA

RNA was extracted from liver and tumour tissue using the TRIzol method, converted into cDNA and used for quantitative RT-PCR (qRT-PCR), as described previously [25]. Briefly, an aliquot of cDNA was used in a qRT-PCR (ABI Prism 7700 Sequence Detector). Probes and primers for mouse tissue were designed by Beacon Designer™ (PREMIER Biosoft International, Palo Alto, CA) (Table 1). To confirm the absence of contaminating genomic DNA, RT-minus and no template controls were performed. Each cDNA sample was analysed in duplicate and assessed using the comparative CT method. The qRT-PCR protocol began

with 50°C incubation for 2 minutes, followed by 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. 18S ribosomal RNA served as the internal control.

Immunohistochemistry

Samples were collected in 4% paraformaldehyde and fixed for 48 hours before embedding in paraffin. Sections were cut at 4µm and mounted on SuperFrost Plus slides (Menzel-Glaser). Sections were dewaxed in two 10 minutes histolene solvent baths and rehydrated by serial progression through three 100% ethanol baths. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in TBST for 5 minutes. An antigen retrieval step in citrate buffer (10 mM, pH 6) for 15 minutes at low microwave power was required. The c-Met (goat anti-mouse, R&D systems, cat#AF527) was applied at 1µg/L; the EGFR antibody (rabbit anti-mouse, Cell Signalling Technology, cat#2232) at 5ng/mL; Ki67 (Rabbit anti-rat, Dako, cat#M7249) at 7.4mg/L. Slides were incubated with primary antibodies at 37°C for 2-3hours and then 4°C overnight. A Horse Radish Peroxidase (HRP) conjugated bovine anti-goat secondary antibody (Santa Cruz Biotechnology, cat # SC-2347) was applied to bind the c-Met primary antibody, HRP conjugated goat anti-rabbit secondary antibody (DakoCytomation, cat#P044801-2) was applied to bind with EGFR and Ki67 primary antibodies for 1 hour at 37°C before visualisation with DAB. Rabbit IgG fractions and goat normal serum provided negative controls for the respective antibodies. Slides were counterstained with Mayer's haematoxylin for 3 minutes and cover slipped.

Cell Proliferation Counting Using Image Analysis Software

Images of both liver and tumour tissue from each experimental group were taken using digital light microscope (Nikon Coolscope®, Nikon Corporation, Japan) at between 40x and 600x magnification (with a scale bar for size calibration) and were analysed using Image-Pro plus

(version 4.5). A random and even distribution covered by each sample was ensured. The number of tumour proliferating cells was obtained in tissues stained for the antigen Ki-67. Positive stained nuclei (dark brown) were counted and expressed as a percentage of the total number of nuclei per field.

Statistical Analysis

Statistical analyses were conducted using SPSS (Statistical Package for the Social Sciences, version 17, USA) or Microsoft Excel (2003). Normally distributed data were assessed by ANOVA. Kruskal-Wallis test was performed on non-parametrical data. Mann-Whitney U tests were performed to assess the statistical difference between individual groups. Quantitative data are presented as means $\pm$ S.E.M. P value (P) ≤ 0.05 was considered statistically significant.

Results

Hepatectomy stimulated tumour growth in our murine model of colorectal liver metastases

In this study, mice underwent colorectal liver metastases induction followed by 70% hepatectomy or sham hepatectomy. Tumour growth was determined by comparing liver/body weight (LBW) ratios in sham-operated and hepatectomised animals once tumours were established in the liver (21 days). LBW ratios of hepatectomised animals with tumour induction were significantly higher than sham-operated animals, $p = 0.02$, denoting an increase in 30% on tumour load (Fig. 1a). In addition, tumour cells from animals submitted to hepatectomy (regenerated liver) showed a significantly higher number of proliferating tumour cells, evaluated by Ki67 staining, compared with tumour cells from sham-operated liver (Fig.

