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Inducing and characterizing liver regeneration in mice: Reliable models, essential “readouts” and critical perspectives

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Abstract

Elucidating the molecular circuitry that regulates regenerative responses in mammals has recently attracted considerable attention because of its emerging impact on modern bioengineering, tissue replacement technologies, and organ transplantation. The liver is one of the few organs of the adult body that exhibits a prominent regenerative capacity in response to toxic injury, viral infection, or surgical resection. Over the years, mechanistic insights into the liver’s regenerative potential have been provided by rodent models of chemical liver injury or surgical resection that faithfully recapitulate hallmarks of human pathophysiology and trigger robust hepatocyte proliferation leading to organ restoration. The advent of mouse transgenics has undeniably catalyzed the wider application of such models for researching liver pathobiology. This article provides a comprehensive overview of the most reliable and widely applied murine models of liver regeneration and also discusses helpful hints, considerations, and limitations related to the use of these models in liver regeneration studies.

Keywords

liver; partial hepatectomy; hepatotoxicity; carbon tetrachloride; hepatocytes; proliferation; regeneration

INTRODUCTION

Liver regeneration--Overview

The mammalian liver is unique in its remarkable ability to adapt to environmental perturbations by readily upholding cellular defense programs against noxious chemical and/or biological agents. Because it is continually exposed to blood-borne toxic particles and pathogens, the liver has developed a diversified arsenal of protective and homeostatic mechanisms that help maintain both its metabolic function and anatomic integrity (Taub, 2004). Crucial to its prime role in human physiology and metabolism is the ability of the liver to mount robust regenerative responses following exposure to insults such as acute toxic injury, viral infection, or surgical resection (hepatectomy) (Fausto *et al.*, 2012; Taub, 2004). Any of these stimuli can effectively trigger tightly regulated hepatocyte proliferation and lead to the activation of cellular and molecular programs that repopulate the remnant liver, while maintaining its metabolic integrity throughout the regenerative process. It is

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generally accepted that liver regeneration occurs in response to the coordinated activation of growth factor-regulated and cytokine-driven pathways that prime quiescent hepatocytes and other non-parenchymal liver cells (such as Kupffer cells, sinusoidal endothelial cells, and stellate cells) to re-enter the cell cycle in a synchronous manner and proliferate (Michalopoulos, 2010; Taub, 2004). Hepatocytes constitute the main parenchymal cell population (more than 90% of all liver cells) that undergoes proliferation after hepatectomy and toxic injury, whereas hepatic stem-like cells (*i.e.*, oval cells) are thought to participate in the process by actively proliferating only in cases in which hepatocyte proliferation is blocked or delayed, such as in the presence of toxic agents. Once the liver mass has been restored, further molecular signaling results in termination of the process, with hepatocytes and non-parenchymal cells halting their proliferative activities, exiting the cell cycle, and reacquiring their quiescent phenotype.

The key molecular “players”

Progression through the cell cycle--beyond the initiation phase--requires growth factors. Among the most important growth factors that influence hepatocyte responses both *in vitro* and *in vivo* are hepatocyte growth factor (HGF), transforming growth factor- α (TGF- α), and epidermal growth factor (EGF) (Fausto *et al.*, 2012). The expression pattern of cyclin D1 is thought to be a crucial factor that determines the stage at which replication becomes growth factor-independent and autonomous (Fausto *et al.*, 2012). Among the most important priming factors that instruct quiescent hepatocytes to re-enter the cell cycle and proliferate is an array of cytokine-dependent prosurvival signaling pathways. *In vivo* studies using mouse and rat models of acute liver injury and/or partial hepatectomy have demonstrated that hepatocyte priming requires the concerted action of pro-inflammatory cytokines such as IL-1 β , TNF- α , and IL-6 (Cressman *et al.*, 1996; Taub, 2004), as well as the upstream activation of Kupffer cells through the engagement of innate immune receptors such as the complement anaphylatoxin receptors C3aR and C5aR (Strey *et al.*, 2003). In addition, extensive remodeling of the hepatic extracellular matrix occurs shortly after partial hepatectomy, and several matrix-degrading enzymes have been implicated as regulators of the liver regenerative process.

Research groups have reached a consensus on the main effector pathways and transcriptional programs that are activated in the early phases of liver regeneration. NF- κ B, STAT3, AP-1, and C/EBP β play major roles in the initiation of liver regeneration, driving the transcriptional activation of hepatic immediate-early genes (Taub, 2004). Gel retardation (band-shift or “gel-shift”) assays that probe the activation profile of these transcription factors in liver nuclear extracts are considered a “gold standard” in the field. In addition to describing partial hepatectomy and acute toxic injury protocols to induce liver regeneration in mice, this unit will also provide succinct protocols that are pertinent to probing hepatic transcription factor activation in response to these procedures.

The translational value of animal models of liver regeneration

Preclinical animal models of liver regeneration constitute the basic platform upon which novel molecular targets can be screened and evaluated for further drug development or therapeutic intervention in liver-associated pathologies (Michalopoulos, 2010; Orlando *et al.*, 2011). In this respect, the translational value of applying murine models of liver regeneration to test novel liver therapeutics and assess the impact of diverse effectors in priming hepatocytes to regenerate is well appreciated and multifaceted. The regenerative capacity of the liver has been the subject of intense investigation because emerging insights from its molecular regulation may offer new perspectives and unprecedented opportunities for therapeutic interventions in liver transplantation, such as prolonging donor graft survival and protecting livers from ischemic damage.

Valuable insight into the molecular basis of liver regeneration has been gained from studies employing animal models that largely rely on triggering hepatocyte cell cycle re-entry and regeneration after **chemical liver injury** (acute hepatic failure models) or **surgical resection** (partial hepatectomy models) (Tunon *et al.*, 2009). These studies have consistently been modeled in rodents (rats and mice), and reliable, highly reproducible animal protocols have been developed over the years, with minor modifications by various research groups, to accurately reflect the basic stages that underlie the complex regenerative program of the liver. In this respect, the *2/3 partial hepatectomy* technique, in which approximately 67–70% of the total liver mass is removed, represents the most widely applied platform for characterizing liver regeneration in rodents (Martins *et al.*, 2008). This surgical technique constitutes a keystone experimental procedure for investigating responses that are pertinent to liver pathobiology, such as tumorigenesis, fulminant liver failure, cirrhosis, and chronic infection, as well as general physiological growth responses. The surgical technique was first described by Higgins and Anderson in 1931 in a seminal study that still serves as the gold standard in the field of liver regeneration (Higgins and Anderson, 1931). After 2/3 partial hepatectomy, hepatocytes are the first liver cells to be primed to exit the G₀ phase and undergo sequential cycles of cell division. In the rat liver, DNA replication begins as early as 16 h after resection. After a 70% hepatectomy in the rat, the weights of the liver remnant at 24 and 72 h are 45% and 70% of the original liver weight, respectively. Between 7 and 14 days, the liver volume is 93%, and by day 20 the liver has completely recovered its original volume through hyperplasia of the remaining lobes (Martins *et al.*, 2008).

In models of chemical liver injury, a series of drugs and toxins has been used by different groups to establish a liver phenotype that resembles fulminant liver failure, liver inflammatory fibrosis, or end-stage cirrhosis in humans (Tunon *et al.*, 2009). These hepatotoxic agents have included acetaminophen, D-galactosamine, thioacetamide, concavalin A, bacterial LPS, and the liver toxin carbon tetrachloride (CCl₄). (Jaeschke *et al.*, 2011) The CCl₄-induced model of acute liver injury has been showcased in the literature as a highly reproducible and well-tolerated model for inducing liver regeneration in mice after a single intraperitoneal injection of CCl₄ (Tunon *et al.*, 2009). Such rodent models of liver injury have been instrumental in helping scientists dissect the contribution of various priming factors (such as cytokines, inflammatory stimuli, and bacterial products) in the early stages of liver regeneration (hepatocyte cell cycle re-entry, activation of the early hepatic transcriptional program, and immediate-early gene expression).

The advent of mouse transgenic technology and the exponential growth of high-throughput gene expression profiling platforms, along with the availability of inbred gene “knock-out” mouse strains backcrossed on various genetic backgrounds, have provided significant thrust to the field of liver regeneration. Such genetically manipulated mouse strains have enabled the systematic investigation of various effectors in the regenerative process.

This article will provide a comprehensive overview of some of the murine experimental models that have been developed as reliable methods of inducing and characterizing liver regeneration. In the following sections, we will describe the basic features and experimental steps followed in two widely applied models of liver regeneration: the CCl₄-induced acute hepatic injury model and the 70% partial hepatectomy model, according to the suture ligation protocol (this protocol requires minimal surgical skills and can be easily reproduced in a short time frame). Furthermore, supporting protocols will be provided for evaluating liver regenerative responses in terms of quantifying liver mitotic activity and DNA replication and also for measuring the activation of transcriptional networks that are crucial to hepatocyte priming and cell cycle re-entry.

Since both experimental models require an appreciable understanding of mouse liver anatomy (particularly by a scientist exposed to mouse surgical procedures for the first time) a short introduction on the main features of the rodent liver anatomy is provided below:

Basic liver anatomy: Rat vs. mouse

The mouse liver, like that of the rat, is divided into four main lobes: the caudate lobe (CL), the right lateral lobe (RLL), the median lobe (ML), and the left lateral lobe (LLL) (Figure. 1). The CL is divided into three parts: the caudate process and anterior (AC) and posterior (PC) caudate lobes. The RLL is divided into the superior right lobe (SRL) and inferior right lobe (IRL). The ML is divided into the right median lobe (RML) and the left median lobe (LML). Although the anatomy of the mouse liver has not been described in as great detail as has that of the rat liver, its lobular anatomy is very similar (Cook, 1965). One unique feature of the mouse liver is that it has a gall bladder, which is absent in the rat. In both species, the inferior vena cava traverses the liver parenchyma, allowing its visible segments--protruding from either side of the organ--to be easily identified.

