4   Discussion

4.1   Analysis of Met function in the adult liver

The mammalian liver has an extraordinary capacity to regenerate after partial hepatectomy or other types of injury. Although the livers' unique regeneration capacity has been well-known phenomenon for centuries, the molecular mechanisms, which underlie liver regeneration, have been not unequivocally established.

Much of the evidence that HGF/SF/Met signaling is involved in liver regeneration is based on data obtained in cell culture (Nakamura et al., 1989). In addition, expression of HGF/SF is up-regulated after liver injury in rats and increases prior to the onset of DNA synthesis. Moreover, elevated blood levels of HGF/SF are found in experimental animals and in patients after liver injury (Zarnegar et al., 1991). More direct evidence for a role for HGF in the control of liver cell proliferation is the capacity of wild-type and recombinant HGF to elevate hepatocyte DNA synthesis *in vivo* in the liver of normal mice (Ishiki et al., 1992; Roos et al., 1995 and Hartmann et al., 1998). Furthermore, intravenous injection of recombinant HGF enhances replication of hepatocytes after liver injuries (Liu et al., 1994). These approaches, however, did not establish whether HGF/SF/Met signaling is essential for liver regeneration and genetics had not been used to test the role of Met in the adult liver. Homozygous deletions of the *Met* and/or *HGF/SF* in mice are associated with embryonic lethality. The mutant mice display a reduced size of the liver, an absence of the hypaxial muscle groups that develop from migrating precursor cells, and an altered development of the placenta that causes the embryonic lethality. This lethality had precluded the genetic analysis of Met functions in the adult. I used cre-loxP technology, and particularly the *Mx-cre* transgenic strain, for an analysis of Met function in the liver of the adult, i.e. at a stage when liver growth is completed.

Ablation of Met in the adult liver had little impact on function of the organ. Conditional *Met* mutant mice had a normal life span, were fertile and did not show any overt abnormalities. However, when Met is lacking in the liver for a prolonged time period, lipids accumulate. A fatty liver is a common pathological condition in humans and has various etiologies, for instance inborn metabolic defects, toxicity of medications,

hepatitis viral or alcoholism (Braunwald et al., 1998). Thus, a long-term loss of Met function in the liver causes stress to the liver and impairs its homeostasis. Accumulation of lipids in the liver could have several causes, for instance increased uptake from the periphery and/or enhanced *de novo* synthesis by hepatocytes. It is possible that Met can prevent abnormal lipid deposition, since HGF/SF was shown to regulate lipid synthesis in rat hepatocytes (Kaibori et al., 1998). Alternatively, Met could protect the liver from harmful insults during postnatal life that result in a pathologic metabolism. The mechanism responsible for the accumulation of lipids in *Met* conditional mutant mice is unclear and requires further investigations. An abnormal lipid accumulation was shown not to interfere with liver regeneration in wild-type animals (Picard et al., 2002). Furthermore, staetosis was also observed in other mutant mice in which liver regeneration is impaired, for instance *c-jun* conditional knockout, or *FGF* transgenic mice (Behrens et al., 2002; Steiling et al., 2003).

I analyzed liver regeneration after partial hepatectomy in the *Met* mutant mice. Interestingly, survival of the *Met* mutant animals and the efficiency of regeneration depended on the procedure used for partial hepatectomy. Thus, liver regeneration was not initiated in *Met* mutant mice and they died after surgery if a well-established protocol was used that allows survival and liver regeneration in control mice. A second protocol, which was provided by Wüstefeld and colleagues (Wüstefeld et al., 2003), allowed liver regeneration and survival of the *Met* mutant animals, and was then used in my further analysis. This phenomenon has been previously described by others that studied liver regeneration in *IL-6* or *gp130* mutant mice. It had led to apparently controversial results, i.e. liver regeneration of IL-6 and gp130 mutant mice were reported to be severely impaired, or to proceed to completion on a normal schedule (Cressman et al., 1996; Wüstefeld et al., 2003; Greenbaum et al., 1998 and Sakamoto et al., 1999). In these studies, identical amounts of hepatic tissue were removed, but differences in the surgical procedure existed such as type of the anesthesia used, the position of the incision and the procedure employed for the ligature of blood vessels. It is possible that variable amounts of stress associated with the surgery might affect the regeneration process and the survival of the mutant animals. Even if partial hepatectomy was performed under optimal conditions, the liver regeneration in conditional *Met* mutant mice was impaired and liver-to-body-weight ratio was not properly restored five to seven days after surgery. The impaired regeneration of the *Met*

mutant liver was accompanied by defective proliferation and cell cycle progression of the hepatocytes.