1b) at days 18 and 21 post-induction ($p = 0.05$ and $p = 0.001$, respectively) when tumours reach the plateau growth phase.

c-Met is upregulated during liver regeneration

c-Met mRNA levels assessed by qRT-PCR in the regenerating liver were significantly increased above normal liver levels at days two and four post-hepatectomy, $p = 0.01$, with peak c-Met levels occurring four days after liver resection. At this timepoint, c-Met mRNA was two-fold above levels in the normal liver and was also significantly elevated above sham-operated liver levels, $p = 0.02$ (Fig. 2a). c-Met levels returned to normal by the end of the process of liver regeneration at day 10. c-Met mRNA levels in the regenerating liver with tumour induction followed a similar expression pattern compared to regenerating liver alone (with no tumour induction). Interestingly tumour induction results in significant elevation in liver c-Met mRNA levels at day two post induction compared to normal or sham-operated livers with no tumour induction ($p < 0.009$) (Fig. 2a). The result indicates that c-Met signalling may be important for the establishment of tumour metastases. Additionally at this timepoint c-Met mRNA levels in the sham-operated tumour induced group were significantly higher than the hepatectomised liver with tumour induction group ($p < 0.05$) and the hepatectomised liver with no tumour induction group ($p = 0.02$) (Fig. 2a). c-Met mRNA declined towards normal liver levels in both sham-operated and regenerating liver with tumour induction day 10 post-induction (Fig. 2a).

c-Met protein assessed by immunohistochemistry was localised in the regenerating liver with and without tumour induction and compared to sham-operated animals (Fig. 2b). Both normal (Fig. 2b-II) and sham-operated (Fig. 2b-III) livers expressed c-Met staining on the membrane of hepatocytes. In response to hepatectomy, c-Met staining intensity increased and was

observed in the membrane of hepatocytes and liver sinusoids, most notably at 4 days after hepatectomy (Fig. 2b-IV). In animals that had undergone tumour induction, c-Met staining was similar in sham-operated (Fig. 2b-V) and hepatectomised animals (data not shown) 4 days post-induction. By day 6 post-tumour induction, c-Met was highly expressed in the cytoplasm of micrometastatic tumours, as well as, in the liver parenchyma in hepatectomised animals (Fig. 2b-VI). The goat negative control showed no staining (Fig. 2b-I).

Hepatectomy increased c-Met expression in both liver parenchyma and liver tumours during exponential tumour growth

The effects of hepatectomy and tumour induction on c-Met expression in both tumours and surrounding liver were investigated during tumour exponential (day 16) and plateau (days 18 and 21) growth phase. The CRC metastases were carefully dissected from the surrounding liver tissue and analysed separately. qRT-PCR analysis showed that c-Met mRNA levels in tumour-bearing resected liver was significantly higher than in sham-operated tumour-bearing liver ($p < 0.009$) by day 21 (Fig. 3a). Tumour-bearing resected livers were significantly higher than normal liver levels at all tumour growth time points analysed (day 16, $p = 0.03$; day 18, $p = 0.01$ and day 21, $p < 0.009$), whereas in sham-operated tumour-bearing liver, c-Met levels were increased above normal liver levels only at day 16 ($p = 0.05$). c-Met levels were significantly higher in tumours from resected livers compared to sham-operated livers at days 18 and 21 ($p < 0.009$ and $p = 0.01$, respectively) (Fig. 3b).

c-Met protein assessed by immunohistochemistry was localised during plateau tumour growth phase. Tumours from both sham-operated (Fig. 3c-I) and regenerated (Fig. 3c-II) livers displayed cytoplasmic c-Met expression; however the staining was stronger in tumours from hepatectomised animals compared with sham-operated animals. Hepatocytes from

hepatectomised animals with tumour induction presented intense c-Met expression particularly at the tumour periphery 21-days post-tumour induction, while sham-operated tumour-bearing livers presented less c-Met.

EGFR is upregulated during liver regeneration and tumour induction further increases this upregulation by day 10.

