

BASIC STUDIES

## Mutant Hras<sup>G12V</sup> and Kras<sup>G12D</sup> have overlapping, but non-identical effects on hepatocyte growth and transformation frequency in transgenic mice

Marxa L. Figueiredo\*, Timothy J. Stein†, Adam Jochem and Eric P. Sandgren

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI, USA

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### Correspondence

Dr Eric P. Sandgren, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI 53706, USA  
Tel: 608-263-8870  
Fax: 608-265-8435  
e-mail: sandgren@svm.vetmed.wisc.edu; sandgren@rarc.wisc.edu

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### Abstract

**Background:** Mouse hepatocarcinogenesis is associated with mutations in Hras, but infrequently in Kras. The effect on carcinogenesis of developmental age at the time of ras mutation remains unknown. **Aim:** We sought to compare quantitatively the effects of expressing mutant H- or Kras genes in fetal vs. adult mouse liver. **Methods:** We established an inducible system of gene expression in mouse liver to define disease pathogenesis associated with activation of oncogene expression. **Results:** Diffuse expression of either oncogene in fetal or adult hepatocytes caused hepatomegaly. For mutant Hras<sup>G12V</sup>, this phenotype was almost fully reversible and accompanied by apoptosis, indicating that maintenance of hepatomegaly requires continuous Hras<sup>G12V</sup> expression. We also examined the effect of ras expression on growth of transplanted hepatocytes in an *in vivo* system that allows us to quantify hepatocyte growth effects in both permissive and restrictive hepatic growth environments. Mutant Kras<sup>G12D</sup> had no effect on hepatocyte growth in this system. In contrast, Hras<sup>G12V</sup> induced increased hepatocyte focus growth in quiescent liver, the hallmark of a cell autonomous growth stimulus. Hras<sup>G12V</sup> also increased the fraction of donor hepatocyte foci that displayed extreme growth, a characteristic of preneoplastic lesions. **Conclusions:** The primary effect of diffuse, whole-liver expression of either mutant ras gene in fetal or adult mouse liver is diffuse and progressive hepatic growth. Hras<sup>G12V</sup> mutation influences hepatocarcinogenesis by conferring cell autonomous growth potential upon foci of expressing cells and by increasing the risk of neoplastic progression. Kras<sup>G12D</sup> does not share these latter carcinogenic effects in mouse liver.

The 21 kD ras proteins are central components of cellular signalling pathways that control cell proliferation, differentiation and survival (1). The mammalian ras genes encode three closely related GTPases, Hras, Kras and Nras. Mutations in ras genes have been identified in 30% of cancers in humans and also in many rodent spontaneous and chemically induced tumours (2, 3). Ras genes are oncogenically activated via single point mutations that cluster at codons 12, 13, 61 or 117. These mutations create amino acid changes in the GTP-binding pocket domain that render proteins constitutively

active. Hras is the most commonly identified activated oncogene in mouse hepatocellular carcinomas (HCC) (4–6). K- and Nras mutations occur less frequently (7, 8). Ras mutations are identified infrequently in human HCC. However, all human HCCs display elevated ras signalling activity compared with adjacent normal liver in association with decreased expression of ras pathway inhibitors (9–11), supporting a role for ras signalling in these tumours.

Transgenic mice have been developed that target expression of mutant Hras<sup>G12V</sup> specifically to hepatocytes (12–14). These studies provide hints about the biological effects of mutant ras in liver. Initial targeting used an albumin enhancer/promoter-mutant Hras<sup>G12V</sup> transgene (AL-Hras<sup>G12V</sup>) (13). In that study, the majority of founder mice died 1–3 days after birth with liver hyperplasia. In a smaller group of founder mice with no evidence of perinatal liver enlargement there was a mild adult hepatic phenotype, including

\*Present address: University of Texas Medical Branch, 301 University Blvd, Department of Pharmacology & Toxicology, Galveston, TX 77550 USA

