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A potential role for $p15^{Ink4b}$ and $p57^{Kip2}$ in liver development

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Abstract Hepatocytes undergo marked changes in proliferation during normal liver development. In order to elucidate the mechanism for these changes, we examined the ontogeny of expression for the known cyclin-dependent kinase inhibitors (CKIs), $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$, $p19^{Ink4d}$, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. All except $p16^{Ink4a}$ were expressed at some time between late gestation and adulthood. The mRNA and protein expression patterns for $p15^{Ink4b}$ and $p57^{Kip2}$ were consistent with a role for these CKIs in the regulation of hepatocyte proliferation. Specifically, $p57^{Kip2}$ may contribute to hepatocyte growth arrest that occurs in term fetuses, while $p15^{Ink4b}$ may contribute to the maintenance of adult hepatocytes in a quiescent state. These results assign a possible role to two CKIs not previously identified as involved in hepatocyte cell cycle control.

Key words: Hepatocyte; Fetal; Cell cycle; $p15^{Ink4b}$; $p21^{Cip1}$; $p57^{Kip2}$

1. Introduction

Over the last 3 days before birth in the rat, the liver triples in size. This remarkable growth is characterized by a surge in fetal hepatocyte proliferation. Rat hepatocytes then undergo a period of temporary growth arrest at the time of parturition [1,2], following which they synchronously re-enter the cell cycle. They then make a gradual transition beyond the first postnatal week to the quiescent state associated with the adult rat hepatocyte phenotype. Both the peripartum growth arrest and the transition to a quiescent adult phenotype are associated with accumulation of hepatocytes in the G1 phase of the cell cycle and a decline in cyclin D-associated cyclin-dependent kinase (CDK) activity [3].

Candidates for mediating this decline in perinatal CDK activity include the CDK inhibitors (CKIs), a group of small proteins that regulate cell cycle progression throughout its various stages [4,5]. There are two families of CKIs based on their structures and the particular CDKs they interact with. The Cip/Kip family, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$, can bind to complexes containing all known CDKs. They therefore have the potential to block progression through all phases of the cell cycle. The Ink4 family, $p15^{Ink4b}$, $p16^{Ink4a}$, $p18^{Ink4c}$ and $p19^{Ink4d}$, bind only CDK4 and CDK6, thereby mediating G1 cell cycle arrest. Whether the individual members of the Ink4 and Cip/Kip families have overlapping versus non-redundant roles is not entirely clear.

We have examined the role of individual CKIs in the control of hepatocyte proliferation by examining their expression during the perinatal transition, the postnatal transition to a quiescent adult hepatocyte phenotype, and the period of hepatocyte growth stimulation induced by partial hepatectomy. Our results indicate a possible role for two CKIs, $p15^{Ink4b}$ and $p57^{Kip2}$, that have previously not been recognized as regulators of the hepatocyte cell cycle.

2. Materials and methods

2.1. Animals

Timed pregnant Sprague–Dawley rats (Charles River Breeding Laboratory, Wilmington, MA, USA; day of conception designated as embryonic day 0 [E0]; term as E21) were used for all fetal studies. Samples were obtained by Cesarean section performed under pentobarbital anesthesia (50 mg/kg, given by intraperitoneal injection). Postnatal studies used offspring of timed pregnant dams that were allowed to deliver spontaneously and nurse their pups. Adult liver samples were from male Sprague–Dawley rats weighing 200–250 g. Partial (two-thirds) hepatectomy was performed on 125–150 g male Sprague–Dawley rats under methoxyfluorane anesthesia. For these studies, control animals underwent sham hepatectomy.

2.2. Analysis of CKI RNA and protein content

For reverse transcriptase-polymerase chain reaction (RT-PCR), isolation of total RNA from frozen liver samples was performed as described previously [6]. cDNA was generated using 3 μg of total RNA in the Superscript™ Preamplification System (Life Technologies, Gaithersburg, MD, USA). Rat-derived sequence data were used for primer design except when not available ($p21^{Cip1}$, $p57^{Kip2}$, $p18^{Ink4c}$, $p19^{Ink4d}$). In these cases, primers were designed to target the areas of greatest homology between mouse and human sequences. PCR primers were designed using the MIT Primer3 design program (http://www.genome.wi.mit.edu//cgi-bin/primer/primer3.cgi). The primer sequences used, PCR conditions and expected product sizes are shown in Table 1. RT-PCR was performed in a semi-quantitative fashion using the 'primer-dropping' method with β-actin as the control RNA [3]. PCR products were sequenced (Yale University Keck Biotechnology DNA Sequencing Laboratories, New Haven, CT, USA) to confirm the specificity of the PCR primers.