EGFR mRNA levels in sham-operated liver did not vary significantly from normal liver levels. In contrast, EGFR levels increased significantly in a parabolic manner in the regenerating livers. EGFR levels in the regenerating liver were significantly elevated above sham-operated liver levels 4 days post-resection ($p < 0.009$), until peak EGFR expression, at 3-fold higher than normal liver EGFR, occurred six days after resection ($p < 0.009$) (Fig. 5A). EGFR mRNA levels were still significantly increased above normal and sham levels 8 days post-resection ($p = 0.01$), but by day 10 no significant difference was found between groups (Fig. 4a). When mice were induced with tumours, EGFR levels were significantly higher than normal liver or sham operated without tumour induction groups at all timepoints analysed ($p \leq 0.05$ for day2 and $p \leq 0.01$ for days 4-8) similar to what was seen with the c-Met above. EGFR levels in tumour induced group did not alter significantly upon liver resection and they were similar to the levels induced by the liver resection alone (Fig. 4a). At 10 days post-tumour induction, EGFR expression peaked at 3.4 and 3.6-fold above normal liver levels in both sham-operated and regenerating livers with tumour induction, respectively ($p < 0.009$) (Fig. 4a).

EGFR protein assessed by immunohistochemistry was expressed in the cell membranes of hepatocytes and liver sinusoids. In response to hepatectomy, EGFR staining increased in intensity in the regenerating liver (Fig. 4b). Developing micrometastases can be seen in the

liver by day 6 after hepatectomy and tumour induction (4b-VI). Tumours showed faint staining for EGFR in the cytoplasm of tumour cells. The surrounding liver parenchyma show strong staining for EGFR after tumour induction in both sham-operated and resected liver (Fig. 4b).

EGFR mRNA is downregulated in the liver parenchyma and liver tumours of hepatectomised animals at tumour growth time points

The pattern of EGFR expression in sham-operated and regenerated tumour-bearing liver was divergent. As tumours became larger, EGFR expression increased significantly in the sham-operated liver at days 18 ($p = 0.01$) and 21 ($p < 0.009$) post-tumour induction (Fig. 5a). Conversely, EGFR mRNA in the regenerated tumour-bearing liver was significantly increased above normal liver levels 16 days post-tumour induction ($p = 0.03$), but then declined by days 18 and 21, where EGFR levels were significantly below the tumour-bearing sham-operated liver ($p = 0.01$) (Fig. 5a). The same pattern was found for tumour tissue from sham-operated and resected animals. EGFR mRNA levels in tumours from sham-operated animals were significantly higher than from hepatectomised animals at 21 days ($p = 0.01$), when EGFR levels had increased by 2-fold (Fig. 5b).

Hepatocytes from sham-operated animals with tumour induction presented intense EGFR expression particularly at the tumour periphery at 21-days post-tumour induction (Fig. 5c-I), while hepatocytes from resected animals presented less intense *EGFR* staining (Fig. 5c-II). EGFR protein was detected in the cytoplasm of tumour cells in sham-operated livers (Fig. 5c-I). At 21 days post-tumour induction EGFR protein was barely detectable in tumours from resected livers (Fig. 5c-II).

Liver IGF-IR mRNA increased in response to tumour induction but not liver resection

IGF-IR expression was also investigated during liver regeneration in the presence or absence of tumours. Sham-operated IGF-IR mRNA levels did not differ significantly from normal liver levels. At the early timepoints following hepatectomy IGF-IR mRNA levels in the regenerating liver were consistently higher than sham-operated control livers, however this difference did not reach significance (Fig. 6). Tumour induction induced similar levels of IGF-IR upregulation in both sham-operated and regenerating liver which only became significantly different to normal liver on day 10 ($p = 0.03$ and $p = 0.01$, respectively) (Fig. 6). IGF-IR mRNA expression increased in regenerated tumour-bearing liver with exponential tumour growth compared to normal liver levels at all three time points, days 16 ($p = 0.05$), 18 ($p = 0.02$) and 21 ($p < 0.009$), and it was significantly upregulated above sham-operated tumour-bearing liver levels 18 days post-tumour induction, $p = 0.02$ (Fig. 7a). Tumour tissues from sham-operated and regenerated liver also showed increasing levels of IGF-IR during exponential tumour growth, which were significant only by day 21 in livers from resected animals compared to normal livers ($p < 0.009$), but not compared to sham-operated livers (Fig. 7b).

