THE START SIGNAL.

In the adult liver, which is not actively growing, most of the cells have exited the cell cycle and are in the quiescent G0 state and only about 1 hepatocyte in 20,000 (0.005%) is dividing. Removal of 70% of the liver initiates a process in the remaining 30% remnant in which each of the remaining cells have to divide on average 1.6 times to restore the liver mass. Thus all the cells can be expected to divide once and two-thirds to divide a second time. However, the nature of the ‘start’ signal remains elusive. The conventional view has been that the ‘start’ signal is generated by the removal of the resected liver mass and the consequent reduction of liver function(s) and physiology. The complexity of the regenerative process is such that multiple signals may independently induce different aspects of the regeneration response and a concerted sequence of events, many of which are not unique to liver regeneration, may be collectively responsible for firstly driving the cells from the quiescent G0 state into the G1 phase of the cell cycle, and then on through the restriction point(s) of G1 into S phase where commitment to division has been reached. The G0 to G1 transition can be reversed and the cells can escape from the regeneration pathway if the appropriate signals are withdrawn before the cells have passed through the restriction point(s) and committed to S phase.

There is a wealth of information from parabiotic experiments and cell culture studies demonstrating clearly that liver growth promoting signals are carried in the circulation during the time that regeneration is occurring. Rats whose circulatory systems are connected together (parabiosis) show that a partial hepatectomy in one of the animals induces a growth response in the liver of the joined animal and suggests that liver regeneration is associated with soluble factors which can be carried in the blood stream. Increases in the levels of key cytokines and growth factors occur relatively early in the remnant liver following hepatectomy, but they are not the earliest recorded events and thus by the conventional understanding of a ‘start’ signal appear to represent a response to the ‘start’ signal rather than the ‘start’ signal itself.

The importance of portal blood flow

The liver is unusual in that it has a dual blood supply, with about 80% provided by the portal vein and 20% by the hepatic artery. This arrangement allows for maintenance of the blood supply and any decreases in portal blood flow are offset by increased arterial supply, the ‘hepatic artery buffer response’. The importance of the portal vein blood supply to hepatic regeneration has been recognised for at least half a century and diversion of the portal blood
flow results in a markedly delayed regenerative response. Regeneration in animals devoid of portal viscera is delayed even more, but can be reversed to some extent by treatment with glucagon. Regeneration is also less vigorous in germ-free animals suggesting a role for an endotoxin-mediated input into the regeneration process.

Studies of this kind have highlighted the importance of interplay between different organs during regeneration and emphasised the hepatotrophic nature of portal blood.

**A Role for Nitric Oxide?**

The possibility that nitric oxide (NO) is the ‘start’ signal has been advocated. It was proposed that following removal of 70% of the liver the increased portal vein blood flow through the remaining 30% remnant is associated increased blood pressure and an increased shear stress at the surface of the cells lining the blood vessels resulting in the formation and release of NO from these cells. In other systems NO is known to act as a signalling molecule and it is assumed that it could act similarly in liver and start off the regenerative mechanism. However, NO is difficult to measure directly and its involvement is usually inferred from the effects of inhibitors, and the exact time course of the NO formation is not absolutely clear. In addition, the possible involvement of NO in the early stages of regeneration has been known since the mid 1990’s (see later) without gaining universal acceptance as a ‘start’ signal.

If the portal blood flow is diverted following hepatectomy so that there is no increase in portal blood pressure (and by implication no stimulus to the increase in NO postulated to be the start signal) then there is a delayed growth response of the remnant liver. However, several of the early changes associated with the normal response (such as increases in IL-6, TNFα and PCNA (proliferating cell nuclear antigen) and HGF- though see later) occur much as normal. Thus, even in the absence of increased portal blood pressure, changes do occur in the remnant liver and regeneration does eventually occur. This highlights the complexity of the overall response and of the start signal mechanism in particular. Nitric oxide may turn out to be a component of the ‘start’ signal, but seems unlikely to be the sole initiating factor for regeneration.

**A possible role for bile acids in the 'Start' signal?**

A recent paper in the journal Science described the effects of bile acids on liver regeneration. (Huang et al, 'Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration' (2006). Science 312, 233-236.) The liver produces bile acids which are used in the digestive process in the gastrointestinal tract and are reabsorbed with the digested material and transported back via the portal vein to the liver. The bile acids in the liver itself constitute less than 5% of the total bile acid pool. High concentrations of bile acid in the liver have a toxic effect and cause apoptosis and necrosis, but relatively small increases cause proliferation and an increase in liver growth. Feeding mice a diet containing a low amount of bile salt (0.2% cholic acid) for 5 days caused a 30% increase in the weight of the liver.
The cholic acid binds to bile acid receptors on the nuclear membrane and stimulates liver growth. Furthermore, bile salts affect the rate of regeneration following partial hepatectomy (see diagram below).

**Effect of 0.2% cholic acid diet or 2% Cholestyramine resin on liver growth following partial hepatectomy in wild type mice or mice lacking the nuclear bile acid receptor FXR**

(From Huang et al, 'Nuclear receptor-dependent bile acid signaling is required for normal liver regeneration' (2006). Science 312, 233-236.)