†Present address: Department of Medical Sciences School of Veterinary Medicine University of Wisconsin-Madison Madison WI 53706 USA

occasional development of HCC. However, these mice developed lung neoplasia at 1–12 months of age. Later, Wang and colleagues also used the mouse albumin enhancer/promoter to target the expression of mutant Hras<sup>G12V</sup> to hepatocytes in mice (14). By 9 months of age, about half the mice developed HCC. Lack of perinatal hepatic hyperplasia suggests that expression was absent, reduced, or not fully penetrant in young mouse liver. Harada and colleagues utilised the cre-recombinase system to induce expression of a mutant Hras<sup>G12V</sup> in hepatocytes (12). In their system, an Hras<sup>G12V</sup> coding sequence was cloned downstream from a lox-flanked stop codon plus polyadenylation sequence and introduced into one endogenous Hras locus using gene targeting. In the absence of cre-mediated recombination the Hras<sup>G12V</sup> sequence was not transcribed. When cre-recombinase was targeted to hepatocytes using an adenoviral vector, hepatocytes that underwent cre-mediated deletion between the loxP sites deleted the stop codon and polyadenylation sequence and expressed the mutant Hras<sup>G12V</sup>. Transgenic mice were inoculated with adenoviral vectors expressing cre-recombinase at 7 weeks of age. By 4 weeks post-inoculation dysplastic hepatocytes were apparent microscopically, but they were not associated with changes in cell proliferation and there was no progression to dysplastic nodules or hepatocellular carcinoma through 17 months of age (12). In contrast, when Hras<sup>G12V</sup> and mutant  $\beta$ -catenin were coactivated in hepatocytes using this approach, focal neoplasia developed.

Collectively, these studies demonstrate diffuse disruption of normal liver growth by mutant Hras<sup>G12V</sup> when it is expressed in fetal liver, and variable increase in risk for Hras<sup>G12V</sup>-mediated neoplastic transformation in adult liver. However, they leave unanswered two important mechanistic questions about the effects of ras mutations in rodent liver. Firstly, are the effects of ras mutants different when expression is restricted to adult vs. fetal hepatocytes? Secondly, do these different activated ras genes have different biological effects during hepatocarcinogenesis? If so, what are the differences? The more frequent detection of mutations in Hras than in other ras family members in HCC could be explained if Hras preferentially causes cancer in this organ. We address these questions in this report using the tetracycline inducible system (15, 16) to regulate hepatocyte expression of transgenes encoding mutant Hras<sup>G12V</sup> or mutant Kras<sup>G12D</sup>. Expression of mutant ras can be turned on or off in hepatocytes of any age, allowing us to identify phenotypic consequences of adult vs. fetal/perinatal mutant ras expression. We then employed these mice together with the newly developed comparative hepatocyte growth assay to quantify hepatocyte growth characteristics and neoplastic progression frequency associated with each mutant ras. These studies define mechanisms by which mutant ras genes influence hepatocarcinogenesis.

## Materials and methods

### Animals

Mice were maintained according to The Guide for the Care and Use of Laboratory Animals in AAALAC-accredited facilities. All experimental procedures were approved by the University of Wisconsin–Madison Animal Care and Use Committee. The transgenic lines used in these studies have been assigned the following genetic designations: MUP-muPA line 350-2, TgN(MupPlau)1Eps; MT-nLacZ line 379-4, TgN(Mt1nLacZ)4Eps; LAP-tTA, TgN(tTALap)5Uh; tetO-hPAP line 1391-4, TgN(tetOpALPP)11Eps; tetO-Hras<sup>G12V</sup> line 1562-1, TgN(tetOpHRAS<sup>G12V</sup>)15Eps; AL-tetO-Hras<sup>G12V</sup> line 1480-3, TgN(tetOpHRAS<sup>G12V</sup>)16Eps; tetO-Kras<sup>G12D</sup> line 1575-11, TgN(tetOpKRAS<sup>G12D</sup>)13Eps.

### Transgene construction and identification of transgenic mice

Mice carrying the metallothionein (MT)-nLacZ marker transgene, encoding nuclear-localized  $\beta$ -galactosidase, have been described (17). MUP-uPA transgenic mice were used as hepatocyte transplant recipients in these studies, and have been described (18). LAP-tTA mice (19) were obtained from Jackson Laboratories (Bar Harbor, Maine) and backcrossed into FVB to establish a uniform strain background for these studies. Data from multiple backcross generations (FVB-3 to FVB-7) were controlled for by simultaneous evaluation of littermate controls.