Discussion

Stimulation of tumour growth after liver resection

Hepatic resection is the procedure of choice for curative treatment of CLM. For patients with multiple bilobar spread, extended hepatectomy might be required to achieve complete margin-free resection. High recurrence rates occur after major resection, especially within the remnant liver. The upregulation of growth factors required for liver regeneration after resection is thought to stimulate the growth of micrometastases. The stimulation of colorectal

tumour growth in the liver after 70% hepatectomy in mice has been previously reported by our group [24] and subsequently confirmed in these experiments. The liver/body weight ratio was used as an indicator of liver tumour burden [29]. 70% liver resection resulted in an increase in 30% on tumour load. Interestingly, tumour cells from resected animals show higher proliferation rates compared with sham-operated animals at tumour growth plateau phase. Supporting the hypothesis that major liver resection stimulates tumour growth. Other authors have also confirmed an increase in tumour cell proliferation and/or tumour volume during liver regeneration after resection in subcutaneous and liver tumours [30,22,21,31]. Therefore, the clinical concern remains as to whether a two-stage resection for patients with multiple bilateral CLM is justified [32,33].

Liver regeneration

c-Met and EGFR are well documented to play a major role in hepatocyte proliferation during regeneration following liver injury or surgical resection [34,12,35]. Our results show that c-Met expression increase after liver resection, peaking at day four, and decline towards the completion of the regenerative process (day 8-10). Ross and co-workers also found that c-Met upregulation and phosphorylation peaks between three and four days post-hepatectomy in mice [36] which coincides with HGF upregulation [37]. EGFR is also upregulated, peaking at 6 days after hepatectomy. Ross *et al.* reported a peak in EGFR protein expression at four and five days post-hepatectomy [36]. These results suggest that EGFR could also have a significant role at later stages post resection during the reestablishment of liver structure. Some authors have suggested that IGF-IR signaling is required for normal liver regeneration [15,38]. Although we observed a trend towards increasing IGF-IR in the regenerating liver, it was not statistically significant. An increase in sample number may be needed to confirm this result.

Liver regeneration and tumour induction

In this study we observed significant upregulation of c-Met and EGFR in liver tissues early upon CLM induction, indicating that signalling through these receptors may have an important role in the establishment of metastases. The strong staining of c-Met and EGFR at the hepatocytes membrane, suggest that the presence of tumour cells may induce their expression by hepatocytes. This reflects the ability of the tumour to manipulate the surrounding tissue in order to favour its growth. c-Met mRNA expression 2-days after tumour induction was upregulated in the liver of sham-operated animals compared to both normal and regenerating liver with tumour induction. This might indicate that, in the absence of a significant injury, such as major hepatectomy, the presence of tumours increase c-Met expression in the immediate days after induction. Liver regeneration might prevent the increase in c-Met expression that would otherwise result from tumour induction. Both c-Met and EGFR levels are the same in tumour induced livers, with and without resection throughout the early timepoints (except for c-Met on day2). Liver resection alone and tumour induction alone increase the expression of c-Met and EGFR; however there is no additive effect when tumour induction and liver resection are performed together. These results suggest that the liver regeneration process may require optimal levels of these receptors which would be tightly controlled and as such it over-rides the tumour stimulation effects. In fact, the liver is known as a “hepatostat” due to its exceptional ability to control its size, not allowing overgrowth [39]. By day 10, the levels of c-Met for all groups are similar to sham-operated levels while the levels of EGFR in the tumour induced groups are at their highest since tumour induction. This increase in EGFR levels at this timepoint coincides with the onset of angiogenesis in our model [26]. EGFR may be involved in the angiogenic switch during tumour development. Indeed, EGFR is known to induce angiogenesis through the

upregulation of VEGF and other mechanisms [40-44]. IGF-IR levels do not change upon tumour induction up to day 8. By day 10 there is a trend of increasing expression of IGF-IR, similar to EGFR levels; however that was not significantly different from sham-operated livers. IGF-IR does not seem to be relevant for the establishment of CLM in our model.