BASIC PROTOCOL 1: Inducing liver regeneration in mice: The 2/3 partial hepatectomy model (suture ligation technique)

The 2/3 partial hepatectomy technique represents the most widely applied platform for inducing liver regeneration and characterizing relevant effector pathways in rodents. This technique was first described by Higgins and Anderson in 1931 in a seminal study that still serves as the gold standard in the field of liver regeneration and receives an increasing amount of citations in relevant studies on an annual basis (Higgins and Anderson, 1931). Higgins and colleagues were the first to demonstrate that surgical removal of 2/3 of the liver lobes in the white rat could stimulate synchronous hepatocyte proliferation, leading to a gradual restoration of liver mass through a process of compensatory liver hyperplasia. After 2/3 partial hepatectomy, hepatocytes are the first cells to be primed into exiting the quiescent state (G_0 phase) and entering the G_1 phase of the cell cycle. Usually, within a time window of 36–48 h after liver resection, most hepatocytes are distributed in the S-phase of the cell cycle and have already initiated DNA replication. This hallmark event in the hepatocyte cell cycle allows for accurate predictions of the regenerative response of hepatectomized mice by means of bromodeoxyuridine (5-bromo-2'-deoxyuridine; BrdU) injection and incorporation of this nucleotide analogue into replicating liver cells. Since partial hepatectomy induces a highly reproducible and synchronous profile of cell cycle re-entry in hepatocytes, it qualifies as an ideal procedure for evaluating liver regeneration at specific time points after surgery. It is also used as a platform to evaluate other replication-dependent responses of the liver in various pathologies. The prototype procedure developed by Higgins *et al.* was eventually adapted to the mouse (Yokoyama *et al.*, 1953); despite the fact that mice and rats share several similarities in liver anatomy (lobular architecture), the unique features presented by these two model organisms have resulted in discrepancies noted in studies by different research groups. The partial hepatectomy procedure includes careful steps of preparation before the actual surgery, a specific protocol for inducing mouse sedation, and a reproducible method of surgical resection of the liver lobes that can be performed by any laboratory member that has previously gone through a training session and an ample observation period.

Particular care has to be taken beforehand to ensure that all animal protocols fully comply with Institutional (IACUC) and National Ethics Regulations, relevant legislation, and Guidelines on the Humane Use of Experimental Animals in Research.

Materials and Reagents

8- to 14-week old mice
Isoflurane for anesthesia (or, alternatively, ketamine/xylazine solution)
Ophthalmic ointment (Fougera)
Iodine/Betadine
70% (vol/vol) Ethanol
0.9% (wt/vol) NaCl solution (sterile saline) or sterile PBS
Surgical instrument cleaner
95% (vol/vol) Ethanol

Equipment

Isoflurane vaporizer (for mouse anesthesia; SurgiVet)
Germinator for instrument sterilization (between surgeries)
Gaymar water pump for heated bed (Kent Scientific)
The use of a water pump with circulating heated water minimizes the rodents' heat loss during surgery and aids post-surgical recovery.
1 Autoclavable case or tray for sterilizing surgical instruments
1 Petri dish
2 Small beakers (~50 mL)
1 15- or 50-mL conical tube for saline/PBS
1 Plastic or styrofoam pad for prep area bed
2 Paper towels per surgery
Tape
2 3-mL syringes
1 Sub-Q or 27-30G1/2 needle
1 Small chamber (*e.g.*, a dessicator vessel) for initial rodent anesthetization
1 Electric razor for shaving mouse fur
4 Cotton swabs per surgery
1 Small tube or thin gauze roll (~12 × 75 mm)
1 Sterile surgical drape per surgery
2 Curved microdissecting forceps (Roboz)
1 Operating scissors (Roboz)
3 Hemostatic forceps (Roboz)
2 Pads or rolled-up aluminum foil (~2 × 2 × 10 cm) to prop up hemostatic forceps
1 Sterile cotton swab per surgery
1 Microdissecting scissors (preferably with curved blades)

- 5-0 Silk suture (Roboz) for occluding blood flow to liver lobes (supplied as a spool)
- 1 Sterile gauze pad per surgery
- 1 Needle holder (Roboz)
- 1 5-0 silk suture (Roboz) with attached 12-mm cutting needle for muscle and skin closure
- 2 Trays for washing instruments

Preparation

- 1 From the spool, cut 2 pieces of 5-0 silk thread~10 cm long for each surgery as sutures for resection and place them in the Petri dish.
- 2 Autoclave sutures for resection, small tube, and surgical instruments for 20 min at ~120°C. Sterilize the small tube with soap and ethanol if it is not autoclavable.
- 3 Turn on the Gaymar water pump for the heated bed and the Germinator for instrument sterilization (*each takes ~30 min to warm up*). Tape a paper towel to the heated bed so the mouse does not lie directly on the plastic. Also tape a paper towel to the prep area bed.
- 4 Check that enough isoflurane is present in the dispenser and that the air pressure is appropriate (usually ~1.75 L/min).
- 5 Fill a small beaker with iodine/betadine and a second beaker with 70% ethanol (~5–10 ml each). Fill a conical tube with sterile saline/PBS (~5 mL per surgery).
- 6 BEFORE EACH SURGERY - Fill two 3-mL syringes (without needles) with 2 mL each of sterile saline/PBS from the conical tube, and add a Sub-Q or 27-30G1/2 needle to one of them. Keep the tips of the syringes/needle sterile.

Surgery

- 7 Weigh the mouse and anesthetize it with isoflurane by placing it into the chamber with a vaporizer setting of ~3.5%.
- 8 Once the mouse is anesthetized (slow breathing and no movement), transfer it to the prep area and put its nose into a nose cone into which isoflurane flows. Reduce the vaporizer flow to ~2–2.5%. Make sure the mouse does not have problems breathing during the procedure.
- 9 Carefully apply ophthalmic ointment to the eyes to keep them from drying out.
- 10 Shave the abdomen (cut in the direction of hair growth except on the last pass—in that case, go against the hair growth).
- 11 Apply iodine/betadine using a cotton swab, starting from the midline of the abdomen toward the edges (Fig. 2A). Apply a second time, using a new cotton swab, then swab twice with 70% ethanol. Remove any loose fur from the mouse.
- 12 Move the mouse to the heated bed (surgery area) and place its nose into a nose cone into which isoflurane flows. Use two pieces of transparent tape to tape the mouse's front legs down onto a paper towel on the heated bed (*so they are reaching towards the side; do not pull tightly*). Place the small tube or thin gauze roll under the mouse so that the sternum is pushed up (creating easier access to the liver). Use two separate pieces of tape to hold down the back legs. Keep the feet flat so that the toe reflex can be used to test for unconsciousness

(step 13). Place a surgical drape over the mouse. Reference Fig. 2B to see how the mouse should be prepared in this step.

- 13 Press firmly on the mouse's back toes with your fingernails to test for the toe pinch reflex. Once the mouse is completely unresponsive (it does not twitch when its toes are pressed), it is ready for surgery.
- 14 Record the starting time. Grab the skin with the curved forceps near the middle bottom of the abdomen (approximately at the level of the back legs) and cut straight up the middle of the abdomen with the operating scissors (do not cut the muscle). Cut up to and slightly above the xiphoid process (white lump near the sternum area, under the muscle). The extent of the skin incision can be seen in Fig. 2B.
- 15 Grab the muscle with the curved forceps just below the xiphoid process and tug up slightly. The linea alba (white line running down the middle of the abdomen) should be visible (Fig. 2B). Make a small incision just below the forceps on this line. Turn the scissors around and cut along the linea alba to the bottom of the skin incision (if done properly, the muscle should not bleed). Pull up gently on the muscle while cutting to avoid hitting any organs. Next, cut above the initial muscle incision to the bottom of the xiphoid process. Carefully cut the muscle on top of the xiphoid process (do not cut the xiphoid process itself), if desired, to allow slightly better access to the abdominal cavity.
- 16 For a sham surgery, place a small piece (~2 cm) of 5-0 thread on the liver (to account for potential biomaterial reactions related to the presence of the sutures in the partial hepatectomy procedure), record the time, and skip to step 28.
- 17 Clamp a hemostatic forceps to the very edge of the skin near the top-middle of the incision on each side. Support the forceps by laying it on the pad or rolled-up aluminum foil placed on the side of the mouse. Clamp a third forceps to the top of the incision (above the xiphoid process) and lay it on the nose cone apparatus. See Fig. 2D for the placement of the hemostats.
- 18 The two largest lobes of the liver (median and left lateral lobes; Fig. 2C) that will be resected should be plainly visible.
- 19 Use the sterile cotton swab to gently pull the liver forward (the swab can be moistened with saline/PBS if desired). The top membrane (falciform ligament) should be visible (Fig. 2D, E). Cut the top membrane completely (be sure not to cut the diaphragm) with the microdissecting scissors. Use the swab to push the left lateral lobe up so that it sticks to the top of the abdominal cavity. Move this lobe or press gently on the stomach until the membrane connecting the left lateral lobe to the small caudate lobe below it is visible (Fig. 2F, G). Cut this membrane.
- 20 Optional: Cut the membrane connecting the back part of the left lateral lobe to the main liver stem (near the diaphragm). This membrane is hard to see. When it is cut, the tip of the lobe near the back pops up. This membrane can be left uncut without affecting the outcome of the surgery.
- 21 With the left lateral lobe pushed up so it is separated from the rest of the liver, place the center of a 5-0 suture on the liver stem between this lobe and the caudate lobe. Use the cotton swab and/or forceps to gently wrap the suture upward around the left lateral lobe (Fig. 2H). Pull the lobe down so the suture is now lying on top. Make sure the suture is wrapped only around the stem of the lobe. Tie the suture in a knot, using one pair of forceps to loop one end of the

suture around top end of a second pair of forceps. Grab the end of the free suture with the second pair of forceps, pull it through the loop and tighten the knot (Fig. 2I). Repeat this step, tying from the opposite direction to create a square double knot (Fig. 2J). Note the time. Hold the ends of the suture with forceps and cut off the excess suture close to the knot.

