HEPATOLOGY

Cloning and expression profile of FLT3 gene during progenitor cell-dependent liver regeneration

Iraz T Aydin,* Zeynep Tokcaer,* Aydin Dalgic,† Ozlen Konu* and Kamil C Akcali*

*Department of Molecular Biology and Genetics, Bilkent University, and †Department of Surgery, Gazi University Medical School, Ankara, Turkey

Abstract

Background and Aim: The liver has a unique capacity to regenerate upon exposure to viral infections, toxic reactions and cancer formation. Liver regeneration is a complex phenomenon in which several factors participate during its onset. Cellular proliferation is an important component of this process and the factors that regulate this proliferation have a vital role. FLT3, a well-known hematopoietic stem cell and hepatic lineage surface marker, is involved in proliferative events of hematopoietic stem cells. However, its contribution to liver regeneration is not known. Therefore, the aim of this study was to clone and examine the role of FLT3 during liver regeneration in rats.

Methods: Partial cDNA of rat homolog of FLT3 gene was cloned from thymus and the tissue specific expression of this gene at mRNA and protein levels was examined by RT-PCR and Western blot. After treating with 2-AAF and performing hepatectomy in rats to induce progenitor-dependent liver regeneration, the mRNA and protein expression profile of FLT3 was investigated by real-time PCR and Western blot during liver regeneration. In addition, cellular localization of FLT3 protein was determined by immunohistochemistry.

Results: The results indicated that rat FLT3 cDNA has high homology with mouse and human FLT3 cDNA. It was also found that FLT3 is expressed in most of the rat tissues and during liver regeneration. In addition, its intracellular localization is altered during the late stages of liver regeneration.

Conclusion: The FLT3 receptor is activated at the late stages of liver regeneration and participates in the proliferation response that is observed during progenitor-dependent liver regeneration.

Introduction

The liver has a great capacity to regenerate after the loss of hepatocytes by toxic or viral injury or secondary to liver resection[1,2] with a process known as liver regeneration, as illustrated by the ancient Greek legend of Prometheus. Liver regeneration is a complex physiological response, and a series of reactions must take place for survival of the organism. Oval cells have a central role in progenitor-dependent liver regeneration and they are activated to proliferate when the replication and functional capacity of the mature hepatocytes is disrupted.[3,4] Oval cells are regarded as bipotential precursors for the two hepatic parenchymal cell lineages, hepatocytes and bile duct cells, and have lineage options similar to those displayed by hepatoblasts in early stages of liver development.[5–8] Several models in rodents have been established for triggering oval cell proliferation.[9] In the model of 2-acetaminofluorene (2-AAF) treatment and partial hepatectomy (PH) operation (2-AAF/PH), the proliferating oval cells form irregular duct-like structures that are connected with preexisting bile ducts.[10] The continuous administration of a low concentration of 2-AAF suppresses the proliferation of hepatocytes and at the middle point of the treatment the animals are subjected to a massive loss of hepatocytes by PH. When the hepatocytes do not respond to growth signals, there is rapid growth of the oval cells.[11]

Oval cells express specific proteins, one of which is thought to be FLT3.[12–14] FLT3 encodes an receptor tyrosine kinase (RTK) of 993 amino acids that belongs to the RTK subclass III family and is a well-known hematopoietic stem cell marker.[15,16] Unstimulated FLT3 receptors are in monomeric form in the plasma membrane. In this inactive state, the conformation of the receptor might result in steric inhibition of dimerization and exposure of phosphoryl acceptor sites in the tyrosine kinase domain (TKD) by the juxtamembrane domain.[16] After stimulation with the ligand FLT3L, membrane-bound FLT3 quickly changes conformation, and forms a homodimer and exposes phosphoryl acceptor sites in TKD.[17] Dimerization stabilizes this conformational change, which further enhances activation of the receptor.[18] The rate of FLT3 production, the speed of degradation of the activated FLT3L–FLT3 complex,
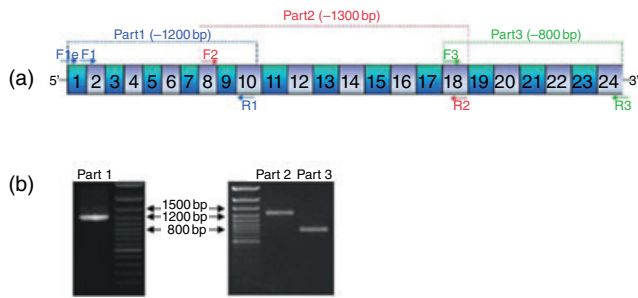
Figure 1 (a) Exons of FLT3 and location of cloning primers. F1e-F1 and R1 primers (blue) were used for amplifying Part 1, F2 and R2 primers (red) were used for amplifying Part 2, and F3 and R3 primers (green) were used for amplifying Part 3. (b) Agarose gel electrophoresis of PCR products of Part 1, Part 2 and Part 3. All three fragments were amplified from thymus cDNA. 100 bp DNA Ladder Plus was used as the molecular size marker.