Growth factor receptors and tumour growth at late timepoints

The expression pattern of c-Met, EGFR and IGF-1R in the tumours from sham-operated livers is very similar. In the absence of liver resection, increased levels of c-Met, EGFR and IGF-1R were detected from day 16 (exponential tumour growth) to day 21 (plateau phase of growth). It suggests that these growth factor receptors play a role in tumour growth in our murine model. Indeed, increased expression and activation of these receptors occur frequently in human colorectal cancer and therefore they have been proposed as targeted therapy [45-49]. c-MET protein expression and activation has been reported as associated with a poor prognosis in human colorectal cancers [45,46]. The importance of c-Met, EGFR and IGF-1R signalling has been recognized in many carcinogenic processes such as tumour cell proliferation, survival, tumour angiogenesis and resistance to treatment in many cancers [50,51,47-49,52]. These results further confirm the relevance of our murine model.

Tumour induction does not change the levels of c-Met and IGF-1R mRNA in the liver parenchyma from sham-operated animals at late timepoints. However, the levels of EGFR increase in the liver of these animals, similarly to what happen in the tumour. This result confirms the relevance of the EGFR signalling in the absence of liver resection and suggests a paracrine contribution of EGFR from adjacent liver. This type of phenomenon has been explored by Pietras *et al.* where they have shown that platelet-derived growth factor (PDGF) ligands expressed by cervical cancerous epithelia stimulate PDGF receptors on stromal cells to up-regulate fibroblast growth factors promoting angiogenesis and tumour growth [53].

Further studies are required to assess this hypothesis in our settings. Interestingly, our group has previously shown that the induction of colorectal liver metastases in this mouse model causes upregulation of TGF- β and VEGF in the liver parenchyma by day 21, compared with normal liver (liver with no tumours)[54]. These findings further support the hypothesis of a cross-talk between tumour and stromal cells, both within the tumour and the adjacent liver.

Liver regeneration and tumour growth at late timepoints

Hepatectomy increased c-Met expression both within the tumours and the adjacent liver parenchyma during the tumour plateau growth phase (day 21). Indicating that c-Met/HGF axis may be an important factor determining recurrence after hepatectomy. The increase in c-Met levels in tumours from resected animals coincides with the increase in tumour cell proliferation. Signalling through c-Met may promote tumour growth by increasing tumour cell proliferation. This is a key finding and supports c-Met as a likely stimulator of tumour growth after hepatectomy. Accordingly Wu *et al.* measured significantly higher HGF levels in serum and c-Met expression in cancerous tissues of patients with liver tumour recurrence or metastasis than those without recurrence or metastasis [55]. In a clinical study Osada *et al* have proposed that CLM with high c-Met expression and under high level of its ligand, HGF, led to recurrence soon after hepatectomy, leading to unfavourable patient prognosis [56]. Therefore, HGF/c-Met axis may be a key measure to prevent tumour recurrence. These data would encourage pursuit of HGF/c-Met axis neutralization strategies as potential adjuvant treatment for CLM, however this would probably inhibit liver regeneration leading to liver failure in at least some of the patients. The recent development of an antibody (LMH 80) which binds to the precursor of c-Met, potentially found exclusively in tumours, may represent an interesting alternative to overcome off-target effects [57]. Additionally, as the use of radiolabeled monoclonal antibodies as adjuvant treatment for CRC patients undergoing

liver surgery has been proposed, radiolabeling the antibody LMH80 may be an even more attractive alternative [58]. Indeed, targeting the HGF/c-Met axis after regeneration is complete, after day 10 in our model, may reduce tumour recurrence.

During exponential tumour growth, EGFR is downregulated in liver and tumour from resected animals but upregulated in sham-operated animals. The expression of EGFR is lost in the established tumour. These results imply that EGFR may not directly stimulate tumour cell proliferation in resected livers, although it is an important factor during tumour growth in the absence of resection. Accordingly, Jong and co-workers found that serum of hepatectomised rat induce proliferation of CRC cells; however EGFR expression was not changed. We may speculate that the intense EGFR activation, by its numerous ligands, during the course of liver regeneration may lead to its downregulation at a later time (ligand-induced EGFR downregulation). To verify this assumption it would be necessary to evaluate liver EGFR expression 21 days after resection in animals with no tumour induction.