- 22 Repeat step 21 to wrap a second piece of 5-0 suture around the median lobe. Place the suture between it and the right lateral lobe (Fig. 2K). The suture should be placed slightly forward on the median lobe so some tissue is left between it and the stem of the lobe when the median lobe is pulled back down (i.e., when viewing the suture on top of the lobe; Fig. 2L); if the suture is too close to the stem, ischemia can result. After the suture is wrapped around the lobe but before tying the knot, pull on it gently and make sure both ends lie parallel to each other to ensure proper placement. Tie the knot (Fig. 2M), remove the excess suture, and note the time. Use the median of the difference between the times when each lobe was sutured to get the time of resection (i.e., if there was an interval of 4 min between resections, add 2 min to the time the left lateral lobe was resected to get the average time of resection).
- 23 Use the forceps to gently grab the median lobe and carefully resect it above the knot with the microdissecting scissors, leaving a small remnant piece (Fig. 2N). Repeat this procedure for the left lateral lobe (Fig. 2O). Be sure not to cut the knot or cut below it, or to rip the lobes with the forceps.
- 24 Make sure there is no bleeding from the remnant pieces or other areas. Slight bleeding may be stopped by gently holding the cotton swab on the area until a clot forms.
- 25 Gently rinse the abdominal cavity with 2 mL saline/PBS (from the 3-mL syringe without a needle). Gently press the sterile gauze pad over the abdomen to absorb as much saline/PBS as possible.
- 26 Remove the hemostatic forceps on the sides, or cut the skin around them to remove them (so no necrotic tissue is left). Unclamp the hemostatic forceps, holding the skin above the xiphoid process (it is difficult to cut away this skin without causing more harm).
- 27 Use the 5-0 suture and needle to close the muscle layer. Grab one side of the muscle near the top of the incision with the forceps and insert the needle through the muscle using the needle holder. Grab the other side and insert the needle through. Pull the suture through, leaving a small amount at the end. Loop the long end of the suture (with the needle) around the top of the forceps, then loop it again. Grab the short end of the suture with the forceps and pull it through the loop to tighten the knot (surgeon's knot). Loop the long suture around the forceps in the opposite direction, but only once, and pull the short end through the loop (double knot). Cut off the short end of the suture (excess) close to the knot. Do not cut the end with the needle, so the muscle can be closed with continuous sutures.
- 28 Insert the needle into same side of the muscle initially used in step 27, slightly below the knot. Then insert it through the muscle on the other side and pull the suture all the way through until it is tight (Fig. 2P).
- 29 Repeat step 28 until the bottom of the incision is reached. For the last stitch, leave a small loop of suture when pulling it through (i.e., do not tighten it completely). Tie the knot as in step 28, using the small loop as the "short end of the suture". Cut off both pieces of the suture near the knot (Fig. 2Q).

- 30** Repeat steps 28 through 30 for the skin layer (Fig. 2R). Note: *Do not leave loose ends when cutting the suture, or the mouse may chew on them and pull out the stitches.* Alternatively, staples can be used to close the skin incision (usually 3–4). Record the ending time.

The entire surgical procedure from the initial incision until closure of the skin should take ~15–20 min if done well.

- 31** When finished closing the incision, untape the legs and turn the mouse over. Inject 2 mL of saline/PBS subcutaneously (using the syringe with the needle) by pulling up on the skin above the scapula and inserting the needle directly under the skin layer.

- 32** Pick up the mouse, supporting its abdominal area (*i.e.*, not by the tail) and place it in a clean cage (*paper towels can be put in the cage temporarily to cover the bedding, but remove them when the mouse is walking normally*). The mouse should wake up within 5–10 min if not sick and if the surgery went well.

A heat lamp should be placed over the cage for warming for up to 1 h post-surgery, but leave part of cage covered to allow the mouse to escape from the lamp if it gets too hot.

- 33** Weigh the removed liver lobes and discard or process them as desired (*e.g.*, place them in 10% formalin for fixation or freeze them in liquid N₂ to make extracts at a later date).

- 34** Place the instruments in a tray with surgical instrument cleaner, let them sit at least 2 min, and rinse them with dH₂O. Place them briefly in a second tray of 95% ethanol.

Once they are dry, sterilize them in the Germinator beads for 30 s–1 min if they are needed again that day. Otherwise, put them back in their case for autoclaving.

BASIC PROTOCOL 2: The CCl₄-induced acute liver injury and regeneration model

In addition to the most widely applicable model of liver regeneration, 70% partial hepatectomy, another murine model produces a similar proliferative phenotype in resting hepatocytes and induces a robust and coordinated regenerative response in the liver parenchyma: the *CCl₄-induced acute liver toxicity model* (Doolittle *et al.*, 1987). While partial hepatectomy induces liver regeneration in the absence of pronounced inflammation, the CCl₄ model is characterized by a prominent inflammatory response in the liver that is triggered by acute toxic damage and widespread hepatic necrosis and is perpetuated by rigorous matrix remodeling.

CCl₄ has been widely used as a means of inducing chronic liver damage, especially as a model of primary hepatic cirrhosis (Tunon *et al.*, 2009). Repetitive weekly injections of CCl₄ in mice or rats, over a period of 5–6 weeks, result in the development of pronounced liver fibrosis and steatosis (lipid accumulation) and gradually reproduce a phenotype closely resembling human cirrhosis and end-stage liver failure (Kovalovich *et al.*, 2000). However, a single injection of CCl₄ can effectively trigger liver regeneration in response to primary parenchymal necrosis, while still allowing liver restoration. This mild dosage of CCl₄ is associated with limited morbidity-mortality (less than 10%) and allows the animal to fully recover from liver damage after a period of 7–10 days. The mechanism by which CCl₄ mediates acute liver injury involves the generation of very reactive [–CCl₃] species through

the catabolism of CCl₄ in the endoplasmic reticulum of hepatocytes and the concerted action of oxidases and isoenzymes of cytochrome P450 (Johnston and Kroening, 1998). The accumulation of such reactive species within hepatocytes causes extensive oxidative damage to proteins and lipids in various membranous structures and leads to rapid parenchymal necrosis that is prominently located around hepatic vessels.

The following section describes the basic steps of the protocol for the induction of liver toxic damage by a single intraperitoneal injection of CCl₄ into C57BL/6 mice (the preferable strain for such studies because of its moderate susceptibility to CCl₄) (Bhathal *et al.*, 1983).

Materials and Reagents

Mice, 14–16 weeks old, (preferable strain: C57BL/6, gender: female)

CCl₄ (Sigma; avoid breathing in vapors)

Mineral oil

70% Ethanol

Distilled water (dH₂O)

100% Ethanol

Equipment

1 15- or 50-mL conical tube for mixing CCl₄ and mineral oil

1 Hamilton microliter syringe with a 0.25–0.5 ml calibrated barrel and 22S/2”/2 needle

1 Gauze pad

3 50-mL conical tubes for holding solutions to wash the syringe

Carbon tetrachloride (CCl₄) injection

1. Weigh all animals to be injected on the same day.
2. Calculate the amount of CCl₄ needed for each animal to receive 2 µL/g of body weight.
3. Make up a 1:1 solution of CCl₄:mineral oil (*i.e.*, equal volumes of each) to a slightly greater volume than the amount calculated (*i.e.*, prepare some extra in order to ensure that there is enough for all injections).
4. Wipe the needle of the Hamilton syringe with a gauze pad wet with 70% ethanol.
5. Fill the Hamilton syringe with a small amount of the CCl₄:mineral oil solution and remove air bubbles (*very small bubbles may still be present*). Continue filling the syringe until it contains the amount of solution needed.
6. Inject the animal by grabbing the mouse by the scruff behind the neck and turning it over so it is upside down and lying on its back in your palm. Hold the tail (and optionally the right hind leg) between your ring finger and pinky to expose the abdomen. Insert the needle of the syringe (*beveled side up*) under the skin near the left hind leg (slightly above it and to the left). Push the needle into the skin parallel to the mouse's abdomen. The needle should be visible under skin and should not pass through the muscle yet. Press the needle down on the muscle at a ~10° angle so that a small dimple forms. Carefully push the tip of needle through the muscle. When the needle goes through, the dimple should disappear, and the needle should

give more easily. Make sure not to push the needle in too far or to insert it at too large an angle, or organs may be punctured. Quickly inject the CCl₄:mineral oil. Slowly pull out the needle until it is out of the muscle but still under the skin. Move it around slightly a few times and slowly withdraw it from the skin.

No leakage of CCl₄:mineral oil should occur. If leakage does occur, the animal should not be used, since the dose of CCl₄ will be inaccurate.