and the downstream effects of activation probably participate in a complex feedback loop that regulates normal receptor activity.[16] A recent study also suggests that FLT3 is a hepatic lineage surface marker.[19] Its existence in oval cells further supports its crucial role in hepatocyte development. However, the expression pattern of FLT3 has not been investigated during liver regeneration.

Previously, our research team has examined the involvement of the bcl-2 family of genes during liver regeneration,[20] as proliferative and apoptotic mechanisms operate to provide a balance for the survival. In this study we focused on the other aspect of this complex process; proliferative response. We aimed to monitor the expression of FLT3 at both mRNA and protein levels, in progenitor cell-dependent liver regeneration. Other than the elucidation the role of FLT3 in the proliferative response, we also aimed to isolate and sequence the cDNA of rat FLT3 and monitor its tissue specific expression.

Methods

Cloning of rat FLT3

The cDNA sequences of mouse FLT3 (NM 010229), human FLT3 (NM 004119), and predicted rat FLT3 (XM 221874) were aligned using ClustalW tool (European Bioinformatics Institute; http://www.ebi.ac.uk/clustalw/). We aimed to clone the full-length FLT3 cDNA in three overlapping fragments. The primers were designed for the regions conserved among three species. The locations and sequences of the cloning primers are shown in Fig. 1a and in Table 1, respectively. Total cDNA from rat thymus was used as template with the following PCR conditions: the initial denaturation step at 95°C, followed by 45 cycles of denaturation for 60 s at 94°C, annealing for 60 s at 55°C and extension for 60 s at 72°C. A final extension at 72°C for 10 min was applied to all the reactions. PCR products were electrophoresed and sequenced.

Animals

Male 9-week-old, 280–300 g Sprague–Dawley rats were used. They were housed under controlled environmental conditions

Table 1 Primers used for cloning of rat FLT3

Primer	Sequence
F1e	5′-TCT AGA ATG CGG GCG TTG CG-3′
F1	5′-CTG CTT GTT GTT TTG TCA GTA ATG-3′
R1	5′-GGT TGT TCT TAT GAT CGC AAA ATT-3′
F2	5′-ACA GCG TTG GTG ACC ATC CTA-3′
R2	5′-GAA TTG AAT TCC CAT TGA ACC CTG-3′
F3	5′-GTT CAA TGG GAA TTC AAT TCA TTC-3′
R3	5′-TCT AGA CTA ACT TTT CTC TGT GAG-3′

(22°C) with a 12-h light-dark cycle in the animal holding facility of the Department of Molecular Biology and Genetics at the Bilkent University. All the animals received care according to the criteria outlined in the 'Guide for Care and Use of Laboratory Animals' prepared by the National Academy of Science, and this study protocol complied with Bilkent University's guidelines on humane care and use of laboratory animals. The animals were permitted unlimited access to food and water at all times.

Experimental groups

A 2-AAF solution (125 mg/mL) was prepared in DMSO, then 50 mg/kg 2-AAF was administrated to each rat for 6 days. On the seventh day, liver surgery was performed after injecting ketamine (Ketalar, Pfizer, Istanbul, Turkey) subcutaneously at a dose of 30 mg/kg. In the partial hepatectomy (PH) group 70% of the liver mass was resected,[21] and in the sham hepatectomy (SH) group the same surgical procedures were carried out but the liver lobes were not resected. Operations were performed between 08:00 and 12:00 hours to minimize diurnal effects. After completion of the procedure, the animals were placed under a lamp to prevent hypothermia and then put into cages (one animal per cage) with continuous supply of food and water.