Liver tissue, but not tumours, from resected animals express more IGF-IR at tumour growth plateau phase than liver from sham-operated animals. Liver IGF-IR may have a paracrine effect on tumour growth, a possibility that deserves future investigation. IGF-IR also increased in tumour tissue during exponential tumour growth, however with no distinction between sham-operated and resected animals. Wu *et al.* found that IGF-I plays a critical role in the induction of a chronic inflammatory state in the liver of obese animals and can thereby indirectly affect tumour growth in this site. Their results suggest that, IGF-I promotes colorectal liver metastasis not only through a direct paracrine effect on tumour cell survival and proliferation but also through indirect effects, likely involving the host microenvironment and pro-inflammatory responses [59].

Additionally to the growth factors receptors that were studied here other molecules may contribute to increased metastatic growth after liver resection, including interleukin (IL)-6,

macrophage inflammatory protein (MIP)-2, tumour necrosis factor (TNF)- and matrix metalloproteinases. Besides, the cause of enhanced local tumour growth in the liver after resection is possibly multifactorial, other factors such as surgical stress and participation of immune cells can be also relevant [21,60,61].

In conclusion, we demonstrate in our orthotopic mouse model that major hepatectomy stimulates engraftment and growth of colorectal liver metastases and that this effect is probably due to the upregulation of c-Met as a result of the liver regeneration process. Liver IGF-IR may also contribute in a paracrine fashion. This study provides support for the role of c-Met in the stimulation of tumour growth after resection possibly through the promotion of tumour cell proliferation. Recurrence after curative resection for colorectal liver metastases remains high. Therefore, the administration of antibodies that exclusively recognizes the unprocessed form of c-Met found in tumours may be a valuable adjuvant treatment option for patients who underwent surgical resection. Future studies need to determine the best therapeutic applications of this antibody in a likely clinical setting of multimodal treatment concepts following neo-adjuvant chemotherapy and/or post-operative adjuvant systemic therapy.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Fig.1 a Liver to body weight ratios were assessed in sham-operated (white bars) and resected (black bars) animals 21 days post-tumour induction. **b** Percentage of proliferating tumour cells (Ki-67 positive cells per total cell number). * $P \leq 0.05$; ** $P \leq 0.01$. Data are presented as mean $\pm$ S.E.M. (n = 6 for each group)

Fig.2 Temporal expression of c-Met in the liver during liver regeneration following resection and resection with tumour induction. **a** c-Met mRNA levels in normal, sham-operated and resected livers with or without tumour induction. qRT-PCR was used to detect c-Met mRNA levels. Resultant amplifications were normalised against an 18S rRNA endogenous control. * $P \leq 0.05$; ** $P \leq 0.01$. (Comparisons shown against normal liver applies for all groups except sham-operated livers). Data are presented as mean $\pm$ S.E.M. (n = 6 for each group). **b** c-Met protein expression and localisation in the liver parenchyma and tumour tissue of resected and sham-operated animals. c-Met protein was detected by immunohistochemistry. **I** Negative control; **II** Normal liver (periportal region); **III** Sham-operated liver (day 4); **IV** Resected liver (day 4); **V** Sham-operated liver with tumour induction (day 4); **VI** Resected liver with tumour induction (day 6). 200x Magnification. Bar = 50 μ m. T = Tumour; L=Liver parenchyma.

Fig.3 Temporal expression of c-Met in tumour-bearing liver (**a**) and tumour (**b**) after resection or sham-operation at days 16, 18 and 21 post-tumour induction. qRT-PCR was used to detect c-Met mRNA levels. Resultant amplifications were normalised against an 18S rRNA endogenous control. * $P \leq 0.05$; ** $P \leq 0.01$. Data are presented as mean $\pm$ S.E.M. (n = 6 for each group). **c** C-Met protein expression and localisation in the liver parenchyma and tumour tissue of resected and sham-operated animals by day 21 post-tumour induction. c-Met protein was detected by immunohistochemistry. **I** Sham-operated liver with tumour induction; **II** Resected liver with tumour induction. 200x Magnification. Bar = 50 μ m. T = Tumour; L=Liver parenchyma.

