The degradation of the extracellular matrix initiated by uPA is thought also to result in the release of matrix-bound growth factors, particularly HGF. Several tissues synthesise and release HGF. In the liver, the non-parenchymal cells, particularly stellate cells, rather than hepatocytes are the major source of HGF. When first synthesised HGF is a single chain protein that is mitogenically inactive. This inactive form is released and bound by the extracellular matrix, which thus serves as a potential HGF reservoir. Following hepatectomy, the increase in uPA activity not only leads to degradation of the ECM and release of single chain HGF but also converts it to the mitotically competent two chain form.

Interestingly, one of the effects of the hepatoporal shunt, which prevents the increase in portal blood pressure, is that the increase in HGF seen following hepatectomy still occurs, but the HGF is not converted from the single chain inactive form as rapidly as normal. This delay or loss of active 2 chain form may indicate a reduction in the early increase in uPA activity since uPA has been shown to cleave the single chain HGF to the active two chain form. This would imply that the increased uPA activity in the liver is somehow related to the early increase in portal blood flow per unit wet weight or to the early increase in portal blood pressure.

Further support for a role for uPA in the regenerative process comes from uPA-deficient mice. In these animals uptake of $[^3]H$–thymidine into DNA and mitotic index were reduced at 44h post hepatectomy (the peak time for control mice) by almost half compared to control animals, suggesting a slower hepatocyte growth response. Nonetheless, at 8 days post hepatectomy liver size was the same in both sets of animals. Interestingly, in uPAR-/- animals there was no effect on $[^3]H$–thymidine incorporation into DNA at 44h, again suggesting that uPA mediates some effects without binding to the receptor.

Evidence that increases in uPA activity occur in man as well as rodents comes from our group and is shown in the Figure below. Note that there appears to be a threshold of about 40% resection below which no increase in uPA activity is observed. With resections larger than 40% there is an approximately linear relationship between the amount of liver removed and the activity of uPA in the remnant liver.
Early Increases in uPA Activity in the Human Remnant Liver Following Resection

Another important component of the uPA/uPAR system is PAI-1 (plasminogen activator inhibitor 1). Although levels of PAI-1 expression and protein are very low in normal liver, PAI-1 has been classed as an immediate early response gene since it is rapidly induced in the hepatocytes of regenerating rat livers. Peak mRNA levels are reached 2h post hepatectomy and return to normal (negligible) levels by 8 h. Similar transient increases have been reported in mice, but the response is delayed relative to the rat. Collectively this data strongly implicates PAI-1 in the in vivo regeneration process and in cirrhosis in rodents as well as in vitro growth of hepatocytes.

Whilst the changes in ECM remodelling described above are necessary for the regeneration process to occur, it seems currently considered to be a ‘priming’ step which allows the liver to respond to growth stimuli generated by assorted growth factors, and is not of itself sufficient to produce hepatocyte proliferation. The proliferation step requires a combination of cytokines and growth factors.

Immediate-early genes and Transcription factor activation

The immediate early genes, many of which encode transcription factors, are generally expressed at low or undetectable levels in quiescent, non-dividing cells, but within minutes of stimulation by mitotic agents, for example by addition of growth factors, transcription of these genes is activated. The process often does not require protein synthesis and activation relies on post-translational modifications and usually results in a burst of expression that lasts for only a few hours at most. The proto-oncogenes c-jun, c-myc and c-fos are activated in many cell systems in response to a variety of stimuli and were among the first to be associated with regeneration after hepatectomy in the rat. The number of genes classed as immediate early genes for liver has since grown considerably. A combination of subtraction and differential screening of cDNA libraries from regenerating livers and H-35 cells stimulated
with insulin allowed identification of 52 immediate-early genes, 41 of which were described as novel. More recent studies have focused on a subset of these which includes the transcription factors STAT3, NFκB and C/EBP, and much attention is currently being directed towards elucidating the genes which are regulated by these transcription factors during hepatic regeneration.

Changes in the levels of β-catenin, which can act both as a transcription factor with the potential to modulate uPAR, cyclin D1 and c-myc expression and also forms part of an adhesion complex with E-cadherin, have recently been described in the rat. A decrease in the rate of β-catenin degradation, leading to transient increases in hepatocyte β-catenin levels, and increased translocation to the nucleus have been observed during the first 5 min after hepatectomy, making these some of the earliest changes recorded. Active β-catenin breakdown was seen shortly thereafter leading to lower than normal levels of β-catenin that did not return to normal until 48 to 72 h. This early modulation of β-catenin may be important in the regenerative process.