7. If the syringe will soon be used for another injection, it can be cleaned by wiping the needle with a 70% ethanol-wetted gauze pad before performing the next injection.
8. When all injections are finished and the syringe is to be stored for an extended period, clean it by washing it with the following solutions (*i.e.*, fill the syringe with each solution and squirt it out):

a) 2 × soapy dH₂O   b) 2 × dH₂O   c) 2 × 100% ethanol

SUPPORT PROTOCOL 1: Harvesting mouse organs and blood

This section outlines the basic steps that need to be followed when harvesting organs and blood from experimental mice subjected to partial hepatectomy or CCl₄-induced toxic injury. Since most of the protocols described in this unit rely on the use of freshly harvested liver tissue, it is advisable to follow a uniform procedure, such as the one outlined below, that allows the simultaneous harvest of multiple organs to be used for measuring different readouts in a single experiment (*i.e.*, partial hepatectomy or CCl₄ acute liver injury study). The procedure should be streamlined to maintain consistency among animals, and the steps should be performed as quickly as possible in order to avoid exposing the harvested organs to enzyme-mediated degradation and further deterioration. Furthermore, this section provides a reproducible protocol for collecting mouse serum or plasma for various liver-associated assays such as liver enzyme quantification and other liver-associated metabolic measurements, including measurements of liver acute phase proteins that are rapidly deactivated upon the formation of a fibrin clot.

Reagents

Isoflurane for anesthesia (or, alternatively, ketamine/xylazine solution)
 70% Ethanol
 10% Formalin or 0.9% NaCl (for liver perfusion)
 0.5 M EDTA (if plasma is desired and no coated tubes are available)

Equipment

1 Plastic or styrofoam pad
 Tape
 1 1-mL syringe with 25G3/8 needle
 1 10-mL syringe with 25G3/8 needle
 1 Operating scissors
 1 Microdissecting forceps
 2 Hemostatic forceps
 1 Microdissecting scissors

1 Microtainer tube for blood collection per mouse (*with or without a serum separator or EDTA coating, depending on whether plasma or serum is desired; a microfuge tube can be used as an alternative*)

1. Anesthetize the mouse with isoflurane and tape it to the table, as described for partial hepatectomy above. When the mouse is completely unconscious (*i.e.*, no reaction when toes are pressed with fingernails), wipe the abdomen with 70% ethanol.
2. If only blood is needed, a 1-mL syringe with a 25G5/8 needle can be inserted at a 45° angle into the right side of the chest, pointing towards the sternum, to pierce the heart. If desired, an incision can first be made in the skin to remove a flap and allow better visibility, as the location of the heart should then be more easily obtainable by noting palpitations in the chest muscle from its beating. Approximately 1 mL of blood should be recovered (the mouse has total of ~2–3 mL), unless the mouse has recently undergone a procedure resulting in some blood loss, such as partial hepatectomy. Note that if plasma is needed and no coated tubes are available for step 6, the syringe should first be filled with 1/10 the expected final blood volume of 0.5 M EDTA (*e.g.*, 100 μ L if 1 mL blood is expected to be collected), to give a final concentration of 0.05 M EDTA after blood collection. Skip to step 6.
3. If tissues are also desired, it is advisable to collect them before collecting blood (*i.e.*, skip step 2). Grab the skin with the microdissecting forceps and cut an incision from the bottom of the abdomen to the top with the operating scissors. Cut a similar incision in the muscle and use the hemostatic forceps on each side to hold the incision open. Cut a third incision through the skin and muscle from the sides of the original incision (slightly below the middle) out towards the sides of the mouse to create flaps of skin that can be further opened for better organ exposure.
4. Use a 1-mL syringe with a 25G5/8 needle to withdraw blood from the inferior vena cava (*the large vein running down the middle of the abdomen from the liver*). Insert the needle, beveled side up, into the vein at a flat angle, ~1–2 cm below the liver. Slowly withdraw blood into the syringe, being careful not to remove the needle until blood can no longer be collected. Note that if plasma is needed and no coated tubes are available for step 6, the syringe should first be filled with 1/10 the expected final blood volume of 0.5 M EDTA, as described in step 2.
5. Alternatively, especially if enough blood cannot be obtained from the vein, blood can also be taken from the right ventricle of the heart after cutting through the diaphragm and rib cage (*the ventricle is light in color because of oxygenated blood and is in front of the atrium, which is the dark, floppy anterior chamber*), or by following the procedure in step 2.
6. Remove the needle from the syringe and transfer the blood to a Microtainer tube. If no Microtainer tube is available, transfer the blood to a microfuge tube (Microtainer tubes generally result in slightly more serum/plasma being recovered, with less chance of contamination). Close the tube and let the blood sit at room temperature for 30 min to clot if serum is desired, or place it immediately on ice for plasma. Proceed with tissue harvesting if desired, otherwise skip to step 10.
7. Perfuse the liver with 10% formalin or 0.9% NaCl (*if collecting tissue for extracts*), if desired, using a 10-mL syringe with a 25G5/8-gauge needle. If

blood was not collected from the vena cava, cut a small hole in it for the perfusate to exit. Insert the needle into the right ventricle of the heart and slowly inject ~3 mL of solution (*this should perfuse the lungs and the abdominal organs*). Transfer the needle to the left ventricle and inject the remaining solution to continue to perfuse the abdominal organs.

8. If collecting tissue for extracts, cut off the required piece of liver with microdissecting scissors, put it in screw-cap tube, and freeze it immediately in liquid N₂. Harvest other organs (*e.g.*, kidney, spleen, pancreas, duodenum, heart, lungs, thymus), as desired. If BrdU incorporation is being measured in the liver (see below), it is advisable to collect part of the duodenum, since this can be used as a positive control for cell proliferation during the assay. Note that the spleen and pancreas can be harvested simultaneously. The heart, lungs, and thymus can be harvested simultaneously by grabbing the heart with forceps and cutting above the thymus down into the thoracic cavity, and then cutting the ligament underneath all three organs. Place the harvested organs in 10% formalin as they are removed, for further fixation for at least several hours.
9. Turn off the isoflurane dispenser and dispose of the mouse carcass appropriately.
10. To process the blood (after clotting if serum is desired), spin it in a microcentrifuge at maximum speed for 4 min if Microtainer tubes were used. If blood is in a microfuge tube instead of a Microtainer tube, spin it at ~800 × g for 10 min. Keep the microcentrifuge at 4°C if processing the blood for plasma. Transfer the supernatant (serum/plasma) to a new microfuge tube. The supernatant should be clear. *A red supernatant indicates hemolysis, and yellow indicates icteric blood (a sick animal).* If blood was spun in a microfuge tube instead of a Microtainer tube, it may need to be spun again after transferring the supernatant to a new tube, in order to remove any red blood cells/particles still remaining.
11. Store serum/plasma and frozen tissues at –70°C. Avoid repeated freeze-thawing. Fixed tissues can be stored at room temperature in 10% formalin for several days, after which they should be transferred to PBS and stored at 4°C until they are processed into slides.

SUPPORT PROTOCOL 2: Quantifying hepatocyte cell cycle re-entry by means of BrdU immunohistochemistry

BrdU is a synthetic nucleotide that is an analogue of thymidine. BrdU is commonly used for the detection of replicating cells in various tissues (Cressman *et al.*, 1996). This nucleotide analogue can be incorporated into the newly formed DNA of the replicating cell during the S-phase of the cell cycle. This incorporation is stable and can subsequently be detected and quantified with the use of a specific anti-BrdU antibody in immunohistochemical staining of paraffin-embedded tissue sections. It has been well documented that after 70% partial hepatectomy, a wave of synchronous hepatocyte cell cycle re-entry sweeps through the remaining liver parenchyma, peaking at approximately 36–48 h post-surgery (Fausto *et al.*, 2012). This prominent regenerative activity can be reliably measured by means of BrdU incorporation into proliferating liver cells (S-phase-gated, BrdU-positive nuclei). This procedure includes: A) a single intraperitoneal injection of BrdU into the animals 1–2 h prior to liver harvest and B) performance of BrdU immunohistochemistry in paraffin-embedded liver sections harvested 36–48 h after partial hepatectomy or CCl₄ administration.

Materials and Reagents

Slide with liver and intestinal tissue, previously fixed with 10% formalin
BrdU (Sigma)
Sterile PBS or 0.9% NaCl (saline) for injections
PBS, pH7.4 (500 mL – 1L) for immunohistochemistry
Horse serum
Xylene
95% Ethanol
80% Ethanol
70% Ethanol
Citric acid buffer (see REAGENTS AND SOLUTIONS)
Methanol
30% Hydrogen peroxide (H₂O₂)
Anti-BrdU antibody (Millipore, clone AH4H7-1/131-14871, cat. #MAB3424)
PBT (see REAGENTS AND SOLUTIONS)
Horse anti-mouse antibody (Vector, cat.# BA-2000, rat-adsorbed)
Histomount

Equipment

1 1-mL syringe with 25–27G3/8 needle
Slide chamber(s)
Large tray or Petri dish for humidified chamber (preferably one commercially available)
Serological pipettes (if commercially available, humidified chamber is not used)
3M Whatman paper
1 Plastic slide rack
1 1-L plastic beaker
Kimwipes (Kimtech) or similar tissue paper
1 Vacuum trap flask with attached Pasteur pipette
1 Hydrophobic ImmunoPen (Calbiochem)
1 Avidin/Biotin Blocking Kit (Vector)
Parafilm (if blocking slides overnight)
1 Vectastain Kit (Vector)
1 DAB Substrate Kit (Vector)
Hematoxylin stain, Gill's Formulation #2 (optional for DNA counterstaining)
Slide coverslips

A. Bromodeoxyuridine (BrdU) injection

1. Weigh all animals to be injected on the same day.
2. Adjust the concentration of the BrdU solution so that no more than 1 mL of solution is injected per animal. Assuming that each animal receives 50 mg/kg BrdU, the concentration should be 2 to 3 mg/mL.
Example: 25 g animal = 0.025 kg → 50 mg/kg of BrdU × 0.025 kg = 1.25 mg BrdU to be injected → If a 2 mg/mL solution of BrdU is used, then 1.25 mg/(2 mg/mL) = 0.625 mL to be injected.
3. Calculate the amount of PBS or 0.9% NaCl (best) that is needed for each animal to receive the proper amount of BrdU (*i.e.*, the total of all amounts of the solutions to be injected for each animal calculated in step 2).
4. Dissolve the BrdU in this amount of solution (it is advisable to make slightly more than the amount calculated, in order to account for any loss during syringe preparation) and rotate the tube at room temperature for at least 30 min to ensure complete resuspension.
5. Fill the 1-mL syringe with slightly more than the amount of BrdU solution needed. Tap the syringe firmly while holding it straight up (needle pointing up) to shake bubbles to the top. Squirt out the bubbles and BrdU solution until the appropriate amount is left in the syringe.
6. Inject the animal 1–2 h before harvesting blood and/or organs by grabbing the mouse as described in step 6 of the CCl₄ injection protocol above. Inject BrdU intraperitoneally into the left abdomen by inserting the needle at a ~10° angle near the left hind leg (above it and to the left). If desired, the needle may be inserted first under skin, then through the muscle (see step 6 of the CCl₄ protocol). *Be sure not to insert the needle too deeply, or organs may be punctured.* No leakage should occur when the needle is slowly withdrawn.