To construct tetO-hPAP, the human placental alkaline phosphatase (hPAP) coding region with an attached 950 base pair (bp) Simian Virus 40 polyadenylation signal was cloned downstream of DNA containing seven tandem copies of the E.coli bacterial tetracycline operator (tetO) followed by the human cytomegalovirus (CMV) minimal promoter (p<sub>min</sub>) region (provided by H. Bujard). The tetO-hPAP transgenic mice were identified using PCR. To construct tetO-Kras<sup>G12D</sup>, the Kras<sup>G12D</sup> coding sequence (provided by Manual Peruch) with hGH polyadenylation sequence was inserted downstream of the tetOCMVp<sub>min</sub> sequence described above. tetO-Kras<sup>G12D</sup> mice were identified by PCR, using the forward primer 5' TAGGCGTGTACGGTGG GAGG 3' and the reverse primer 5' CTACGCCACA AGTCCAACTACCAC 3'. Twenty-five  $\mu$ l of reaction mixture containing genomic DNA purified from tail biopsies was subjected to the following conditions: (i) 92°C for 2 min; (ii) 35 cycles of: 45 s at 92°C, 90 s at 60°C and 110 s at 72°C; and (iii) 72°C for 7 min. To construct the tetO-Hras<sup>G12V</sup> transgene, a human activated H-ras<sup>G12V</sup> gene (provided by R. Palmiter) was cloned downstream of the tetOCMVp<sub>min</sub> sequence described above. To construct the ALtetO-Hras<sup>G12V</sup> transgene, the human activated Hras<sup>G12V</sup> coding sequence was cloned downstream of a different tetOp<sub>min</sub>, which contained two tetO repeats down-

stream of a minimal promoter from the mouse albumin gene ( $Alp_{min}$ , 300 bp) (pAlbtetO2; provided by William Wilkison).  $Hras^{G12V}$  transgenic mice were identified by PCR as described for  $Kras^{G12D}$ , using the forward primer 5' CCTGTCTCCTGCTTCCTCTAG 3' and the reverse primer 5' CTCACGGGGTTCACCTGTACTG 3'. All transgenes were generated and maintained on the FVB strain background except MUP-uPA, which was in the (FVB6)F1 strain background.

#### Comparative hepatocyte growth assay (CHeGA)

Hepatocytes were isolated from LacZ- or hPAP-marked transgenic mice to facilitate identification of donor hepatocytes in recipient mice. Donor hepatocytes were isolated using a modified two-step ethylenediaminetetraacetic acid (EDTA)/collagenase A protocol (18). Approximately 80% of the cells isolated using this method are hepatocytes (20). The concentration of viable hepatocytes was determined by trypan blue exclusion using a hemacytometer. Cells were maintained at 4°C until transplantation. Hepatocytes were transplanted as a 1:1 mixture in 10  $\mu$ l via intrasplenic injection within 5 h of isolation, as described (21).

Recipient mice initiate expression of the hepatotoxic mouse major urinary protein-urokinase type plasminogen activator (MUP-uPA) transgene at 3–4 weeks of age. The resulting liver disease provides a growth-permissive environment for the healthy donor cells. Donor hepatocytes travel from spleen then proliferate and expand as clonal foci within recipient livers for approximately 4 weeks, at which time the liver is repopulated by a mixture of donor cell clones and healthy endogenous hepatocyte clones derived from progenitors that stably inactivated transgene expression. Recipients are euthanized at 4 or 12 weeks post-transplant. To detect hPAP-marked donor hepatocyte foci in liver, recipient livers are fixed in 4% paraformaldehyde for 1–2 h at 4°C, and samples are treated to detect hPAP activity by incubation with the hPAP substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) as described (18). The hPAP-expressing donor hepatocyte foci are visible on the surface of the liver lobes, and display a blue colour. To detect LacZ-marked donor hepatocyte foci in liver samples, recipient mice are administered 0.1 mg/kg cadmium sulphate intraperitoneally to induce expression of the MT-nLacZ transgene 16–24 h prior to euthanasia. Livers are fixed in 4% paraformaldehyde for 1–2 h at 4°C, and samples are treated to detect  $\beta$ -galactosidase activity by incubation with the substrate 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal, United States Biological), as described (17). The  $\beta$ -gal-expressing donor hepatocyte foci are visible on the surface of the liver lobes, and display a blue colour product as a result of the  $\beta$ -galactosidase enzymatic reaction.