An interesting link between β-catenin and HGF has recently been demonstrated in cultured primary hepatocytes. HGF was shown to induce a redistribution of β-catenin to the nucleus independently of the more widely recognised Wnt signalling pathway. The β-catenin involved in this translocation was not derived from the E-cadherin/β-catenin complex associated with Wnt signalling but instead came from a separate complex formed between Met and β-catenin (c-Met is the receptor for HGF). In normal rat liver, approximately 80% of Met was associated with β-catenin and about 30-40% of β-catenin was associated with Met on the inner side of the hepatocyte membrane, suggesting this Met/β-catenin complex represents a functionally important β-catenin pool. Treatment of the primary hepatocyte cultures with HGF led to phosphorylation of tyrosine residues in both the Met and β-catenin proteins and subsequent dissociation of phosphorylated β-catenin from the Met/β-catenin complex that then translocated to the nucleus. HGF was without effect on the E-cadherin/β-catenin complex. The possible involvement of this newly recognised pool of β-catenin in the changes following partial hepatectomy described above is particularly interesting, especially when viewed against the changes reported for Met phosphorylation. In control rat liver Met is largely unphosphorylated, but after two thirds partial hepatectomy Met undergoes a biphasic increase in phosphorylation with peaks at 1-5 minutes and 60 minutes after resection. This suggests that HGF mediated signal transduction is initiated within one minute of partial hepatectomy. Intriguingly, this is well before any reported changes in HGF levels in the liver or blood and the nature of the ligand causing the early tyrosine phosphorylation of c-met is unknown at present.

Another transcription factor which has been the focus of attention after hepatectomy is STAT3 (a member of the ‘Signal Transduction and Activators of Transcription’ family). Active STAT3 can be detected 30 minutes after partial hepatectomy and peaks with an increase greater than 30 fold at about 3h, and remains activated for several hours thereafter. The IL-6 family of cytokines, which act through the gp130 receptor to activate JAK intracellular tyrosine
protein kinases, are major activators of STAT3. Once phosphorylated and activated, STAT3 translocates to the nucleus where it modifies gene expression.

Not all immediate-early genes are transcription factors. Itih-4, Inter-alpha-trypsin inhibitor-4, is a member of the serine protease inhibitor family found in liver with possible roles in stabilisation of the extracellular matrix and as an anti-apoptosis agent, and has recently been identified as an immediate-early gene. Expression of this protein following hepatectomy is bimodal, showing peaks at 30 minutes and 12 hours post hepatectomy. Two other enzymes related to key signalling pathways and showing early changes following hepatectomy are protein kinase B/Akt and a protein tyrosine phosphatase known as PRL-1 (phosphatase of regenerating liver-1). PRL-1 is highly induced in regenerating rat liver by an IL-6 independent mechanism, showing a 15 fold increase at 30 min and returning to basal levels at 6h post hepatectomy. Plasma levels of insulin-like growth factor binding protein 1 (IGFBP1) are increased about 100X shortly after hepatectomy. Upregulation of IGFBP-1 expression is IL-6 dependent, requiring IL-6 induction of STAT-3 and AP-1 (c-Fos/c-Jun) and the endogenous hepatocyte nuclear factor-1 (HNF-1).

The expression of the TGF-β receptor genes is interesting in that there is a decrease protein and mRNA for all three types of TGF-β receptor immediately after partial hepatectomy in rats. This decrease is maximal at 24 h post hepatectomy and thus represents a class of immediate-early genes which is negatively, rather than positively, modulated. This decrease in receptor expression may be the mechanism by which hepatocytes become resistant to the inhibitory effects of TGF-β on mitosis following hepatectomy.

Plasma noradrenalin levels rise within 1 h of partial hepatectomy. In primary hepatocyte cultures noradrenalin augments the mitogenic activity of both EGF and HGF and counter acts the growth-inhibitory effects of transforming growth factor beta (TGF-β) and it is likely that similar co-mitogenic effects occur in vivo.

Changes in cytokines.

Much of the work on cytokine changes after hepatectomy has focused on TNFα (tumour necrosis factor α) and IL-6 (Interleukin –6). Increases in the levels of these two cytokines, normally associated with inflammation, are observed shortly after partial hepatectomy in rodents. Liver and plasma levels of IL-6 and TNFα rise rapidly after partial hepatectomy, with maximal levels of TNFα preceding maximal levels of IL-6, but these levels are only transient. Strategies which suppress expression of these cytokines delay or inhibit liver regeneration.

IL-6 knockout mice show an impaired regenerative response correctable by IL-6 injection. The proposed signalling pathway is that TNF binds TNFR-1 leading to the activation of NFκB, and increased IL-6 expression. IL-6 is then presumed to activate multiple signalling pathways, including the Jak/STAT pathway, leading to the activation of STAT3. A recent
immunohistochemical study in rats showed STAT3 activation in Kupffer cells preceded activation in hepatocytes. The available evidence thus suggests that TNF acts to prime the hepatocytes for division by increasing sensitivity and responsiveness to growth factors, but is not of itself capable of inducing mitosis.