B. BrdU Immunohistochemistry (*citric acid denaturation method*)

Preparation—Amounts given here and throughout the protocol are for the use of 1 large incubation chamber (100-mL capacity, for 6–10 slides); amounts in parentheses are for the use of a smaller incubation chamber (50-mL capacity, for 5 slides or less).

- 1 Prepare horse serum/PBS: 80 μ L of horse serum (4% final) in 2 mL of PBS.
- 2 If blocking the slides overnight, mix 4 mL (2 mL) of horse serum in 96 mL (48 mL) of PBS.
- 3 Prepare a humidified chamber:
Use a commercial chamber or large plastic tray (or large Petri dish for fewer than 5 slides) and line the bottom with 3M Whatman paper. Moisten the paper with water or PBS and dump out any excess liquid. If a non-commercial chamber is used, place serological pipettes on the Whatman paper to support the slides so they don't lie on the paper. Cover the chamber with a lid during incubations.

Procedure: Rehydration (perform this step in a hood, recycle the solutions, and use separate chambers for xylene, leaving them in the hood to air dry):

- 4 Incubate the slides in xylene 3 times for 5 min each (3 × 5 min). Replace the solution after each incubation.

- 5 Incubate the slides in 95% ethanol 2×5 min.
- 6 Incubate the slides in 80% ethanol 1×5 min.
- 7 Incubate the slides in 70% ethanol 1×5 min.
- 8 Rinse the slides in running dH₂O for 5 min.

Denaturation

- 9 Place slides in a plastic slide rack (every other space) and immerse them in 700 ml of citric acid buffer in a 1-L plastic beaker. Heat them in a microwave at full power for approximately 13 min, which should allow about 7 min to reach boiling, followed by a 6-min incubation.

This step enhances the availability of antigens by breaking aldehyde cross-links and denaturing dsDNA, which is released from the histones so that BrdU is exposed. It also quenches widespread endogenous alkaline phosphatase activity.

- 10 Remove the beaker from the microwave and allow the solution to cool at room temperature for 10 min.
- 11 Rinse the slides in running dH₂O for 10 min.

Peroxidase Quench (to quench endogenous tissue peroxidase activity)

- 12 Incubate the slides for 10 min at room temperature in freshly mixed 95 mL (47.5 mL) methanol + 5 mL (2.5 mL) 30% H₂O₂.
- 13 Rinse the slides in running dH₂O for 5 min.

Blocking Step

- 14 Blot the slides dry by wiping off the back of each slide with a Kimwipe or similar tissue paper and letting the front drain (hold the slide vertically), then quickly drying the slide by dabbing around the tissue. Use a vacuum (a Pasteur pipette attached to a vacuum trap flask) to remove the liquid around and between the tissue, but do not let the tissue dry out.
- 15 Use a hydrophobic pen to outline the tissue on each slide.
- 16 Immediately add 2–3 drops of undiluted Avidin Blocking Solution. Do not let the tissue dry out. Make sure all of the tissue is covered.
- 17 When all of the slides are ready, incubate them for 15 min at 37°C in the humidified chamber.
- 18 Rinse the slides briefly in PBS.
- 19 Blot each slide dry as in step 1 above and immediately add 2–3 drops of undiluted Biotin Blocking Solution. Do not let the tissue dry out. Make sure all of the tissue is covered.
- 20 When all of the slides are ready, incubate them for 15 min at 37°C in the humidified chamber.
- 21 If the slides will be blocked overnight, add them to a slide chamber containing horse serum/PBS. Cover the chamber with Parafilm and incubate the slides at 4°C (*i.e.*, in a cold room or refrigerator) overnight. Skip to Primary Antibody Incubation. Otherwise, rinse the slides briefly in PBS and continue with step 9.

- 22 Blot each slide dry and immediately add horse serum/PBS (100 μ L or enough to cover the tissue). Do not let the tissue dry out.
- 23 When all of the slides are ready, incubate them for 20 min at 37°C in the humidified chamber.

Primary Antibody Incubation

- 24 Dilute anti-BrdU primary antibody in PBT at a 1:500 dilution.
- 25 Blot each slide dry (DO NOT RINSE) and immediately add antibody (100 μ L or enough to cover the tissue). Do not let the tissue dry out.
- 26 When all of the slides are ready, incubate them for 45 min at 37°C in the humidified chamber.
- 27 Rinse the slides in PBS 2 $\times$ 5 min at room temperature.

Secondary Antibody Incubation

- 28 Dilute biotinylated horse anti-mouse secondary antibody in PBT at a 1:200 dilution.
- 29 Blot each slide dry and immediately add antibody (100 μ L or enough to cover the tissue). Do not let the tissue dry out.
- 30 When all of the slides are ready, incubate them for 30 min at 37°C in the humidified chamber.
- 31 Prepare ABC Reagent while the slides are incubating:
Add 1 drop of Reagent A from the Vectastain kit to 2.5 mL of PBS, followed immediately by 1 drop of Reagent B. Mix the solution and incubate it for 30 min at room temperature.
- 32 After the incubation with secondary antibody, rinse the slides in PBS 2 $\times$ 5 min at room temperature.

Antibody Labeling

- 33 Blot each slide dry and immediately add ABC Reagent (100 μ L or enough to cover the tissue). Do not let the tissue dry out.
- 34 When all of the slides are ready, incubate them for 30 min at *room temperature* in the humidified chamber.
- 35 Prepare Substrate while the slides are incubating:
Add 1 drop of Buffer Stock Solution from the DAB Substrate Kit to 2.5 mL of dH₂O and vortex for 10 s to mix. Then add 2 drops of DAB Stock Solution and vortex another 10 s to mix. Make sure the solution is yellowish-brown and not purple. If the solution is purple, make it again using less DAB. Finally, add 1 drop of Hydrogen Peroxide Solution and vortex for 10 s to mix. If a gray-black stain is desired instead of reddish-brown, add 2 drops of Nickel Solution and vortex 10 s to mix (addition of Nickel Solution is optional).
- 36 After the incubation with ABC Reagent, rinse the slides in PBS 2 $\times$ 5 min at room temperature.

Detection

- 37 Blot each slide dry and immediately add DAB Substrate (100 μ L or enough to cover the tissue). Do not let the tissue dry out.
- 38 Incubate the slides for 5 min at room temperature (no humidified chamber). Start timing as soon as DAB is added to the first slide and let each slide incubate for 5 min (*i.e.*, time each slide separately).
- 39 After 5 min, move each slide to a slide chamber filled with dH₂O. When all of the slides are done incubating, rinse them in running dH₂O for 5 min.

Counterstain (Optional step that stains non-replicating DNA blue)

- 40 Dip the slides briefly (~10 s) in 200 mL of filtered hematoxylin stain (note that used solution can be recycled). Dip the slides briefly in dH₂O to rinse them, dump out the dH₂O, and repeat the rinse 2 or 3 times until no more stain is present in the dH₂O.

Dehydration—*Perform this step in a hood, recycle the solutions, and use separate chambers for xylene, leaving them in the hood to air dry*:

- 41 Incubate the slides in 70% ethanol for 1 min.
- 42 Incubate the slides in 95% ethanol 3 $\times$ 1 min.
- 43 Incubate the slides in xylene 2 $\times$ 3 min.
- 44 Place the slides on a paper towel (in a hood), but do not let the slides dry out.
- 45 Add a coverslip by adding a small amount of Histomount to the slide over the tissue section. Hold the coverslip so one edge is against the slide below the tissue and drop it gently so it covers the tissue. Flip the slide over and gently press near the ends of the coverslip to spread the Histomount around the tissue (the tissue should be covered). Avoid bubbles (remove them by pressing down on the coverslip to push them out or using forceps to do so by pressing near the bubble and gently closing the forceps to push it out). Let the Histomount dry several hours to overnight.

SUPPORT PROTOCOL 3: Preparation of nuclear protein extracts from mouse liver

Characterizing the regenerative response of the liver after partial hepatectomy or acute liver injury relies on a series of *in vitro* assays using freshly prepared mouse protein extracts. A focal point in all liver-related protocols is the quantification of early hepatic gene expression in terms of transcription factor activation (Fausto, 2000). Active transcription factor complexes are recruited from the cytoplasm, enter the cell nucleus, and associate with target gene-promoter regions. Therefore, the reliable quantification of their activity requires obtaining highly enriched nuclear protein extracts from liver cells. This section describes the basic steps that need to be followed to prepare nuclear extracts from mouse liver.