Cross-sectional surface areas occupied by single blue-staining donor clones are determined on the liver surface under low-power magnification by measuring the major axes (a, b) of each stained focus, then calculating the area of an oval using the formula  $A = \pi[1/4(a + b)]^2$  (18). For each recipient liver, we measure 50 foci of each donor type.

#### Quantification of hepatocyte proliferation and apoptosis

One hour prior to euthanasia, all mice were administered 200 mg/kg bromodeoxyuridine (BrdU), a nucleotide analogue incorporated into the DNA of cells in the synthesis phase of cellular replication. Cells undergoing replication were identified using immunohistochemistry to detect BrdU incorporation as described (18). The BrdU labelling index was determined by expressing the number of BrdU-positive hepatocyte nuclei as a percentage of the total number of hepatocyte nuclei counted. Apoptotic indices in foci were calculated similarly, using morphological criteria to identify apoptotic cells: (i) chromatin condensation and nuclear fragmentation into apoptotic bodies, (ii) eosinophilic cytoplasm and (iii) cell shrinkage.

#### Evaluation of gene expression

Total RNA was isolated from approximately 30 mg of mouse liver, quantified and subjected to cDNA synthesis as described (22). Fitness of cDNA templates was determined using the SPUD assay (23) and the 3':5' GAPDH assay (24). GAPDH, HPRT1 and TBP were selected for mouse liver from a group of 10 commonly used housekeeping genes (25). RT-qPCR reactions were performed using the Biorad iQ5, and data quantification using iQ5 software. Primers used were as follows (5' to 3'): *Hras* mutant (human) F: ggacgaatcagccccactatagag, R: acaca ggaagccctccccggtg; *Hras* total F: tttgccatcaacaaccaagtc, R: gcagccaggtcacactgtt; *Kras* mutant (human) F: ggac gaatgatccaacaatagag, R: acaagaagccctccccagtc; *Kras* wildtype (mouse) F: ggatgagtacgacctacgatagag, R: acaa agaaagccctccccagtt; *Kras* total F: acacagcaggtcaagaggag tacag, R: cacaagaaagccctccccagtc; *Gs* F: gtgagcccaagtgtt ggaa, R: gaaggggtctcgaaacatggc; *Glt1* F: tgcctatcctcctctt atcatct, R: ggccgtgctttagcat; *Oat* F: gccctttctggcggtttat ac, R: tggtttaatggtcagcattatctca; *Otc* F: accttctttcttaccacaaa, R: ttgagagagccatagtgttctctg; *Ass1* F: ctgaaggacaag gctatgatg, R: gagagaggtgccaggagatag; *Asl* F: tgttttggg cttctctgtgatg, R: aggtgtctgggttttctct; *Cps* F: ctaccctg cttactgagacctt, R: ccaacagcgtccatctctact; *Arg1* F: ctccaa gccaaagtccttagag, R: aggagctgtcattaggacatc; *G6p* F: cgac tcgctatctccaagtga, R: gttgaaccagtctccgacca; *Khk* F: atgtg tgggcaaaataaccaga, R: caagcaaggaaaggacagtc; *Acaca* F: atgaggatgacaggcttgc, R: tgccatgctcaaccaagta; *Alb* F: aga taagtgtgtgccattccaa, R: tgtgtgcaggaaacattcgt; *Afp* F: aac tctggcgatgggtgttta, R: acactgatgtctttccactcca; *Cyp2e1* F: ggctgtcaaggagggtctac, R: ctaccctctgtttcccca; *Cyp7a1* F: atgtccactcatcacaactcc, R: ctgtgtccaatgcctctgc; *Cyc-*

linD1 F: ccctgacaccaatctcctcaac, R: gcatgtaggcacaatctcct; Ccnnb1 F: cgttgagttgacaagctctctc, R: cctgtctgtagccaagaggttc; Bax F: ttgctacagggttctatc, R: tgtccagttcatctccaa.

### Evaluation of serum chemistries

Serum chemistries were performed to determine if hepatocyte-targeted expression of activated H- or Kras had an identifiable effect on overall liver function or health. Serum was transported on ice and evaluated using a Hitachi 912 Chemistry Analyzer (Roche Diagnostics, Indianapolis, IN, USA).