Three randomly selected animals from the PH and SH groups were killed by cervical dislocation at 2 h, 12 h, 18 h, 24 h and 7 days after the operation. The remnant liver lobes were excised and washed in DMEM medium, then immediately frozen in liquid nitrogen.

Total RNA isolation and reverse transcription

Total RNA was isolated from tissue samples using Tripure solution (Roche-Boehringer Mannheim, Germany) according to the manufacturer's protocol. The integrity of the isolated RNA samples was determined by denaturing- (formaldehyde-) agarose gel electrophoresis. The cDNA samples were synthesized from the total RNA samples with the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada) according to the manufacturer's protocol.

Real-time RT-PCR

The primers that were used for real-time RT-PCR are shown in Table 2. Before performing the real-time RT-PCR reactions for experimental samples, the amplification efficiencies of FLT3 and cyclophilin (CYC; internal control) primers were calculated using a standard dilution series. The initial denaturation step was at 95°C

Table 2 Primers used for real-time RT-PCR

Primer	Sequence
FLT3 Forward	5′-TGG TGA CCT GCT CAA CTA CCT A-3′
FLT3 Reverse	5′-AGT CAC AGA TCT TCA CCA CCT T-3′
CYC Forward	5′-GGG AAG GTG AAA GAA GGC AT-3′
CYC Reverse	5′-GAG AGC AGA GAT TAC AGG GT-3′

for 10 min, followed by 50 (for FLT3) and 32 (for CYC) cycles of denaturation for 60 s for FLT3 and 30 s for CYC at 94°C, annealing for 60 s at 60°C for FLT3 and 30 s at 55°C for CYC and extension for 60 s for FLT3 and 30 s for CYC at 72°C. A final extension at 72°C for 10 min was applied to all the reactions. The real-time PCR reactions followed by a melting curve analysis were carried out in iCycler (Bio-Rad, Hercules, CA, USA). The threshold cycle (Ct) values for FLT3 and CYC for each of the 2-AAF-treated SH and PH samples (normal liver, 0 h, 2 h, 12 h, 18 h, 24 h and 7 days) were obtained. Fold change in the expression of FLT3 was estimated based on the comparative Ct ($2^{-\Delta\Delta Ct}$) method[22] using the normal liver sample as calibrator.

Protein isolation and quantification

For protein isolation, 100 mg of tissue samples were homogenized in 1 mL of Camiolo Buffer and centrifuged at 12 000 g for 20 min at 4°C. In order to check the equal loading, 12 µg of protein samples were run on 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 5 µL PageRuler Prestained Protein Ladder (Fermentas) was used as protein molecular weight marker. After electrophoresis, the gel was stained with Coomassie staining solution for 5 min and then destained with Coomassie destaining solution overnight.

Western blotting

The proteins were separated on 8% SDS-PAGE and transferred to polyvinylidene fluoride membrane. The membrane was blocked with blocking solution for 1 h at room temperature. FLT3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted to a concentration of 0.2 µg/mL in blocking solution, and the membrane was incubated in antibody solution for 1 h at room temperature. Then, the membrane was incubated in 1/5000 diluted antirabbit-horseradish peroxidase (HRP; Sigma, St Louis, MO, USA) secondary antibody solution for 1 h at room temperature. Finally, the membrane was incubated with ECL Plus Detection Reagent (Amersham Biosciences, Piscataway, NJ, USA) for 5 min and placed in a X-ray film cassette and developed.

Immunohistochemistry

Paraffin sections (5 µm thick) were incubated for 30 min in 0.3% hydrogen peroxide in methanol to quench endogenous peroxidase activity. After washing three times with phosphate-buffered saline (PBS) for 10 min each, slides were incubated with preblocking serum (normal goat serum 1.5%, bovine serum albumin 2%, triton-X 0.1%) for 1 h at room temperature. Primary antibody of FLT3 (Santa Cruz Biotechnology) was applied at a concentration

of 5 µg/mL in preblocking solution and kept at 4°C for overnight. After washing three times with PBS, tissue sections were incubated for 15 min each with biotinylated antimouse and antirabbit Ig and streptavidin-HRP (all Dako, Glostrup, Denmark) at room temperature. After washing 10 min with PBS, slides were rinsed in 0.5% Triton-X 100/PBS for 30 s. Color developments were achieved by incubation with Liquid DAB+ (Dako). The slides were then counterstained with hematoxylin and mounted using Faramount Aqueous Mounting Medium (Dako).