Materials and Reagents

- Mouse liver tissue portion (snap-frozen)
- PBS
- Homogenization buffer (see REAGENTS AND SOLUTIONS)
- 95% Ethanol

Distilled H₂O (dH₂O)

Buffer C (see REAGENTS AND SOLUTIONS)

Buffer D (see REAGENTS AND SOLUTIONS)

Protease and phosphatase inhibitors (see REAGENTS AND SOLUTIONS for suggested inhibitors)

Equipment

1 50-mL conical tube

1 Glass petri dish per liver

1 Razor blade per liver

1 Large glass Dounce homogenizer tube and pestle per liver (or reuse after cleaning)

Overhead stirrer

Beckman ultracentrifuge with SW41 rotor

Swing buckets for SW41 rotor

1 Plastic SW41 ultracentrifuge tube (14 × 89 Ultra Clear) per liver

1 Spatula per liver

1 Small plastic homogenizer tube and pestle (Kontes) per liver

Beckman TL-100 tabletop ultracentrifuge with TLA-45 rotor and tubes (if this is not available, a Beckman J2-21 centrifuge with JA-20 rotor or similar, with 15-mL Corex tubes and screw-top microfuge tubes can be used)

Dialysis tubing membrane (20,000 MW or smaller)

Microdialyzer (Spectrum)

Parafilm

Preparation

- 1 Add protease and phosphatase inhibitors to aliquots of all buffers.
- 2 Pre-cool all solutions and buffer aliquots, a large (15-mL) and small Dounce tube and pestle, glass Petri dish, razor blade, 50-mL conical tube, and all ultracentrifuges, rotors, buckets, and tubes to 4°C.

Keep all buffers and tubes on ice during the procedure.

Procedure

- 3 Remove the liver and place it in ice-cold PBS in a 50-mL conical tube (fill the tube about halfway).
- 4 Rinse the liver with cold PBS 2–3 times to remove blood.
- 5 Optional—Tare a Mettler balance with a Petri dish, discard the final PBS wash, and dump the liver onto the dish to weigh it.
- 6 Add the liver and 1 mL of homogenization buffer to a Petri dish (use a pipette to pour the buffer over the liver).
- 7 Mince the liver finely with a razor blade.

- 8 Transfer the liver tissue to a large Dounce tube (use the razor blade to scrape the liver off the Petri dish).
- 9 Add 3 mL of homogenization buffer to the Dounce tube.
- 10 Screw a large pestle into the overhead stirrer. Set the stirrer to 2.0 and slowly push the tube onto the pestle until it reaches the bottom of the tube, or as close to the bottom as possible (*it should take about 30 s to reach the bottom*). Set the stirrer to 2.6 and hold the tube for a few seconds until large pieces of the liver are all homogenized. Set the stirrer back to 2.0 and slowly pull the tube off (*it should take about 1 min to pull off the tube – if a vacuum space occurs between the pestle and the homogenate, then the tube is being pulled too fast*). Turn off the stirrer after the pestle is out of the homogenate but before pulling the tube completely off it.

Do not over-homogenize the tissue, or nuclei will be lysed.

- 11 Optional: Check a drop of the liver homogenate under a microscope. At least 90% of the nuclei should be intact.
- 12 Place the Dounce tube back on ice and add 6 mL of homogenization buffer to an SW41 ultracentrifuge tube.
- 13 Use the same pipette to mix the liver homogenate and scrape pieces off the side of the Dounce tube. Layer the homogenate on top of the buffer in the SW41 tube with a pipette. Leave the pipette in the Dounce tube while using a new pipette to add 2–3 mL of homogenization buffer to the sides of the Dounce tube. Use the first pipette to mix up any remaining homogenate and transfer it to the SW41 tube (*continual reuse of the pipette maximizes the amount of homogenate transferred to the SW41 tube*). If it is not already full, fill the SW41 tube to near the top with homogenization buffer.

The homogenate should not sit more than a few hours before centrifugation; the sooner it is centrifuged, the better.

- 14 Centrifuge the homogenate in an ultracentrifuge at 27,000 rpm ($90,000 \times g$) for 55 min at 4°C. Wash the large Dounce tube and pestle by rinsing them with soap and hot water, dH₂O, and 95% ethanol. Let them drain and air dry.
- 15 When the centrifugation is finished, use a spatula to remove the top layer of tissue clumped in the tube and discard the supernatant. Let tube drain for 10 min on ice (*pack ice over the bottom of the tube*), then cut off the bottom of the tube above the pellet with a razor blade.
- 16 Resuspend the pelleted nuclei in 150 µL of Buffer C per g of liver

If the pellet is small, resuspend it in 50–100 µL of Buffer C. The resuspended pellet can be left at 4°C until all of the extracts are read.

- 17 Transfer the extract to a small homogenizer tube. Screw a small pestle into the overhead stirrer. Homogenize the pellet at speed 2.0 until a change in consistency becomes visible (30 s–1 min). Turn off the stirrer, and pull off the tube.

If the pellet is not sufficiently broken up, lyse the nuclei by hand with the same small pestle, using strokes and twists. Alternatively, a hand-held stirrer, if available, can be used instead of the overhead stirrer (keep the tube on ice).

- 18 Transfer the extract to a tabletop ultracentrifuge tube.
First spin down the homogenizer tube to transfer as much extract as possible). Also, transfer as much of the homogenate as possible from the pestle to the ultracentrifuge tube. If a tabletop ultracentrifuge is not available, transfer the homogenate to a screw-top microfuge tube instead.
- 19 Optional: Spin down the ultracentrifuge tube and tape it to the side of a shaker at 4°C (e.g., in a cold room). Shake the tube at high speed for 20–30 min (until all of the extracts are ready). If a shaker is unavailable, rotate the tube as fast as possible.
- 20 Centrifuge the extract in a tabletop ultracentrifuge at 45,000 rpm ($136,000 \times g$) for 20 min at 4°C.
If a tabletop ultracentrifuge is not available, put the screw-top microfuge tube in a 15-mL Corex tube and centrifuge it in a J2–21 centrifuge using a JA-20 rotor at 18,000 rpm ($39,000 \times g$) for 30 min at 4°C (use forceps to remove the screw-top tube after spinning).
- 21 Prepare the microdialyzer by cutting dialysis tubing membrane in half (cut off each side so there are 2 pieces). Keep a single layer of the membrane, wash it under dH₂O, and rinse it in Buffer D. Add it to the microdialyzer and fill the chamber with Buffer D. Make sure no bubbles are present.
- 22 When centrifugation of the extract is complete, transfer the supernatant to a well on the microdialyzer.
- 23 When all of the extracts have been added, cover the wells with Parafilm and place the microdialyzer on a stir plate in a cold room (4°C) set so the stir bar rotates slowly. Dialyze the extracts for 1 h to overnight.
- 24 After dialysis, transfer each extract from a well to a microfuge tube and measure the protein concentration
Protein concentration should be greater than 6 mg/mL.
- 25 Aliquot 25–50 µL into microfuge tubes and store them in liquid N₂ or at –70°C.

SUPPORT PROTOCOL 4: Probing the activation of transcriptional networks that promote liver regeneration (*electrophoretic mobility shift assay*)

To assay for transcriptional activation of immediate-early gene expression in the regenerating liver, one can set up gel mobility shift assays (“gelshift” assays) that will quantitatively probe the ability of key hepatic transcription factors to bind to prototype (consensus) oligonucleotide probes. Generally, these synthetic DNA probes correspond to the transcription factor-responsive elements that are located within the promoter regions of target genes. Additionally, a “super-shift” assay can be performed, in which antibody against the transcription factor is added to confirm its identification. Unlabeled (“cold”) probe can also be added for competition assays to verify the specific binding of the transcription factor to the labeled oligonucleotide. This section provides a detailed outline of the basic protocol for the performance of *electrophoretic mobility shift assays* (EMSAs, or gel-shift assays) using nuclear extracts prepared from mouse liver. The procedure includes: i) labeling of the oligonucleotide probe and ii) the gel shift reaction in the presence of liver nuclear extracts, followed by native PAGE analysis of reactions and exposure of the polyacrylamide gel to autoradiography film.

Materials and Reagents

HPLC-purified oligonucleotide probe
 5'-end DNA labeling kit (Promega)
 ATP, [γ - ^{32}P]
 G25 Sephadex spin column
 Gel Shift Reaction buffer (see REAGENTS AND SOLUTIONS)
 30% Acrylamide
 0.5 $\times$ and 10 $\times$ TBE
 80% Glycerol
 10% Ammonium persulfate (APS)
 TEMED
 Poly dI:dC
 3M Whatman paper

Equipment

Scintillation counter
 Electrophoresis equipment (gel box and power supply)
 Gel dryer
 Autoradiography film
 Automatic X-ray film developer (for autoradiography) *or alternatively, developer/fixer solutions for manual processing of the film in a darkroom*

Label the Probe

1. Use a double-stranded, annealed, HPLC-purified oligonucleotide.
2. Label 250 ng:
  - 5 μL oligo DNA (50 ng/ μL stock)
  - 2 μL 10 $\times$ polynucleotide kinase buffer (from 5'-end DNA labeling kit)
  - 9.5 μL dH₂O
  - 2.5 μL ATP, [γ - ^{32}P]
  - 1 μL T4 polynucleotide kinase
  -
  - Total reaction volume = 20 μL
3. Incubate mixture at 37°C for 1 h.
4. Add 10 μL dH₂O to the mixture.
5. Purify the mixture by running it through a G25 Sephadex spin column according to the manufacturer's instructions.
6. Count the probe's specific activity, which should be greater than 100,000 dpm, on a scintillation counter.