4.2   Cell cycle progression in conditional *Met* mutant mice

After liver injury, hepatic cells synchronously re-enter the cell cycle and replicate. The partial hepatectomy model represents thus an excellent example of controlled cell proliferation *in vivo*, where a precise balance between proliferative and anti-proliferative signals results in an accurate restoration of liver mass. In the normal liver, hepatocytes are quiescent cells, and need to exit G0 before they can enter the replicative cycle, i.e. the have to be primed for replication. HGF/SF/Met signaling has been implicated in priming, which was based on two observations: (i) the very rapid increase in the levels of circulating HGF/SF after partial hepatectomy was considered to be one of the first events triggering regeneration; (ii) transgenic mice that express HGF/SF under the albumin promoter display an up-regulated expression of the *c-myc* and *c-jun* proto-oncogenes. *c-myc* and *c-jun* are immediate early genes important for the exit from G0. Furthermore, *c-jun* and *Met* mutant mice display similar defects in liver development, an indication that c-jun might act as an essential component downstream of Met signaling. Surprisingly, in the regenerating liver of *Met* mutant mice, the activation of c-jun and expression of c-fos were not changed, which indicated that certain aspects of priming occurred correctly in conditional *Met* mutants.

The expression of immediate early genes during liver regeneration does not lead to DNA replication unless the cells receive further signals that allow them to progress through the cell cycle. Such signals are provided by growth factors, and lead to the expression of cyclin D1. In hepatocytes, the induction of cyclin D1 marks the exit from G0 and progression through G1 phase (Albrecht et al., 1995; Loyer et al., 1996). Compared to control mice, the protein levels of cyclin D1 were reduced in conditional *Met* mutants, indicating that cell cycle progression was already impaired at the stage of the G0-G1 transition. Even though the expression or activation of some immediate early genes was not changed, the exit from quiescence was impaired in *Met*-deficient livers indicating that signaling cascades for instance PI3K or MAPK can also regulate the expression of cyclin D during liver regeneration.

The cyclin D/cdk4/6 complex is thought to be a focal point of the signal transduction pathway that integrates growth-stimulatory and inhibitory signals into the cell cycle machinery (Sherr, 1996). This complex together with cyclin E/cdk2 is responsible for the phosphorylation of pRb and thus the entry into S-phase. Over-expression of cyclin D1 in primary hepatocytes results in DNA replication even in the absence of growth factors (Albrecht and Hansen, 1999). Cyclin E as well as another S-phase cyclin, cyclin A, appear on schedule in conditional *Met* mutant mice, but their expression was reduced. Therefore, not only the exit from quiescence, but also the progression through G1 and the entry into S-phase is impaired in conditional *Met* mutant mice.

Concomitantly with lower levels of cyclin D and E protein, the phosphorylation of pRb was significantly reduced in the conditional *Met* mutant when compared to control mice. pRb phosphorylation releases the E2Fs heterodimes, a family of transcription factors that control numerous S-phase molecules, for instance PCNA, cdk1, cyclin A. Many growth inhibitory signals (like TGF-β) mediate their effect by blocking the phosphorylation of pRb. Thus, pRb monitors positive and negative growth signals and determines whether or not the cell will divide. The fact that phosphorylation of pRb is reduced is a further indication that cell cycle progression and entry into S-phase are impaired in the liver of *Met* mutant mice. This is in agreement with the observed reduction in hepatocyte proliferation as assessed by BrdU incorporation two days after partial hepatectomy.

The overall amount of phosphorylated pRb was lower in the regenerating liver of the *Met* mutant mice. Further, phosphorylated pRb persisted over a prolonged time period, and was observable five days after partial hepatectomy in *Met*-deficient livers. This correlated well with the prolonged expression of some E2F-responsive genes, and high levels of PCNA and phosphorylated cdk1 were observed five days after partial hepatectomy. PCNA plays an important role in DNA replication as a part of the DNA polymerase complex, while cdk1 activity is indispensable during mitosis. Prolonged pRb phosphorylation did however not correlate with sustained replication, and instead the growth of hepatocytes had already ceased five days after partial hepatectomy in the liver of *Met* mutant mice. The lack of hepatocyte replication despite the presence of signals for S-phase entry might be caused by an up-regulation of counteractive inhibitory pathways. Indeed, five days after partial hepatectomy, a strong up-regulation