Direct injection of TNF into the portal vein of normal rats sensitises hepatocytes to the effects of growth factors. Interestingly, binding of tumour necrosis factor, causes apoptosis in normal liver, but stimulates hepatocyte growth during regeneration and it has been postulated that this difference in response relates to the prevailing level of reactive oxygen species (ROS).

It is not absolutely clear at present if the changes in TNFα and IL-6 are mediated by inflammatory cells brought to the liver via the blood, or from cells within the liver (such as Kupffer and Stellate cells) or if there is a mixture of both. Early studies suggested that endotoxin, a major inducer of TNFα production by Kupffer cells, had a role in liver regeneration. The available evidence suggests that TNFα stimulates IL-6 production by the Kupffer cells and this then leads to the early activation of at least 4 transcription factors (namely, NFκB, STAT3, AP1 and C/EBP) supporting a role for Kupffer cells. A recent study in which livers were depleted of Kupffer cells by pre-treatment with liposome- encapsulated dichloromethylene-diphosphonate (Cl2MPD-liposomes) abolished changes in IL-6 (and IL-10) and reduced the expression of TNFα, HGF and TGF-β. Although the regeneration was significantly delayed and DNA synthesis significantly decreased, loss of the Kupffer cells (confirmed by immunohistochemistry) did not abolish the regenerative response completely.

The figure below illustrates how IL-6 and STAT3 interact and also shows that IL-6 can activate the MAPK pathway, though the precise mechanism by which the MAPK pathway is stimulated is still uncertain.

THE PROGRESSION PHASE
For the purpose of this review, the progression phase covers events initiated between about 1 h and 6 h post hepatectomy in the rat partial hepatectomy model. This corresponds to the early to mid G1 period after which the hepatocytes pass through the G1 phase restriction point(s) and become committed to S phase and subsequent mitosis.

The figure below summarises the progression phase, cell cycle phase and stop signal events.

LEGEND: EVENTS OF THE PROGRESSION AND CELL CYCLE PHASES.
The events from the end of the first hour through to 48 hours following hepatectomy are illustrated. The caveats outlined in the legend for Figure showing the Priming Phase apply here also.

Changes in Growth Factors
Hepatocytes isolated from adult liver do not readily divide in culture unless supplemented with mixtures of growth factors at high concentration. Hepatocytes from foetal liver are generally much more responsive. For both types, hepatocyte growth factor (HGF) appears the most potent mitogen. HGF (also known as ‘scatter factor’) is produced by a variety of
mesenchymal cells (but not by epithelial cells, including hepatocytes) and mediates its effects by binding to a specific receptor (c-Met) on the hepatocyte cell surface. Plasma levels of HGF increase rapidly after partial hepatectomy (greater than 20 fold increase within 1 h) and elevated levels are maintained for about 3 days. There appear to be at least 3 potential sources of this HGF. The first, increased production of HGF by liver Stellate cells, does not occur until 3-6h post hepatectomy in the rat and presumably cannot account for the early observed increases in HGF. A second source of HGF is interaction of the uPA/uPAR proteolytic system with the bound single chain HGF associated with the ECM as discussed earlier. A third possible source for HGF, (which may be thought of as a priming phase event), is release of HGF from non-hepatic tissues, particularly the lungs. On the basis of studies with cultured MRC-5 human embryonic lung fibroblast it was proposed that nor adrenaline (which shows early increases in plasma following hepatectomy) is the stimulus for increased HGF production by the lung and that effects were mediated primarily through β-adrenergic receptors. A recent study looked at the forms of HGF (single chain inactive or two chain active forms) present during rat liver regeneration and showed resting liver contained both forms, but the single chain inactive form was predominant. Immediately post hepatectomy was a consumptive phase where both forms decreased within the liver, (but only the active two chain form appeared in plasma), followed by a productive phase where liver HGF levels increased 5-fold over sham operation control levels, and both forms were being produced.

Although HGF can serve as a mitogen for cells in culture, injection of HGF into the portal vein of rats has very little effect on hepatocyte cell division. Two other mitogens (EGF, epidermal growth factor, and TGF-α, transforming growth factor alpha) which can promote in vitro cell division were also notably ineffective when injected via the portal vein. The effect of HGF or TGF-α could be enhanced by subjecting the animals to a relatively smaller partial hepatectomy in which only about 30% of the liver was removed which is about the threshold necessary to affect liver function, and a negligible DNA response is evoked. However, sensitivity to HGF and TGF-α is stimulated and marked DNA synthesis occurs when these mitogens are then injected. A similar effect is produced by injection of collagenase into the liver before the HGF administration. The mitotic effect of HGF was markedly enhanced and more than 60% of the hepatocytes showed increased DNA synthesis (the collagenase alone had minimal effect). This suggests a ‘priming step’ related to reorganisation of the hepatic ECM is necessary before the hepatocytes can respond to growth factor stimulation.

End of Lecture 3