Perform the Reaction

Prepare the appropriate Gel Shift Reaction buffer (see REAGENTS AND SOLUTIONS below).

1. Prepare a 5% native acrylamide gel and store it at 4°C. The recipe given here is for a 1.5 mm-thick gel (40-mL volume), which should give good results. Halve the volumes for a 0.75-mm gel.

6.65 mL 30% acrylamide (5% final concentration)

2 mL 10× TBE (0.5× final concentration)

500 µL 80% glycerol (1% final concentration)

30.3 mL dH₂O

500 µL 10% APS (0.125% final concentration)

50 µL TEMED

2. Mix:

4 µL liver extract (1–10 µg protein)

1 µL poly dI:dC (1–2 µg)

5 µL 2× Gel Shift Reaction Buffer (make fresh)

Total volume = 10 µL

Note that the reaction may be adjusted up to a 100-µL volume, but adjust the gel well size accordingly. If a cold competitor sequence will be included in the assay, add the desired amount during this step. Cold competitor is usually added at 50×–100× the concentration of the labeled probe (250 ng/total µL volume after labeling) so that the chance of protein binding to the unlabeled oligonucleotide over the labeled probe is 50–100 to 1. Make sure to adjust the amount of 2× Gel Shift Reaction buffer so that it is half of the new total volume, with the cold competitor included.

3. Incubate the mixture at room temperature for 15 min.
4. If performing a “super-shift,” add 1 µL of the appropriate antibody and incubate the mixture at 4°C for 1 h. If the antibody might interfere with probe binding, add it and incubate the mixture after adding and incubating the DNA probe (below).
5. Dilute the probe to 100,000 dpm/µL and add 1 µL to the mixture.
6. Incubate the mixture at room temperature for 15 min. The gel can be pre-run for 10–15 min during the incubation.
7. Load the mixture onto the 5% acrylamide gel.
8. Run the gel at 300 V in approximately 1 L of 0.5× TBE in a cold room (4°C).

The run time depends on the protein being examined. The protein/DNA complex should run approximately 1/4 the length of the gel (NF-κB=1.5 h; Stat3 and E2=2 h). If performing a super-shift, it may be desirable to run the gel an extra hour to allow room for shifting.

9. Dry the gel on a gel dryer. Cut a piece of 3M Whatman paper large enough to cover the gel and press it onto the gel after separating the glass plates. Peel the gel off of

the plate by slowly lifting one end of the Whatman paper and rolling it back. Put the gel (still on the Whatman paper) on the gel dryer for 4 h (2 h for a thin gel).

10. Expose the gel directly to autoradiography film at -70°C overnight (*do not cover the gel with any material such as plastic wrap*) and develop.

REAGENTS AND SOLUTIONS

1. Citric acid buffer (for BrdU immunohistochemistry)

0.4 g citric acid (10 mM final concentration)

Double-distilled H_2O (dd H_2O) to 3500 mL

Adjust pH to 6.0 with NaOH

dd H_2O to 4 L.

2. PBT (for BrdU immunohistochemistry)

5 mL 10 $\times$ PBS (1 $\times$ final concentration)

500 μL 10% bovine serum albumin (BSA; 0.1% final concentration)

1 mL 10% Triton X-100 (0.2% final concentration)

43.5 mL d H_2O (up to a final volume of 50 mL)

Filter and store at 4°C .

3. Homogenization buffer

(Use approximately 15 mL per liver; prior to preparation, filter spermine and spermidine, autoclave other stocks except glycerol, and add together)

4 mL 1 M HEPES, pH 7.6 (10 mM final concentration)

10 mL 1 M KCl (25 mM final concentration)

800 μL 0.5 M EDTA (1 mM final concentration)

320 mL 2.5 M sucrose (2M final concentration)

120 μL 0.5 M spermine (0.15 mM final concentration)

200 μL 1 M spermidine (0.5 mM final concentration)

40 mL glycerol (10% final concentration)

24.9 mL d H_2O (up to a final volume of 400 mL)

Store at 4°C .

4. Buffer C

(Use approximately 200 μL per liver)

1 mL 1 M HEPES, pH 7.6 (20 mM final concentration)

20 μL 0.5 M EDTA (0.2 mM final concentration)

4.2 mL 5 M NaCl (420 mM final concentration)

75 μL 1 M MgCl_2 (1.5 mM final concentration)

44.7 mL d H_2O (up to a final volume of 50 mL)

Autoclave and store at 4°C .

5. Buffer D

(Use 100 mL per microdialyzer)

20 mL 1 M HEPES, pH 7.6(20 mM final concentration)

400 μ L 0.5 M EDTA (0.2 mM final concentration)

100 mL 1 M KCl (0.1 M final concentration)

200 mL Glycerol (20% final concentration)

679.6 mL ddH₂O (up to a final volume of 1 L)

Autoclave (*add glycerol after autoclaving*) and store at 4°C.

Note: Prior to use, add protease and phosphatase inhibitors to aliquots of Homogenization buffer, Buffer C, and Buffer D. Suggested inhibitors and final concentrations are: 1 mM DTT, 0.1 mM PMSF, 0.1 mM Na₃VO₄, 1 mM Na₂MoO₄, 1 mM NaF, 1 mM β -glycerophosphate, 2 μ g/mL Antipain Dihydrochloride, 2 μ g/mL Aprotinin, 2 μ g/mL Bestatin, and 2 μ g/mL Leupeptin.

6. Gel Shift Reaction buffers for gel shift assays:

See Tables 1, 2, 3 below

COMMENTARY

Background Information

The 2/3 partial hepatectomy technique represents the most widely applied platform for inducing liver regeneration and characterizing relevant effector pathways in rodents. This technique was first described by Higgins and Anderson in 1931 in a seminal study that still serves as the gold standard in the field of liver regeneration and receives an increasing amount of citations by relevant studies on an annual basis (Higgins and Anderson, 1931). Higgins and Anderson were the first to demonstrate that surgical removal of 2/3 of the liver lobes in the white rat could stimulate synchronous hepatocyte proliferation, leading to a gradual restoration of liver mass through a process of compensatory liver hyperplasia. However, Higgins and colleagues did not provide detailed information on the surgical procedure itself in this paper. Since then, several research groups have introduced modifications to the original surgical technique in an effort to streamline and adapt the procedure to the distinct anatomy of the mouse liver, with two approaches being the most prevalent in this research field: i) the “suture ligation” protocol that removes 2/3 of the liver by performing two separate ligatures in the left lateral and median lobes, respectively and ii) the “hemostatic clip” protocol that is a slight modification of the classical technique, in applying titanium clips to the liver pedicle instead of sutures. Liver microsurgical techniques have also been developed but require specialized microsurgical skills and are more complicated and time-consuming than the 70% partial hepatectomy protocol outlined in this unit.

Critical Parameters

Critical Parameters: Partial Hepatectomy

When planning a 2/3 partial hepatectomy, one has to take into consideration the following factors that may affect critical readouts and parameters of the experimental procedure: In addition to variations that may arise from the different handling of mice throughout the operation in various laboratory settings, including slight modifications of the surgical procedure itself, the impact of anesthesia (*e.g.*, the selection of tolerable anesthetic agents), circadian rhythm variations, access to food or fasting, mouse age, and gender can also

influence the outcome of the partial hepatectomy and thereby compromise the reproducibility of the technique and the reliability of the anticipated results.

Particular care has to be taken to consistently remove the same amount of liver tissue, since the extent of the liver proliferative response is directly proportional to the amount of liver resected.

Anesthesia

Some types of anesthetics can influence postoperative morbidity and mortality. It has been well documented that several anesthetic drugs exert toxic effects in the liver (depending on their metabolic profile and by-products). In addition, drug-induced hepatotoxicity can be exacerbated in mice of certain genetic backgrounds and haplotypes (Tunon *et al.*, 2009). For all these reasons, isoflurane has been selected as the least toxic of the commonly used anesthetic agents (Mitchell and Willenbring, 2008). Isoflurane is an inhalant anesthetic widely used in veterinary surgery because of its safety and low toxicity and the quick recovery of the animal after surgery. Its use, however, requires special equipment, such as a vaporizer. An alternative to isoflurane anesthesia is the use of ketamine/xylazine solutions.

Circadian rhythm and surgery

It is generally accepted that liver mitotic activity fluctuates according to the animal's inherent circadian rhythm and tends to be elevated during early daytime (Bucher, 1963). Cell-cycle progression and the G₂/M phase transition in hepatocytes have been shown to be dependent on proteins that regulate the circadian clock. Therefore, it is highly advisable to plan and consistently perform partial hepatectomy surgeries during the morning hours.

Fasting and metabolic state

The metabolic state of the animals is closely associated with the capacity of their livers to withstand surgery and respond with robust hepatocyte regeneration. A quite common misconception in the field is that subjecting the animals to fasting before partial hepatectomy will ensure synchronization of the metabolic state of their hepatocytes and thereby promote a more effective regenerative response. However, fasting induces hepatic steatosis (abrupt accumulation of lipids in the liver) (Heijboer *et al.*, 2005). Several studies have indicated that the lipid content within hepatocytes can exert variable effects on their regenerative response and even cause impaired regeneration (DeAngelis *et al.*, 2005). Therefore, it is recommended not to fast the animals before surgery.

Gender issues

Although it appears that the gender of wild-type animals has no important effect on regenerative process, one cannot exclude the possibility that certain genetic modifications could induce sex-dependent effects and produce different outcomes after partial hepatectomy, depending on the gender used (Desbois-Mouthon *et al.*, 2006). Therefore, it is advisable to design partial hepatectomy studies using animals of the same sex.