of the cdk inhibitor, p21Cip1/Waf1, was observed in livers of *Met* mutant mice. p21Cip1/Waf1 inhibits numerous cdks involved at all stages of cell cycle progression. For the control of cyclin/cdk activity, the amount of p21Cip1/Waf1 seems to be critical, since one molecule of p21Cip1/Waf1 per cdk-cyclin complex appears to permit the assembly and activity of the complex, while two molecules of p21Cip1/Waf1 inhibit cyclin-cdk activity. Consistently, in the regenerating livers of *p21Cip1/Waf1* mutant mice, hepatocytes progressed more rapidly through the cell cycle, indicating that p21Cip1/Waf1 plays a growth-inhibitory role during this process (Albrecht et al., 1998). The elevated levels of p21Cip1/Waf1 might thus account for the defective cell cycle progression in the regenerating livers of the *Met* mutant mice. An abnormal up-regulation of p21Cip1/Waf1 has been also reported in other mutant mice with impaired liver regeneration, for instance in c-jun conditional mutant mice, or in *FoxM1b* and *p18/INK4c* mutant animals (Behrens et al., 2002; Luedde et al., 2003; Wang et al., 2002b).

The molecular mechanism that is responsible for the up-regulated expression of p21Cip1/Waf1 in conditional *Met* mutant mice five days after surgery is unclear. Previous studies have shown that p21Cip1/Waf1 expression in the liver can be controlled in a p53-dependent and –independent manner. A p53-dependent up-regulation of p21Cip1/Waf1 was observed in response to DNA damage, and provides an important mechanism by which p53 orchestrates growth arrest (el-Deiry et al., 1993). However, the expression of p53 is not altered in conditional *Met* mutant mice, indicating that a p53-independent pathway causes the increase in p21Cip1/Waf1. p21Cip1/Waf1 can be regulated independently of p53 during development and differentiation, for instance by TGF-β signaling, after serum stimulation, or in response to stress (Macleod et al., 1995). The up-regulated expression of p21Cip1/Waf1 in the regenerating liver of the *Met* mutant mice might therefore reflect a stress response, or the presence of an unidentified, anti-mitotic signal. In addition, the protein stability has been considered as a mechanism that changes the concentration of p21Cip1/Waf1 protein. For instance, the transcription factor C/EBPα enhances the stability of the p21Cip1/Waf1 protein, which results in a growth inhibition of fibrosarcoma cells (Timchenko et al., 1996).

I conclude therefore that five days after partial hepatectomy, hepatocytes in conditional *Met* mutant mice encounter two sets of contradictory signals. On the one side, there are signals that promote entry into S-phase like the prolonged presence of phosphorylated pRb, and on the other side signals that inhibit cell cycle progression like high levels of p21Cip1/Waf1 protein. Since the hepatocytes do not continue to proliferate in the *Met*-deficient livers, the inhibitory signals override those that promote proliferation.

A further mechanism that might interfere with liver regeneration in *Met* mutant mice is an increase in apoptosis. Met, like other receptor tyrosine kinases, provides anti-apoptotic signals by activating the Akt kinase. Furthermore, previous studies reported that Met can directly interacts with Fas and can therefore prevent the liver and other organs from Fas-induced apoptosis (Wang et al., 2002a). However, apoptosis rates in the regenerating livers of control and conditional *Met* mutant mice were comparable, indicating that the lack of the anti-apoptotic function of Met is not a dominant mechanism that accounts for the impaired liver regeneration. Not only the number of cells, but also cell size determine the size of organ. However, the cell size was similar in the liver of control and mutant mice. Therefore, defects in hepatic mass restoration after liver injury cannot be accounted for by a change in the size of the individual hepatocytes.

4.3   Cytokines and growth factors are increased in the blood during liver regeneration

The molecular mechanism of liver regeneration is complex and various intra- and extra-cellular pathways orchestrate the restoration of hepatic tissue. A deficit in metabolic capacity of the liver, for instance a result of tissue loss, results in an immediate release of cytokines (TNF-α and IL-6) and of growth factors (HGF/SF, EGF and TGF-α) from the liver. Growth factor and cytokine signaling cascades co-operate, and for instance TNF-α stimulates DNA replication in cultured hepatocytes but requires the presence of other growth factors to accomplish this (Kirillova et al., 1999). In addition, cytokines also stimulate extra-hepatic tissues to release factors that amplify the growth response, for instance TNF-α induces synthesis of IL-6 (Ohira et al., 1996).