Results

Cloning of rat FLT3

We aimed to clone rat homolog of FLT3 cDNA in three overlapping fragments (Part 1, Part 2, and Part 3) from thymus (Fig. 1a). However, cloning of part 1, the most 5′ fragment, could not be accomplished using F1e and R1 primer pair. Therefore, a second forward primer, F1 that recognizes a sequence located approximately 35 base pairs downstream of the coding region was designed. All three fragments were amplified individually (Fig. 1b); sequencing of these fragments revealed a partial cDNA sequence for rat homolog of FLT3 gene (Fig. 2a). Alignment of this sequence with mouse FLT3 (NM010229) and human FLT3 (NM004119) by BCM Search Launcher: Multiple Sequence Alignments tool (Baylor College of Medicine) showed 90% homology with mouse and 81% homology with human sequences (Fig. 2b).

Tissue specific expression of rat FLT3

We analyzed the expression of FLT3 in different rat tissues at both the mRNA and the protein levels. Our results showed that FLT3 mRNA was expressed variably in the entire tissues examined (Fig. 3). In uterus, spleen, cerebellum and lungs, the expression of FLT3 was high, whereas in brain, ovary and skeletal muscle FLT3 level was found to be relatively low. In contrast to the presence of FLT3 mRNA in heart and kidney (Fig. 3), we did not detect any expression of FLT3 at the protein level in these tissues (Fig. 4a, first two lanes). Our Western blot experiments revealed the presence of FLT3 protein in other tissues (Fig. 4a).

Expression of FLT3 mRNA during progenitor cell-dependent liver regeneration

The primer efficiencies of FLT3 and CYC were 1.95 and 1.90, respectively. As these values did not differ greatly from each other and approximated the expected value, the $2^{-\Delta\Delta Ct}$ formula was used in calculation of the fold differences among groups. Our real-time PCR results showed that FLT3 mRNA expression decreased at all time points in the PH groups compared to that of normal liver during progenitor cell-dependent liver regeneration (Fig. 5a). The expression of FLT3 also decreased in the SH groups when compared to normal liver although not to the extent observed in the PH groups, except at the 24 h after the operation. When we examined the expression in liver regeneration after 70% hepatectomy in rats without 2-AAF administration, we observed a similar pattern to the PH group, except at 2 h after PH (Fig. 5b). The difference between these two groups may reflect the effect of 2-AAF.

(a)