For Northern blots, total RNA (10 μg) was separated on a 1% formaldehyde-agarose gel, followed by transfer to nitrocellulose membranes (Amersham, Piscataway, NJ, USA). Filters were probed with either radiolabeled cDNA probes (human $p15^{Ink4b}$, mouse $p16^{Ink4a}$) or riboprobes prepared using CKI PCR products. cDNA probes were labeled with 5′[α-^{32}P]CTP to a specific activity of 2.0×10^8 cpm/μg. Following hybridization, membranes were analyzed by phosphorimaging.

Western immunoblotting was performed as described previously [7]. Primary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-$p57^{Kip2}$ was a rabbit polyclonal IgG directed at an internal region of human $p57^{Kip2}$. Several $p15^{Ink4b}$ antibodies were used, including a rabbit polyclonal IgG directed at the amino terminus of human $p15^{Ink4b}$, a goat polyclonal IgG directed at the carboxy terminus of mouse $p15^{Ink4b}$, and a goat polyclonal IgG directed at the amino terminus of human $p15^{Ink4b}$. In all cases, detec-

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Abbreviations: CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; RT-PCR, reverse transcriptase-polymerase chain reaction; TGFβ, transforming growth factor-β

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tion employed an enhanced chemiluminescent system (ECL; Amersham, Arlington Heights, IL, USA).

2.3. Immunohistochemistry

Formalin-fixed, paraffin-embedded samples of livers were sectioned (5 μm), deparaffinized, rehydrated and stained for $p15^{Ink4b}$ or $p57^{Kip2}$. Primary antibodies were goat polyclonal IgG directed at the amino terminus of human $p15^{Ink4b}$ and at the amino terminus of mouse $p57^{Kip2}$ (both obtained from Santa Cruz Biotechnology). Secondary antibodies and detection methods were the same as those employed previously [2]. In both cases, the specificity of our results was confirmed by the ability of the respective peptide immunogens to block staining.

3. Results and discussion

To establish the presence or absence of the CKIs in rat liver, RT-PCR for all CKIs was initially performed under non-quantitative conditions using total RNA from several developmental time points. For all of the CKIs except $p16^{Ink4a}$, a specific PCR product could be detected for at least one of the time points tested (not shown). No PCR product could be detected for $p16^{Ink4a}$ in rat liver samples from any developmental time point. Multiple primer sets were used for these experiments. One primer set in particular (Table 1), aimed at exon 1, detected the expected 160 bp product using RNA from the D9 mouse mesothelioma cell line. This product was not detected in cDNA preparations from fetal, newborn or adult liver, nor was it detected using cDNA from regenerating liver or cultured fetal or adult rat hepatocytes. However, it was present when amplified from rat placenta cDNA (not shown). Based on these results, we concluded that $p16^{Ink4a}$ is not expressed in normal rat liver during the developmental period.

We proceeded to examine the ontogeny of the six expressed CKIs using semi-quantitative RT-PCR (Fig. 1). Of the Ink4 CKIs, $p15^{Ink4b}$ was overexpressed in adult liver while $p18^{Ink4c}$ was expressed at relatively constant levels at all time points. $p19^{Ink4d}$ was expressed in fetal liver but was barely detectable in adult liver. Examination of Cip/Kip expression also indicated developmental variation. $p21^{Cip1}$ expression followed a pattern that was inversely related to hepatocyte proliferation, with the highest expression at term. In contrast, $p27^{Kip1}$ was expressed at nearly constant levels. Like $p21^{Cip1}$, $p57^{Kip2}$ expression peaked at term. However, no increase in $p57^{Kip2}$ expression was seen in the adult.

Given the absence of a previously assigned role for $p15^{Ink4b}$ and $p57^{Kip2}$, additional experiments were performed in which RNA preparations from multiple developmental time points were analyzed by semi-quantitative RT-PCR (Fig. 2). $p15^{Ink4b}$ expression increased during and beyond the first postnatal week to levels that were highest in adult animals. In contrast, expression of $p57^{Kip2}$ was highest in near-term (E20) and term (E21) animals with a marked decrease by 24 h after birth.