The postulated sources of HGF/SF in the liver are Ito and sinusoidal cells. HGF/SF expression in these cells increases around 6 hours after partial hepatectomy and lasts for 24 hours (Zarnegar et al., 1991). This time window does not correlate with the up-regulation of circulating HGF/SF protein, which appears earlier. However, hepatic extra-cellular matrix that is mainly localized around portal triads contains large amounts of HGF/SF. Matrix proteolysis was proposed to account in part for the rapid increase in the blood levels of HGF/SF. During liver regeneration, an increased HGF/SF mRNA level is also observed in the mesenchymal cells of lung, spleen and muscle (Yanagita et al., 1992). Compared to control mice, increased levels of HGF/SF are observed in the blood stream six hours after partial hepatectomy in conditional *Met* mutant mice. The mechanisms for this further increase in HGF/SF levels are not clear. The liver uses most of the circulating HGF/SF, and the absence of Met in the liver could lower the clearance rates. HGF/SF binds however also to heparin sulphates with high affinity. Heparin sulphate proteoglycans are very abundant extra-cellular matrix proteins and provide the majority of the HGF/SF binding sites in cultured cells or in tissues. Heparin sulphates are considered to be an important determinant in the clearance of HGF/SF, and act independent of Met. Other factors than changes in clearance rates, for instance increased transcription, might therefore be responsible or contribute to the up-regulated levels of HGF/SF in *Met* mutant mice.

Similarly, several sources of cytokines have been discussed during hepatic regeneration. It is not clear if TNF-α and IL-6 are produced by inflammatory cells brought to the liver via the blood or by the macrophage-Kupffer cells within the liver. Alternatively, both sources might contribute to the generation of these cytokines (Datto et al., 1995 and Akashi et al., 1999). In the conditional *Met* mutant mice, elevated levels of IL-6 are found over prolonged time period. Persisting high levels of IL-6 have been also reported in other mutant mice in which liver regeneration is impaired (Wüstefeld et al., 2003). Interestingly, the deficits in liver regeneration in *TNFR1* knockout mice can be reversed by injections of IL-6 (Yamada et al., 1997), indicating that the elevated IL-6 levels represent a compensatory physiological response that attempts to ensure liver regeneration. Increased amounts of IL-6 might also affect HGF/SF expression directly, since IL-6 responsive elements and binding sites for IL-6 specific transcription factor NF-IL-6 have been identified in the murine HGF/SF promoter (Zarnegar, 1995).

4.4   Signaling of cytokines and growth factor during liver regeneration

During liver regeneration, Met and other receptors are activated and these receptors induce signaling cascades, partially in an overlapping and partially in a specific manner. HGF/SF and IL-6 transmit signals through different classes of receptors, the Met tyrosine kinase and gp130 cytokine receptor, respectively. However, both factors were shown to activate immediate early genes. Nevertheless, the phosphorylation of c-jun and thus activation of JNK as well as the expression of c-fos were not changed in conditional *Met* mutant mice after partial hepatectomy. Therefore, HGF/SF/Met signaling does not significantly contribute to activation of c-jun or induction of c-fos. Instead, other signaling pathways, for instance IL-6, might regulate these transcription factors. In accordance with this, the activation of immediate early genes was substantially impaired in *gp130* and *IL-6* mutant mice (Cressman et al., 1996; Wüstefeld et al., 2003). Another potential regulator of immediate early genes is the TNF-α pathway, since the binding activity of the AP-1 transcription factor was severely reduced in *TNFR1* mutant animals (Yamada et al., 1997).

STAT3 is an important substrate of the activated IL-6 receptor. STAT3 is phosphorylated by Jak kinases, translocates to the nucleus and is an essential signaling component that mediates gp130 induced proliferation (reviewed by Hirano et al., 2000). The Met kinase has been also reported to recruit and phosphorylate STAT3 during tumor progression and formation of branches and tumorigenesis (Boccaccio et al., 1998; Zhang et al., 2002). STAT3 phosphorylation levels were not reduced in the regenerating liver of conditional *Met* mutant mice, and Met signaling does therefore not appear to contribute to the STAT3 activation. Instead, STAT3 activation was prolonged, which parallels the prolonged up-regulation of IL-6 in the blood stream of these mutant mice.

Growth factors, cytokines or integrins use mainly the MAPK pathway to elicit proliferative responses. These factors, including HGF/SF, IL-6, EGF and TGF-α, recruit adapter proteins containing SH2-SH3 domains to the phosphorylated tyrosine of their specific receptors to activate MAPK/Erk signaling. However, the individual contribution of each of these factors to the activation of the MAPK/Erk pathway during liver regeneration is not clear. Although there are many routes leading to Erk phosphorylation, they all involve the Grb2 adapter, which recruits the GDP-GTP

exchange factor, SOS and induces the Ras/MAPK/Erk cascade. Grb2 can bind to Met directly, or is recruited indirectly through the interaction with Shc or SHP2. The adapter SHP2 is also recruited to the phosphorylated gp130 receptor and enables IL-6 to activate MAPK/Erk kinases. The phosphorylation of Erk1/2 was abolished in conditional *Met* mutant mice, indicating that HGF/SF signaling exclusively regulates the activation of these kinases during liver regeneration. The Erk kinases can regulate progression through G1 by different mechanisms. Sustained activity of Erk induces *cyclin D1* expression, and enhances the stability of c-myc protein (reviewed by Roovers and Assoian, 2000).