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TTCAACCAAGAATCTGCCTGGTGATCAAGTGGTGGTTTTAATCATTCATAAGAACAATGGCTCGGCGGCGGG
AAAGTCATCGTCGCACGTCATGGTTCCAGAATCCCCAGAGGACCTCCGGTGTGCCCCGAAGCATCCGGAGTGA
AGGGGTGGTCTATGAAGCTGTCACTGTGGAGGTGGCCCAGTCTGCGTCCTTCACATTGCAAGTACAGCTCAC
CACCCCGGGGGATGTTTCTTGCCTCTGGGTCTTTAAGCACAGCTCGCTGGGCTGCCAGCCACACTTTGATTT
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TATTCAGAGTGAAGCCACCAACCACACGGTGCTGTTCACAGTGAATGTAAGAGATACACAGCTGTACGTGCT
AAGAAGACCTTCCTTCAGAAAGATGGAGAACAGGAGCGCCCTGCTCTGCGTCTCTGAGGGTGTCCCAGGGCC
CACCGTGGAGTGGGTGGTCTGCAGTTCCCACAGGGACAGCTGTAGAGAAGAAGGCTCTGCCGTTGTCAAAA
GGAGGAAAAGGTACTTCACGAGCTATTTGAAACAAACATCAATGCTGTGCGAGAAATGCACTGGGCCGAGA
ATGCACCAAGCTGTTCACCATAGATCTAAACCAGGCTCCTCAGAGCACACTGCCCCAGTTATTTCTGAAAGT
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TTCTCTCAAACATCGTTTCCTTGTGAACAAAGTGGCCTGGAGGATGGGTACAGCATTTCTAAATTTTGCGAT
CATAAGAACAACCCAGGAGAATACATATTCCATGGAGAAATGATGACGCCCAGTTCACCAAAATGTTCATA
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GGAGTTTGGAATAAAAAGGCTAACAGAAAAGTGTTTGGACAGTGGGTGTCGAGCAGTACCCTGAATATGAGT
GAGGCCGGTTTCATCCAAGACAACATCTCCTTCTATGCAACCATCGGGCTTTGCCCGCCTTTCATTGTCGTC
CTCACTGTGCTGATCTGTCACAAATACAAAAGCAATTTAGGTACGAAAGCCAGCTGCAGATGATCCAAGTG
ACCGGCCCCCTGGATAACGAGTACTTCTACATTGACTTCAGGGACTACGAATATGAACCTCAAGTGGGAGTTT
CCAAGGGAAAACTTAGAGTTTGGGAAGGCCTGGGGTCCGGCGCTTTTGGGAGAGTGATGAATGCCACGGCC
TACGGGATTAGTAAAACAGGAGTCTCAATTCAGGTGGCGGTGAAGATGCTAAAAGGAAAAGCTGACAGATGT
GAAAGAGAGGCGCTCATGGCTGAGCTCAAAATGATGACCCACCTGGGGCACCATGACAACATCGTGAACCTG
CTGGGGGCATGCACACTGTCAGGGCCAGTGTACTTGATTTTTGAATATTGTTGCCATGGTGACCTGCTCAAC
TACCTAAGAAGCCAAAAGAGAAAAGTTTCACAGGACGTGGACAGAGATTTTTAAGGAACATAATTTTCAGTTTT
TACCCCACCTTCCAGTCACATTCAAACTCCAGTATGCCGGGTTCACGAGAAGTTCAGATATACCCGCCCGTG
GATCAGGTCTCAGGGTTCAATGGGAATTCAATTCATTCTGAAGATGAGATTGAGTATGAAAACCAGAAGAGG
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ACGTCATTTTAGGCTGTCAGCTCGCAGAGGCAGAAGCAGCGATGTATCAGAACATGGACGGCGATGTCCCA
CTGGAGCACCCAGCCATCTACCAAAACAGGCGGCCTGCCAGCAGAGAGGCAGGCTCAGAGCCGCCATCACCA
CAGGTTCAGGAGAAGACTCACAGAGAAAAGTTAGTCT
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(b)

Expression of (a) FLT3 and (b) CYC mRNA in rat tissues. M, 100 bp DNA Ladder Plus; (–), negative control.

(a) FLT3

(b) CYC

M – Thymus Liver Brain Uterus Spleen Ovary Tongue Heart Kidney Cerebel. Lung Muscle

← 407 bp

← 211 bp

Heart Kidney Thymus Liver Uterus Lung Spleen Ovary Brain Testicle

(a)

(b)

Figure 4 Expression of FLT3 protein in rat tissues by (a) Western blotting with anti-FLT3 antibody and (b) SDS-PAGE with Coomassie staining.
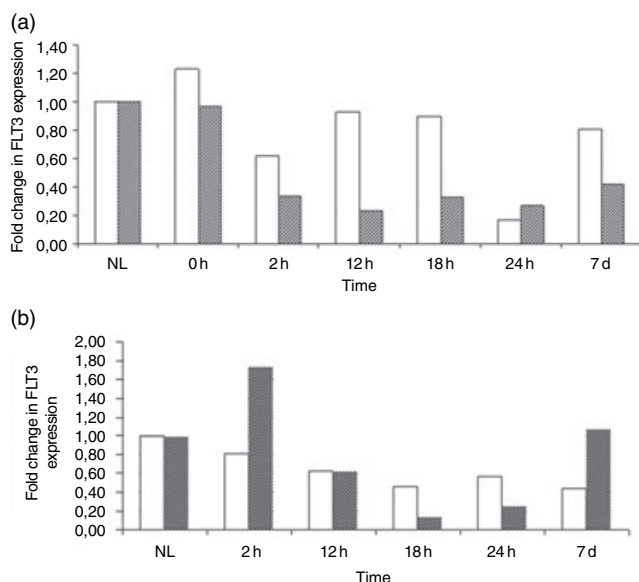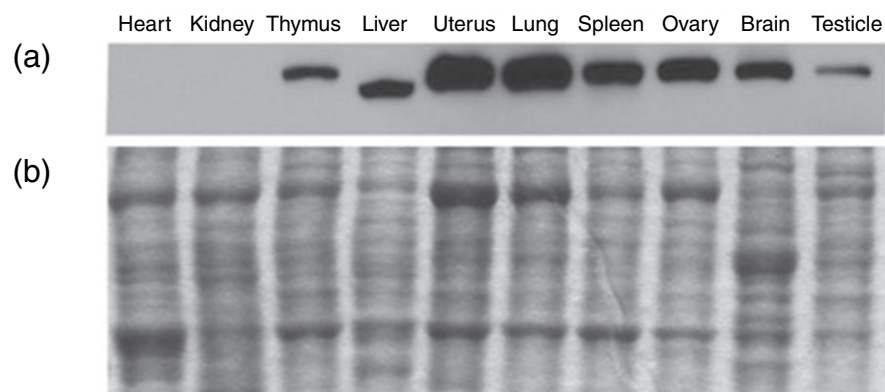