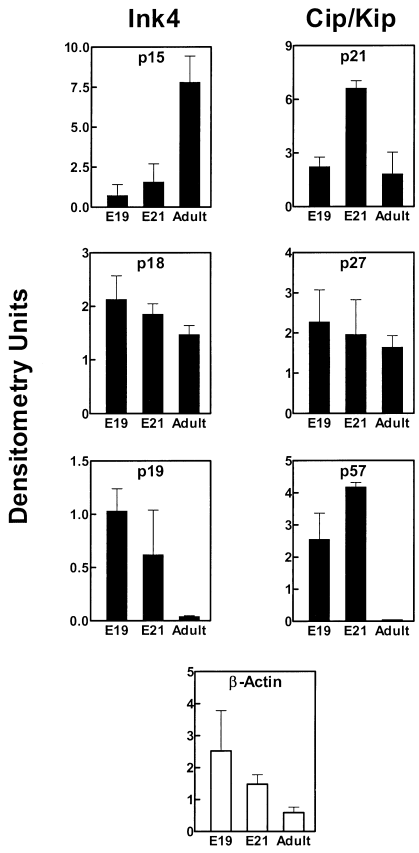

Fig. 1. CKI expression in late gestation fetal and adult liver. Triplicate total liver RNA samples from E19 fetal rats, E21 fetal rats and adult rats were used for semi-quantitative RT-PCR analysis of relative CKI mRNA content. Parallel β-actin analyses were carried out in separate PCR reactions. Following gel electrophoresis, product content was quantified by densitometry of the ethidium bromide-stained bands. Results are presented as the mean plus 1 S.D. for each group of triplicate analyses.

Further detailed studies of the remaining CKIs (not shown) showed a modest perinatal rise in $p21^{Cip1}$ expression, while $p27^{Kip1}$ and $p18^{Ink4c}$ expression levels remained constant across all developmental ages studied (E17, E19, E20, E21, P1, P2, P3, P4, P7, P14, P21, P28, adult). The expression of $p19^{Ink4d}$ fell over the first 4 postnatal days, achieving levels nearly as low as those seen in adult liver by the end of the first postnatal week.

Table 1
PCR primer sequences and conditions for amplification of rat CKIs

	Forward primer	Reverse primer	T_A (°C)	No. of cycles	Product size (bp)
$p21^{Cip1}$	5′-AGCAAAGTATGCCGTCGTCT-3′	5′-GAGTGCAAGACAGCGACAAG-3′	56	24	318
$p27^{Kip1}$	5′-TAACCCGGGACTTGGAGAAG-3′	5′-CATTTTCTTCTGTTCTGTTGGC-3′	56	28	401
$p57^{Kip2}$	5′-AGGAGCAGGACGAGAATCAA-3′	5′-TCTGGCCGTTAGCCTCTAAA-3′	56	26	267
$p15^{Ink4b}$	5′-GGCTCAGAGACGAGCCCTGTA-3′	5′-AGATAGGGCTGGGGAGAAAA-3′	55	30	524
$p16^{Ink4a}$	5′-GCAGATAGACTAGCCAGGGC-3′	5′-TAGGAGAGCAGGAGAGCTGC-3′	54	*	160
$p18^{Ink4c}$	5′-GACCCTAAAGAATGGCCGAG-3′	5′-GGTGTCCCCCTTATGGTTC-3′	56	26	430
$p19^{Ink4d}$	5′-CTTCTTCACCGGGAGCTG-3′	5′-CACCAAAAGGGGTGAGAAAA-3′	56	27	611

The number of cycles is that figure for which semi-quantitation was optimal, except for $p16^{Ink4a}$ amplification (*), which was set at 35 cycles to maximize product synthesis. T_A, annealing temperature.