Fig.4 Temporal expression of EGFR in the liver during liver regeneration following resection and resection with tumour induction. **a** EGFR mRNA levels in normal, sham-operated and resected livers with or without tumour induction. qRT-PCR was used to detect EGFR mRNA levels. Resultant amplifications were normalised against an 18S rRNA endogenous control. * $P \leq 0.05$; ** $P \leq 0.01$. Comparisons shown against normal liver (horizontal bars) applies for all groups except sham-operated livers. Vertical bar on day 2 show the comparison sham x sham with tumour. First vertical bars on days 4-8 show the comparison sham x sham with tumour and the second vertical bar show the comparison sham x resected. Data are presented as mean $\pm$ S.E.M. (n = 6 for each group). **b** EGFR protein expression and localisation in the liver parenchyma and tumour tissue of resected and sham-operated animals. EGFR protein was detected by immunohistochemistry. **I** Negative control; **II** Normal liver; **III** Sham-operated liver (day 6); **IV** Resected liver (day 6); **V** Sham-operated liver with tumour induction (day 8); **VI** Resected liver with tumour induction (day 8). T = Tumour; L=Liver parenchyma. 200x Magnification. Bar = 50 μ m

Fig.5 Temporal expression of EGFR in tumour-bearing liver (a) and tumour (b) after resection or sham-operation at days 16, 18 and 21 pos-tumour induction. qRT-PCR was used to detect EGFR mRNA levels. Resultant amplifications were normalised against an 18S rRNA endogenous control. * $P \leq 0.05$; ** $P \leq 0.01$. Data are presented as mean $\pm$ S.E.M. (n = 6 for each group). c EGFR protein expression and localisation in the liver parenchyma and tumour tissue of resected and sham-operated animals by day 21 post-tumour induction. EGFR protein was detected by immunohistochemistry. I Sham-operated liver with tumour induction; II Resected liver with tumour induction. T = Tumour; L=Liver parenchyma. T = Tumour; L=Liver parenchyma. 200x Magnification. Bar = 50 μ m

Fig.6 Temporal expression of IGF-IR in the liver during liver regeneration following resection and resection with tumour induction. IGF-IR mRNA levels in normal, sham-operated and resected livers with or without tumour induction. qRT-PCR was used to detect IGF-IR mRNA levels. Resultant amplifications were normalised against an 18S rRNA endogenous control. ** $P \leq 0.01$. (Comparisons shown against normal liver applies for tumour induced groups only). Data are presented as mean $\pm$ S.E.M. (n = 6 for each group)

Fig.7 Temporal expression of IGF-IR in tumour-bearing liver (a) and tumour (b) after resection or sham-operation at days 16, 18 and 21 post-tumour induction. qRT-PCR was used to detect IGF-IR mRNA levels. Resultant amplifications were normalised against an 18S rRNA endogenous control. * $P \leq 0.05$; ** $P \leq 0.01$. Data are presented as mean $\pm$ S.E.M. (n = 6 for each group)

Table 1 Probe and primer sequences for qRT-PCR.

	Probe 5'→3'	Primer Sense 5'→3'	Primer Anti-sense 5'→3'	GenBank Accession Number
c-Met	TG GCT CCG AGG GCA CTC ACT ACA C	GCT GAC ATC CAG TCT GAG GTC	CGG TCC TTT TCC GAC AGG AG	NM_008591
EGFR	CG AGG GCT TGG TCC ACA TCC TGC T	TGA GCA ACA TGT CAA TGG ACT TAC	TTT GGT CAA TTT CTG GCA GTT CTC	NM_207655
IGF-IR	TC TCC TCC TCC CCA GCA GCT TCC A	CTG AAG CGC CTG GAA AAC TG	TCG GTG ATG ACT GTG AGC TTG	U00182

Fig. 2

Fig. 3

Fig. 4

Fig. 5