Mice fed the cholic acid diet for 5 days prior to the hepatectomy showed a faster rate of regeneration than the control mice on the normal rat chow. Feeding a diet containing 2% cholestyramine resin (which binds bile salts in the gastro-intestinal tract and results in a lowered blood bile salt concentration) caused a slowing of the regeneration process. In mice lacking the FXR nuclear bile receptor liver growth was strongly inhibited in the early stages of regeneration. Note however that the regeneration rate increases in the FXR/- mice and the liver weight in wild type and knockout mice did not differ at 7 days. The loss of the nuclear bile acid receptor also prevented the acceleration induced by cholic acid and the delayed effect of the cholestyramine. The FXR/- mice also had an increase in mortality (30% ) compared to the wild type (5%). Thus a case can be made for implicating bile salt stimulation of the FXR nuclear receptor in both a 'start' and possibly a 'stop' signal. The removal of 70% of the liver means that the remaining 30% is exposed to an increased bile acid flux from the gut and that this acts through the FXR receptors to stimulate liver growth. As the liver increases in size, the relative flux from the gut decreases and at some point the bile acids cease to stimulate liver growth. This mechanism could also be part of the 'Stop' signal.
THE PRIMING PHASE
The priming phase may be thought of as that part of the process when the previously quiescent hepatocytes exit from the G0 state and re-enter the cell cycle at the level of G1. For the purposes of these lectures the priming phase encompasses events occurring mainly during the first hour of regeneration in the rat 70% hepatectomy model and are summarised in the figure below.

LEGEND: EARLY EVENTS.
The documented events occurring during the first 60 minutes after partial hepatectomy are outlined. It should be remembered that the recorded timings are derived from multiple sources and are limited by the experimental protocols used, and in some cases 30 min was the earliest time point studied. It should also be borne in mind that the division into the separate phases is somewhat arbitrary and it is difficult in most cases to be precise about the exact temporal sequence.

Changes in the urokinase plasminogen activator (uPA) system and extracellular matrix related proteins.
Increases in the activity or amounts of the urokinase-like plasminogen activator (uPA) and its receptor (uPAR), are amongst the earliest documented changes to be seen in the remnant liver following hepatectomy. uPA was originally thought of as part of the fibrinolytic cascade which degrades fibrin and small clots in the circulation. uPA specifically cleaves plasminogen (which is proteolytically inactive) at a Arg-Val bond to generate the active protease plasmin. uPA is now known to have a wider range of actions, including a possible role in metastatic invasion of tissues by tumour cells. In liver regeneration it may be involved in remodelling of the extracellular matrix to allow subsequent cell division, the release of bound single chain form of HGF (and possibly other mitogens) from the ECM and conversion to the active two chain form and the possible activation of a signalling pathway involved in mitosis.

Probably the earliest documented events following partial hepatectomy in rats are increases in uPA (urokinase-like plasminogen activator) activity and in uPAR (uPA receptor) described by George Michalopoulos’ group in Pittsburgh. Increased uPA activity was detected at 1 minute post hepatectomy, and continued to increase up to at least 60 min. When this activity returns to normal levels is less clear. The levels of uPAR (the receptor for uPA) are usually low in normal liver and are not readily detected by Western blotting, but increased uPAR (as detected on Western blots, see diagram below) was seen at 1 min post hepatectomy, and more clearly at 1 hour. This had decreased by 6h and was back to basal levels by 24 h.

**Early Changes in UPAR (Rats)**


The mechanism by which this uPAR appears (presumably on the cell membranes) so quickly following hepatectomy is unclear. The increased uPA activity associated with the liver is
thought to be due to the uPAR binding uPA from the circulating blood and forming a uPA/uPAR complex on the cell surface.

**Early changes in uPA activity (Rats)**

Separate experiments have shown that the enzymatic/proteolytic activity of uPA is increased considerably when it binds to the uPAR receptor. Thus it appears that a very early event in the regeneration process is the localisation of enzymatically active uPA to the cell surface.

Collectively these changes are thought to be associated with, or to initiate alterations in the extracellular matrix which permit the subsequent division firstly of the hepatocytes and then the other liver cell types. uPA is a key initiator of the metalloproteinase cascade leading to matrix degradation. Increased conversion of plasminogen to plasmin has been detected 15 min after partial hepatectomy in rats, and increased fibrinogen breakdown observed at 15 to 30 min. Immunohistochemical staining for fibrinogen and fibronectin shows these proteins decrease in amounts in the periportal regions of the liver 5 to 15 min after hepatectomy, whilst vitronectin and integrin showed little change, suggesting there is a selective degradation of particular ECM proteins early in the regeneration process.

Increases in the inactive forms of the matrix metalloproteinases pro-MMP-2 and pro-MMP-9 have also been reported 30 min after partial hepatectomy in rats, but the active form of MMP-2 is seen 6 to 12 hours later, and MMP-9 is active 3 to 6 hours later.

One may speculate that the degradation of at least some of the extracellular matrix proteins may be necessary for cell division. Freeing the links between the cells and the matrix may be a prerequisite for division and if the cell is too tightly held down then division may physically
impossible. Clearly complete digestion of the extracellular matrix would be problematic and result in complete loss of any liver architecture and result in the equivalent of liver digest for isolated cell production. The matrix digestion and modification thus needs to be carefully controlled. The maintenance of the vitronectin component of the ECM may be significant since PAI-1 (plasminogen activator inhibitor-1, an inhibitor of uPA) binds to a somatomedin-like domain in vitronectin and inhibits uPA-dependent cell adherence and migration. Thus maintenance of a vitronectin binding site for PAI-1 may provide a means of limiting the degradative process.

End of lecture 2