(a)

(b)

Figure 5 Real-time PCR of FLT3 mRNA during progenitor-dependent liver regeneration in (a) 2-AAF-treated and (b) untreated rats after 70% partial hepatectomy (PH). Expression of FLT3 in regenerating (□) sham hepatectomy (SH) and (▦) PH livers was measured at different time points and compared to normal control liver.

Expression of FLT3 protein in progenitor cell-dependent liver regeneration

Our Western blotting data with anti-FLT3 antibody showed relatively constant levels of FLT3 protein during the early stages of liver regeneration compared to that of normal liver, but its level decreased starting from 18 h through to 7 days postoperatively (Fig. 6a). In contrast, there was an increase at 18 h and 24 h in the SH groups (Fig. 6a).

As we observed FLT3 protein in all samples, next we performed immunohistochemistry to examine the cellular location of FLT3 protein in PH and SH groups (Fig. 7). In parallel with Western blot, FLT3 protein was detected in both PH (Fig. 7f–j) and SH groups (Fig. 7k–o) by immunohistochemistry. Interestingly, the cellular localization of FLT3 was different among our samples (Fig. 8). In the SH groups (Fig. 8c,d) and the early PH groups (Fig. 8a), FLT3 protein showed a membranous staining, but in the late PH groups the staining was mainly seen in the cytoplasm (Fig. 8b).

Discussion

From the work described in this article we were able to achieve the following: (i) cloning and characterization of rat FLT3 cDNA, which showed extremely high homology with mouse and to a lesser extent with human; (ii) possible explanation of the mechanism of FLT3 during progenitor-dependent liver regeneration.

Three different types of cells are believed to regenerate hepatocytes: hepatocytes, oval cells and bone marrow derived stem cells.[23] Among these cell types, there has been debate on the role of bone marrow derived stem cells. These are multipotent cells and have a very long proliferation potential. However, they rarely constitute more than 1% of the total hepatocytes in humans,[24] and their role in rodents is still controversial, if any.[25] In contrast, the involvement of hepatocytes and oval cells during liver regeneration is well established.[13] If the liver damage is less severe, mature hepatocytes respond rapidly to liver injury.[24] Finally, oval cells are also the source of new hepatocytes, and these cells are activated when the liver damage is extensive and chronic, or if proliferation of hepatocytes is inhibited. Oval cell precursors located in the canal of Hering represent likely candidates for liver-repopulating stem cells, and can be considered a facultative liver stem cell.[24,26,27]