Other signaling cascades, for instance PI3K, also regulate the levels of cyclin D1. The major component of the PI3K pathway, Akt kinase, contributes to cyclin D1 expression and regulates the stability of cyclin D1 protein (Diehl et al., 1998). Activation of Akt kinase occurred in conditional *Met* mutant mice, but it was delayed, indicating that some other signaling cascades besides HGF/SF/Met contribute to Akt phosphorylation during liver regeneration. A possible candidate for this is IL-6, which was reported to activate Akt via recruitment of PI3K to the phosphorylated gp130 receptor. The main function of Akt is to promote cell survival by inhibiting important apoptotic molecules, such as Bad and caspase-9. A change in apoptosis that would reflect the altered activity of Akt in the *Met* mutant mice was not apparent.

GSK-3β is another kinase that can mediate cell cycle progression. For instance, GSK-3β phosphorylates cyclin D1 and c-myc and thus targets these proteins for ubiquitinylation and subsequent proteolytic degradation (reviewed by Cohen and Frame, 2001). Although GSK-3β is a major target of Akt, the phosphorylation of GSK-3β was not impaired in *Met*-deficient livers. However, other kinases besides Akt have been implicated in GSK-3β regulation, for instance MAPK-activated kinase 1 that is activated by growth factors, or ribosomal protein S6 kinase (S6K) activated via mTOR. In the regenerating livers of *Met* mutant mice, GSK-3β phosphorylation was not altered. Met signaling is thus not essential for GSK-3β inhibition, and other pathways do therefore control the activity of this kinase during liver regeneration.

The analysis of signaling cascades in conditional *Met* mutant mice indicated that Met

co-operates with other signaling pathways during liver regeneration. Met signaling is essential for the activation of the MAPK/Erk pathway, and contributes significantly to the activation of Akt in the regenerating liver. However, STAT3 activation and GSK-3β inhibition are not regulated by Met, and other signaling receptors are responsible for this.

The cross talk of Met with other signaling receptors seems to play an important role during liver regeneration. The putative Met partners in this interaction are gp130 and other cytokine receptors like TNFR. Cytokine receptors were shown to act synergistically on activation of STAT3 during liver regeneration. In addition, cytokine signaling can contribute to regulation of Akt and GSK-3β activity after liver injury.

Although liver regeneration is severely impaired in mutant mice for HGF/SF receptor, it is not completely abolished. Other growth factors whose blood levels increase after liver injury are EGF and TGF-α, although their plasma concentrations rise only slightly, for instance less than 30% in case of EGF. All these growth factors induce a mitogenic response in cultured hepatocytes and it is not clear whether hepatocytes preferentially respond to one of them or the growth factors act in parallel or sequentially during liver regeneration. In fact, there is no mutant mouse described in which liver regeneration is completely abolished. The EGF family of growth factors might display some redundancy with HGF/SF during liver regeneration. However, the compensatory effect of other growth factors is questionable since the major mechanism by which they induce cell proliferation is activation of the MAPK pathway, and Erk phosphorylation was not observed in conditional *Met* mutant mice.

4.5   Outlook

The analysis of liver regeneration in conditional *Met* mutant mice indicates that Met and gp130 receptors interact with each other during hepatic tissue restoration. Moreover, the defects in liver regeneration observed in conditional *Met* mutant mice partially resemble those described for *gp130* mutant animals. Therefore if IL-6 can compensate for the loss of HGF/SF/Met signaling then it would be interesting to analyze liver regeneration in mice mutant for both *Met* and *gp130*.

In addition, the mechanisms responsible for the abnormal lipid accumulation in the aged livers of conditional *Met* mutant mice remain unclear. Met signaling has not yet been shown *in vivo* to regulate any metabolic processes. Therefore, this putative novel function for a receptor tyrosine kinases has to be evaluated in further studies.

The crucial contribution of HGF/SF/Met signaling to cell cycle progression during liver regeneration raises the question about the significance of Met in tumor formation. In addition, HGF/SF is a mediator of epithelial-mesenchymal interactions, which are critical during metastasis/invasion. So far, most of the studies on the role of Met in tumor, concentrated on constitutive active variants of the receptor and little is still known about role that the normal HGF/SF/Met signaling plays in cancer.