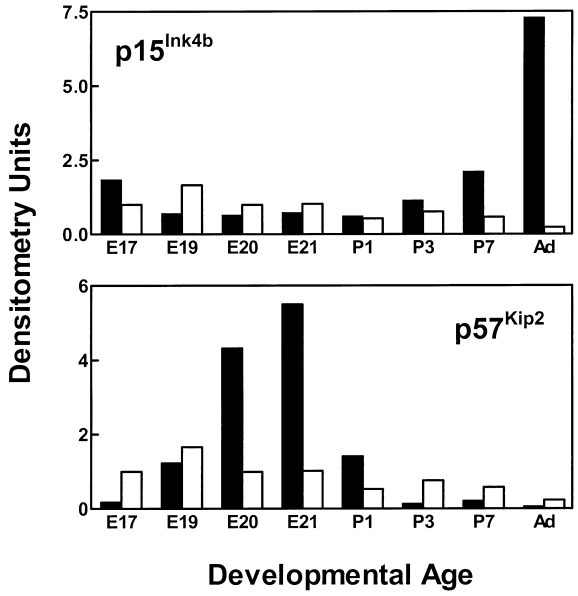

Fig. 2. $p15^{Ink4b}$ (upper panel) and $p57^{Kip2}$ (lower panel) expression during perinatal and postnatal liver development. Total liver RNA preparations from fetal, newborn and maturing rats were analyzed for CKI expression (solid bars) and β-actin expression (open bars) using RT-PCR. E, embryonic day; P, postnatal day; Ad, adult. The results of these representative experiments were confirmed in several additional analyses.

To test the hypothesis that CKI expression maintains the adult hepatocytes in a quiescent state, we studied livers from adult animals that underwent two-thirds partial hepatectomy. We hypothesized a decline in CKI expression following partial hepatectomy. Time points of 6, 24, 48 and 72 h were chosen to capture the phases of cell cycle entry and exit [8]. Results (not shown) demonstrated that $p21^{Cip1}$ was the first CKI to be expressed following partial hepatectomy, being induced by 6 h relative to the level seen in sham hepatectomy controls. By 24 h, partial hepatectomy induced an increase in the expression of all of the CKIs except $p27^{Kip1}$, which was unchanged, and $p57^{Kip2}$, which could not be detected in any of the samples. The expression of all CKIs except $p18^{Ink4c}$ returned to baseline by 72 h. Of note, in no case did partial hepatectomy produce a decrease in CKI expression.

Northern analysis using a sub-cloned rat $p57^{Kip2}$ PCR product revealed a 4.5 kb signal (Fig. 3A) that confirmed the results obtained using semi-quantitative RT-PCR results. Western immunoblotting of whole liver extracts (Fig. 3B) showed that $p57^{Kip2}$ protein levels during the perinatal period paralleled RNA expression. Immunoreactive $p57^{Kip2}$ was not detected following partial hepatectomy, consistent with the RT-PCR results (not shown). We next employed immunohistochemistry to obtain further confirmation of a hepatocyte growth regulatory role for $p57^{Kip2}$ (Fig. 3C). This method allows confirmation that CKI expression is present in hepatocytes and not restricted to non-parenchymal liver cells or the hematopoietic element in fetal liver. $p57^{Kip2}$ staining was prominent in the hepatocellular compartment and demon-