Oval cells express not only markers of the hepatocyte and bile duct lineage but also markers of hematopoietic stem cells such as FLT3.[28,29] As FLT3 is a subclass III RTK, it is logical to assign a role to FLT3 during liver regeneration. However, its expression pattern during liver regeneration is not known. Therefore, we
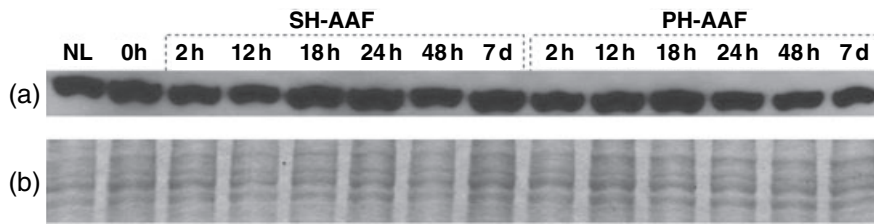
Figure 6 Expression of proteins extracted from 2-AAF-treated sham hepatectomy (SH) and partial hepatectomy (PH) groups by (a) Western blotting with anti-FLT3 antibody and (b) SDS-PAGE with Coomassie staining.
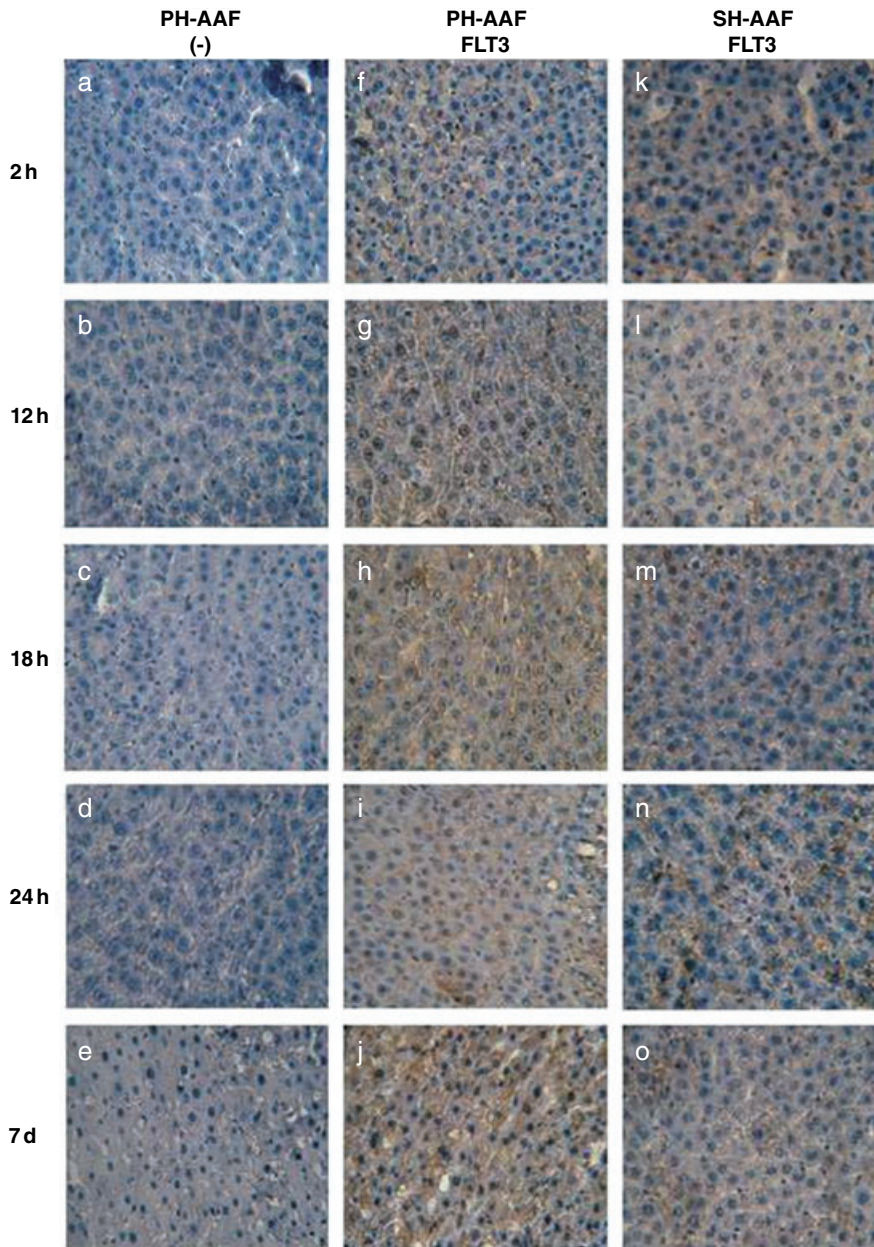

Figure 7 Immunohistochemistry of 2-AAF-treated sham hepatectomy (SH) and partial hepatectomy (PH) groups: (a–e) untreated (control) PH group, (f–j) PH group; and (k–o) SH group.