Age of experimental animals

It is well established that age critically influences the capability of the liver to regenerate and restore its mass following surgery. Younger animals (4–6 weeks old) respond to hepatectomy more effectively by mounting a more robust regenerative response than older animals (8–16 weeks old) of the same genetic background (Bucher, 1963). Therefore, it is highly advisable to design such experiments using a cohort of age-matched animals, usually within an age range of 8–14 weeks (slightly older animals have the advantage of

being larger, with larger livers, somewhat facilitating visibility and access during the surgical procedure).

Variations in surgical technique

The mainstream approach in setting up a partial hepatectomy surgery is to perform large abdominal incisions well below the xiphoid process that allow the researcher to comfortably operate on the liver, while maintaining ample visibility of the lobes that need to be resected (left lateral, median lobes). However, this procedure requires a greater abdominal incision and is therefore lengthier. Alternative surgical strategies have been developed that significantly reduce the duration of the surgery, such as the smaller sub-xiphoid incision that allows for the extrusion and extra-abdominal ligation of the lobes (Wustefeld *et al.*, 2000). These surgical variations require smaller incisions but might complicate the interpretation of results because of limited visibility and inconsistency during surgery (*potential complications include stenosis of the vena cava and subsequent necrosis of the remaining liver lobes*).

Critical Parameters: The CCl₄-induced acute liver injury and regeneration model

1. To date, a simple model that accurately reproduces the pattern of human acute liver failure has not been reported. The rodent models currently in use (such as acetaminophen-, galactosamine-, or CCl₄-mediated liver injury) have significant limitations resulting from the variability in the metabolic machinery of various mouse strains and the differential effects that the same drug or toxin exert in the livers of different strains, through its distinctive biotransformation, catabolic profile, and toxic by-products.
2. CCl₄ metabolism in the liver can be incomplete, leading to distal side effects of the non-metabolized toxin in other vital organs, such as the lungs and kidneys. The metabolic profile of CCl₄ can be altogether different in different mouse strains, especially those that are genetically manipulated, leading to greater variability in the results and inconsistency in the interpretation of data.
3. There is great variation in *species-dependent susceptibility* and *age sensitivity* to CCl₄, largely as a result of distinct genetic control among the different species and different levels of development and effectiveness of the cytochrome P450 detoxifying system. Indeed, it has been demonstrated that BALB/c mice are the most susceptible to the liver-necrotizing effects of CCl₄. C57BL/6 mice are more resistant to CCl₄ and exhibit an intermediate liver toxicity profile with respect to the extent of the liver damage that they sustain after CCl₄ injection (Bhathal *et al.*, 1983).
4. There are several indications from the literature that the susceptibility of the mouse liver to CCl₄-mediated toxicity may be gender-dependent. Sex hormones appear to regulate the activity and expression profile of various P450 isoforms in the mouse (Honkakoski *et al.*, 1992). Furthermore, a reduction in the metabolizing capacity induced by liver injury can render secondary target organs (*e.g.*, kidneys) susceptible to toxic damage in a gender-specific manner (Kim *et al.*, 2007). Therefore, CCl₄-induced liver injury experiments should be planned using single-sex mice.

Troubleshooting

When setting up partial hepatectomies, one might choose to expedite the surgery by performing a single ligature around the pedicles of the median and left lateral liver lobes. This variation of the technique entails the following potential complications:

stenosis of the vena cava (*i.e.*, compression of the vein due to mass ligation) and liver congestion may lead to necrosis of the remaining liver lobes (especially the caudate lobe) and failure of the liver to regenerate. Similar complications can arise when the hemostatic clip technique is applied.

When partial hepatectomy is performed using two separate ligatures for liver lobe resection, one has to take care not to tie the second knot too close to the suprahepatic vena cava; doing so can lead to vein stenosis and liver congestion. These livers soon become necrotic and appear pale in color at 36–48 h post-surgery. Mice whose livers have this appearance at sacrifice should be excluded from the study.

Anticipated Results

Both models of liver regeneration are well-tolerated, reproducible, and consistently associated with high survival rates (more than 90–95%) when the mice are not concomitantly challenged with another drug or treatment (*e.g.*, liver ischemia, infection) that might compromise the liver regenerative response. Survival rates remain high, provided that the mice are not genetically manipulated/engineered (*e.g.*, gene knock-out) in any way that might render them more susceptible to surgical- or toxin-induced liver damage. Mice are expected to recover within minutes after the surgery is complete when isoflurane is used as the anesthetic of choice. Post-operative mortality is negligible or absent if the partial hepatectomy protocol is accurately performed according to the steps described in this unit. Minimal morbidity is associated with this surgical protocol. However, if present in some mice, morbidity usually lasts for only the first 12 h after surgery. The BrdU incorporation profile in the liver cells of wild-type mice is very reproducible, and the peak of DNA synthesis (expressed as a percentage of BrdU-positive nuclei) is normally recorded in the liver parenchyma within 36–48 h post-surgery. The time window within which DNA synthesis reaches its maximum levels may be shifted toward later timepoints in some mouse strains that bear mutations affecting the rate (kinetics) and extent of hepatocyte proliferation.

Time Considerations

Partial hepatectomy—According to the suture ligation technique, the entire surgical procedure, including the preparatory phase, should last no more than 20–25 min per mouse, once sufficient training has been completed. However, variations of this technique that significantly reduce the duration of the surgery have been developed. A common variation that relies on a small, sub-xiphoid incision allows for the extrusion and extra-abdominal ligation of the liver lobes and thereby lowers the duration of the surgery to ~10 min per mouse.

CCl₄-induced acute liver injury—There are no particular time considerations in this model. The entire procedure from prepping the mouse for injection to placing the injected mouse in the cage for recovery is less than 5 min.

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Figure 1. Schematic diagram of the anatomy of the mouse liver

This diagram illustrates the basic anatomical features of the mouse liver, including its distinct lobular architecture, intrahepatic circulation, and the positioning of the ligatures used in the partial hepatectomy technique. TF, transcription factor.

Figure 2. Stages of the partial hepatectomy procedure

(A) Iodine treatment and the dashed line delineate the area shaved in preparation for the surgery. (B) Forceps are used to pull up on the muscle layer, clearly showing the linea alba (yellow arrow) for the incision. (C) The left lateral lobe (LLL) and two segments of the median lobe (right [RML] and left [LML]) to be resected are outlined. The gallbladder, which is also removed, is indicated by the yellow arrow. (D, E) A distant (D) and closeup (E) view of the top membrane (yellow arrow) to be cut. The gallbladder is also clearly visible. (F, G) A distant (F) and closeup (G) view of the second membrane (yellow arrows indicate the approximate start and end) to be cut is shown. Also delineated are the right lateral (RLL) and caudate (CL) lobes (each of which consists of two or three segments, not delineated) that will be left intact after the surgery, and which are visible when the median lobe (ML) and LLL are lifted. (H) Posterior view showing placement of the first suture at the base of the LLL. (I) Anterior view of the suture wrapped around the stem of the LLL. (J)

View of the LLL after tightening the knot of the suture in (I). Note the darker color of the now ischemic LLL (yellow asterisk) after blood flow has been restricted. **(K)** Posterior view showing placement of the second suture at the base of the ML. **(L)** Anterior view of the suture wrapped around the stem of the ML. Note that in this case the suture is not placed at the very base of the stem, but tied somewhat forward of it, pinching in some of the surrounding tissue. **(M)** View of the ischemic ML after tightening the knot of the suture in (L). **(N)** A small remnant piece of tissue remains after resecting the ML. **(O)** View of the remnant tissue after resecting both the ML and LLL, with the remaining RLL and CL clearly visible. Note that the source of the pooled blood in (N) and (O) is the resected lobes, and not bleeding from the remnant tissue. This blood should be cleaned out by the saline/PBS wash in step 19 of the protocol. **(P)** Closeup view of the initial sutures for the muscle layer, showing the beginning knot and first continuous suture. Note the lack of blood around the incision due to a proper cut along the linea alba. **(Q)** Distant view of the muscle layer after it has been closed by continuous sutures. **(R)** Distant view of the skin after it has been closed by continuous sutures.

Table 1

NF-κB Reaction Buffer	Stocks	2× Vol. (μL)	5× Vol. (μL)
20 mM HEPES/KOH, pH 7.9	1 M	40	100
60 mM KCl	1 M	120	100
5 mM MgCl ₂	1 M	10	25
0.2 mM EDTA	0.5 M	0.8	2
10% glycerol	80%	250	500 (100% stock)
0.5 mM PMSF	100 mM	10	25
0.5 mM DTT	1 M	1	2.5
1% NP-40	20%	100	250
ddH ₂ O	-	468.2	-
Final volume		1 mL	1 mL

Table 2

uE3 Reaction buffer	Stocks	2× Vol. (μL)	5× Vol. (μL)
25 mM HEPES/KOH, pH 7.9	1 M	50	125
50 mM KCl	3 M	33	83
0.1 mM EDTA	0.5 M	0.4	1
1 mM DTT	1 M	2	5
10% glycerol	80%	250	625
ddH ₂ O	-	664.6	161
Final volume		1 mL	1 mL

Table 3

STAT3 Reaction buffer	Stocks	2× Vol. (μL)	5× Vol. (μL)
10 mM HEPES/KOH, pH 7.9	1 M	20	50
50 mM NaCl	5 M	20	50
1 mM EDTA	0.5 M	4	10
10% glycerol	80%	250	625
ddH ₂ O	-	706	265
Final volume		1 mL	1 mL

Note: All reaction buffers should be made fresh, immediately before use; E2 transcription factor reactions can be performed in either NF- κ B or STAT3 Reaction buffer.