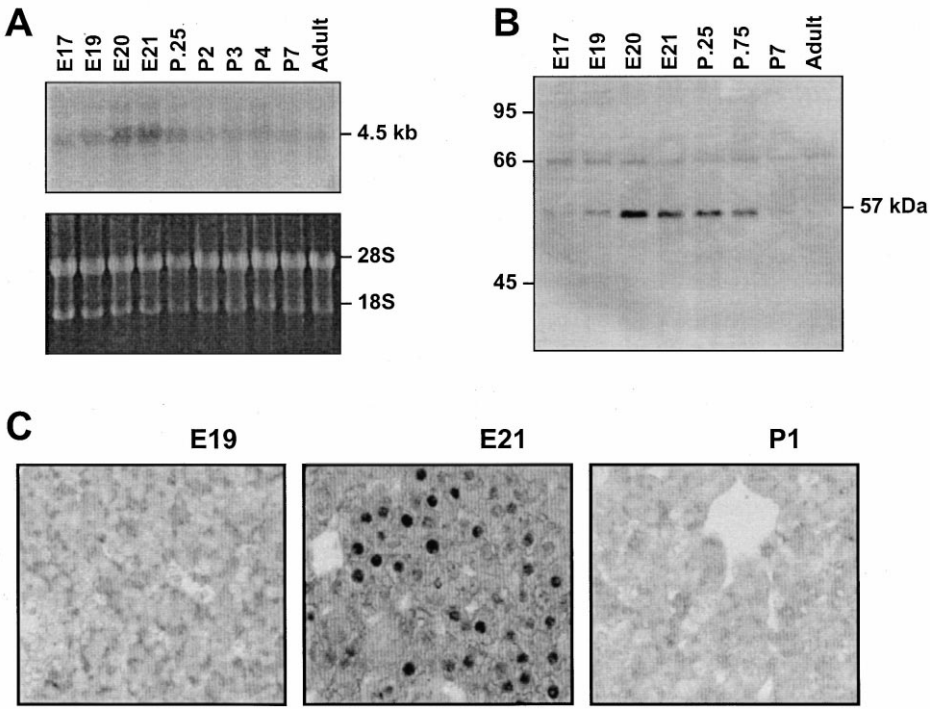

Fig. 3. Analysis of $p57^{Kip2}$ mRNA and protein content during liver development. A: Total RNA was obtained from animals sacrificed at the developmental ages shown (P, postnatal day; P.25 represents 6 h after birth). The upper portion of the panel shows the autoradiogram obtained by Northern blot, while the lower portion shows a photograph of the ethidium bromide-stained gel prior to transfer. B: Protein samples were prepared from livers harvested at the developmental ages shown. The P.25 and P.75 samples were obtained 6 and 18 h after birth, respectively. Numbers to the left of the resulting Western blot represent the position of molecular weight markers, while the position of $p57^{Kip2}$ is shown to the right of the autoradiogram. C: Tissue samples from rats ranging in age from E17 to adult were analyzed for $p57^{Kip2}$ using immunohistochemistry. Results for E19, E21 and P1 are shown. The findings represented by these three photomicrographs were confirmed in several independent experiments.

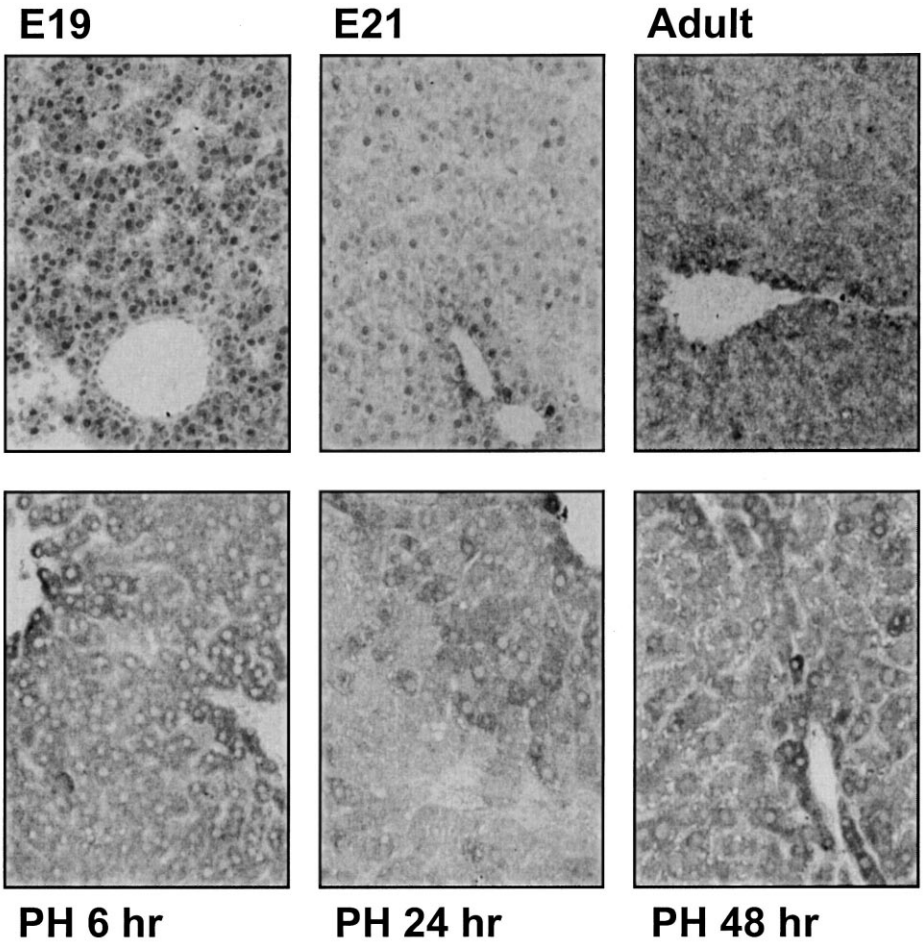

Fig. 4. Immunohistochemistry for $p15^{Ink4b}$. Tissue samples from rats ranging in age from E17 to adult were analyzed for $p15^{Ink4b}$ using immunohistochemistry. Results for E19, E21 and adult rats are shown, as are representative sections of regenerating liver obtained 6, 24 and 48 h after partial hepatectomy. These findings were confirmed in several independent experiments.

strated a nuclear distribution pattern. In agreement with the RNA expression and Western immunoblotting results, levels were higher in term (E21) than in pre-term (E19) liver. The staining was nearly gone by the end of the first postnatal day.