aimed to investigate the expression profile of FLT3 during progenitor cell-dependent rat liver regeneration, which was triggered by 2-AAF-treatment in combination with PH. Our results showed that there was a reduction at the mRNA level of FLT3 after PH. In both the PH and SH groups, the anesthetic used might have had an effect on the expression profile of liver cells. The reduction in the mRNA transcript level of FLT3 in the PH groups also may be related to the massive loss of cells. The observed pattern of FLT3
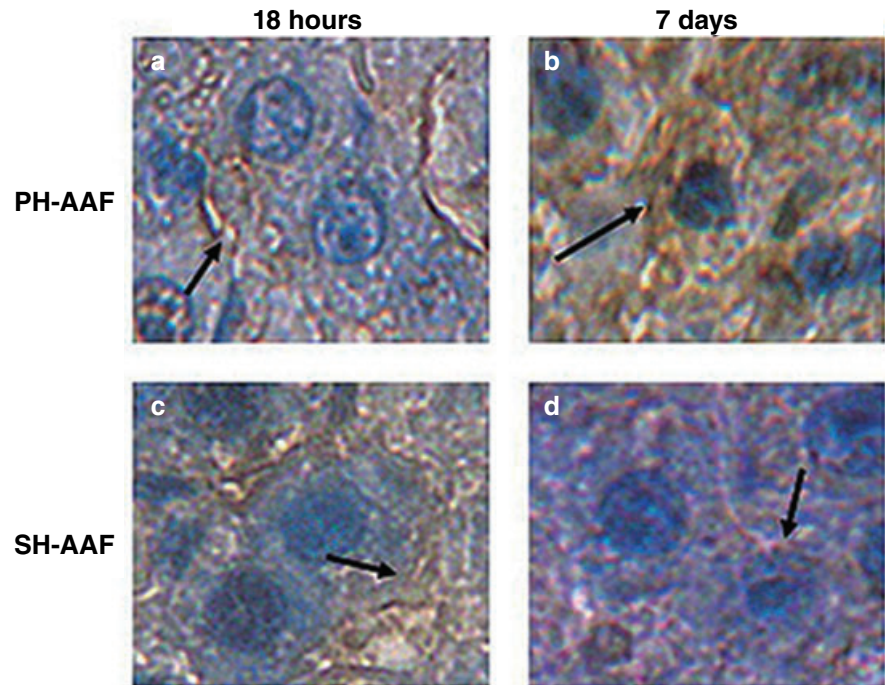
Figure 8 Cellular localization of FLT3 protein in (a,b) partial hepatectomy (PH) and (c,d) sham hepatectomy (SH) groups at 18 h and 7 days. The location of FLT3 protein is shown with black arrows.

expression and lack of a tight correlation between the mRNA and protein levels of FLT3 suggests that an increase in FLT3 expression may not always be a prominent feature of liver regeneration. In addition, the FLT3 protein level was almost equal in all groups.

Our findings indicated that post-translational mechanisms, such as translocation of FLT3 between cellular compartments, might also play important roles. Therefore, we performed immunohistochemistry experiments in order to examine the localization of FLT3 protein in different experimental groups. Our results revealed that in all 2-AAF-treated SH groups and in early groups of 2-AAF-treated PH samples (2 h, 12 h and 18 h), FLT3 expression was seen exclusively on the plasma membrane. Interestingly, in later AAF-treated PH groups, especially in the 7 days after PH group, there was strong cytoplasmic staining. The change in the cellular localization of the FLT3 during early and late stages of liver regeneration strongly suggests alterations in the activation status of the FLT3 receptor. Under normal conditions (normal liver and SH groups), FLT3 resides in the plasma membrane in an inactive monomeric form. However, when liver injury occurs and the liver undergoes a regeneration process, FLT3 is activated and internalized in order to relay signals for proliferative events. Activation and internalization of receptors ultimately results in the degradation of receptors as shown previously.[17] Therefore, the cytoplasmic staining of FLT3 protein in late 2-AAF-treated PH groups may reflect the presence of internalized FLT3 receptors. Western blotting results of the 24 h and 7 days PH groups in which FLT3 protein levels were relatively lower support this model of increased FLT3 receptor degradation.

Taking all our findings together, a likely scenario would be as follows: under normal conditions (normal liver and SH groups), FLT3 is expressed on the plasma membrane in its monomeric inactive form. The situation is also the same in the early PH groups. However, in the late PH groups, in addition to other factors, FLT3 is activated and internalized in order to potentiate cellular proliferation.

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