We further characterized a possible growth regulatory role for $p15^{Ink4b}$ by studying its protein expression pattern. Northern analysis for $p15^{Ink4b}$ resulted in multiple transcripts that were also identified using a mouse $p16^{Ink4a}$ probe. This lack of specificity prevented us from confirming the RNA expression pattern obtained using RT-PCR. In addition, $p15^{Ink4b}$ was not detectable from whole liver samples by Western analysis or by immunoprecipitation. However, it was readily detected in fetal liver sections by immunohistochemistry (Fig. 4). Results showed nuclear staining of $p15^{Ink4b}$ that was prominent at E19 but markedly decreased by E21. This was followed by a gradual increase in staining after birth. However, postnatal staining was not confined to the nucleus. In fact, adult liver showed intense, diffuse staining for $p15^{Ink4b}$. By 6 h after partial hepatectomy, there was clearing of $p15^{Ink4b}$ from nuclei. By 24 h, there were patchy areas showing more extreme loss of $p15^{Ink4b}$ staining. This persisted to 48 h. By 72 h (not shown), staining was again intense and diffuse, similar to that seen in unoperated adult animals.

While the role of CKIs has been intensely investigated in a number of model systems, there previously has not been a systematic examination of the pattern of CKI expression during liver development. Our results indicate that two CKIs not previously assigned a role in liver growth regulation, $p15^{Ink4b}$ and $p57^{Kip2}$, may be physiological regulators of the hepatocyte cell cycle.

$p57^{Kip2}$ has not been previously identified as relevant to liver biology. Prior studies did not show hepatic expression [9,10], perhaps owing to the brief developmental window during which it is induced in liver. Transfection of $p57^{Kip2}$ into osteosarcoma cells induces G1 arrest [10], and it can inhibit the kinase activities of cyclin D- and cyclin E-associated CDKs. Recent data indicate a broad physiological role for $p57^{Kip2}$. It appears to contribute to the terminal differentiation of several cell types, including lens fiber cells [11], myoblasts [12] and osteoblasts [13]. $p57^{Kip2}$ knockout mice display severe developmental defects and die immediately after birth due to dyspnea from severe cleft palate, abdominal muscle defects, and skeletal abnormalities [14]. No changes in liver anatomy or histology were noted in the $p57^{Kip2}$ nullizygous animals. Based on mapping of the $p57^{Kip2}$ gene to the Beckwith–Wiedemann locus, $p57^{Kip2}$ has been assigned a role in the development of Wilms' tumors and lung carcinomas in

these patients [15,16]. Thus, a potential role in hepatocyte proliferation raises the possibility that $p57^{Kip2}$ is involved in the process of hepatic carcinogenesis.

Data regarding the role of $p15^{Ink4b}$ in development are limited. Quelle et al. [17], in cloning the murine $p15^{Ink4b}$ gene, found that it was ubiquitously expressed in postnatal tissues. Our data demonstrate, through both mRNA and protein studies, a novel expression pattern for hepatic $p15^{Ink4b}$ and suggest a putative role for $p15^{Ink4b}$ in the maintenance of the quiescent state in adult rat hepatocytes. This is consistent with the notion that, in the rat, adult hepatocytes are growth-arrested and 'primed' for proliferation following injury [18]. The functional significance of the high level of expression in adult liver is further supported by the finding that partial hepatectomy is associated with a rapid loss of nuclear $p15^{Ink4b}$. In addition, Santoni-Rugiu et al. [19] have shown that c-*myc*-induced hepatocarcinogenesis in transforming growth factor (TGF)-α transgenic mice is associated with disrupted TGF-β induction of $p15^{Ink4b}$. Finally, $p15^{Ink4b}$ has been shown in one study to be deleted in nearly a quarter of cases of hepatocellular carcinoma [20].

In summary, the developmental pattern of expression of $p15^{Ink4b}$ and $p57^{Kip2}$ indicates a role in hepatocyte biology. The two CKIs may be involved in the induction of hepatocyte growth arrest at two distinct points during liver development, the perinatal period and the postnatal transition to a quiescent adult hepatocyte phenotype. This apparent involvement in the physiological control of hepatocyte proliferation raises the possibility of involvement of $p15^{Ink4b}$ and $p57^{Kip2}$ in disorders associated with pathophysiological hepatocyte proliferation.

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