## Results

### Regulated gene expression in mouse liver

The tet-inducible system uses two transgenes. LAP-tTA targets expression of the tetracycline transactivator (tTA) to hepatocytes via the liver-enriched activating protein promoter (19). tTA is a chimeric protein containing the herpes simplex virus viral protein 16 transactivator domain and the tet repressor (tetR) moiety from the tet operon of *Escherichia coli*. The tetR portion contains a DNA-binding domain that allows the protein to bind to tet operator (tetO) sequences in target genes, thereby activating transcription. Target transgenes contain tandem repeats of the 19 base pair tetO sequence adjacent to a minimal promoter ( $P_{min}$ ) from cytomegalovirus (CMV) or the mouse albumin gene, cloned upstream of hPAP, mutant Hras<sup>G12V</sup>, or mutant Kras<sup>G12D</sup> gene coding sequences. When both tTA and target transgenes are present in the same mouse, tTA binds to tetO as a dimer and transactivates the expression of hPAP and/or ras genes in hepatocytes. However, tetracycline or more potent analogues, such as doxycycline (Dox), bind to the tetR moiety, thereby inhibiting binding of tTA to the tetO sequences and eliminating expression of target genes. For these studies, DOX was administered in the drinking water at 5 mg/mL at the schedules noted in text and tables.

To evaluate the kinetics of target transgene activation and silencing, LAP-tTA/tetOCMV-hPAP bitransgenic mice were subjected to different Dox treatment regimens, and multiple tissues evaluated for hPAP expression (data not shown). In the absence of Dox, liver stained uniformly blue following incubation in the hPAP substrate BCIP. Some staining also was observed in the renal medulla. Monotransgenic LAP-tTA or tetOCMV-hPAP mice displayed no liver staining. Additional animals were administered Dox in drinking water until adulthood, and then Dox was removed. By 1 week post-Dox, faint staining was present in liver, and by 2 weeks staining was intense, indicating that Dox clearance and activation of target transgenes occurs within 1 week. In the converse experiment, livers of initially untreated mice administered Dox for 1 week stained a faint blue, and after 2 weeks of treatment were

**Table 1.** Effect on liver growth of hepatocyte-specific expression of mutant H- or Kras

Transgene line	Ras expression* (# mice)	Endstage, weeks post- induction, mean ± SD	Liver/body weight %, mean ± SD
Non-transgenic	None (16)	–	5.3 ± 0.6
tTA/Hras	Continuous (15)	6.4 ± 4.2†	15.6 ± 2.0
1562-1	On at birth (4)	6.0 ± 1.0	18.4 ± 4.6
	On at 6 weeks (13)	13 ± 6	16.7 ± 3.6
tTA/Hras	Continuous (17)	9.5 ± 4.8*	17.4 ± 3.6
1480-3	On at birth (7)	8.9 ± 2.0	16.2 ± 2.8
	On at 6 weeks (5)	15 ± 4	14.7 ± 0.6
tTA/Kras	Continuous (1)	0.14*	–
1575-11	On at birth (4)	4.6 ± 1.0	33 ± 13
	On at 6 weeks (10)	13 ± 7	16.6 ± 5.1

\*Ras expression was initiated in mice by removing Dox at the stated age. Ras expression was continuous during development in mice that did not receive Dox *in utero* or after birth.

†Age in weeks.

unstained, indicating that Dox suppressed most hPAP activity by 1 week after drug administration.

### Mutant ras expression in hepatocytes reduces mouse longevity, increases liver weight and alters hepatic gene expression

Non-transgenic mice and mice carrying a single transgene displayed normal liver weight as a per cent of body weight both in the presence and absence of Dox (data not shown). To identify the effects of Hras<sup>G12V</sup> and Kras<sup>G12D</sup> on liver growth *in vivo*, we established bitransgenic mice carrying LAP-tTA and an inducible mutant ras transgene (Table 1). Without Dox treatment (target gene on), survival of bitransgenic mice was reduced. The most extreme effect was observed in animals never exposed to Dox. For the Kras<sup>G12D</sup> line, only one bitransgenic animal could be obtained, and it required euthanasia 1 day after birth. Onset of lesions in this mouse resembled perinatal disease in AL-Hras<sup>G12V</sup> transgenic mice (13). Bitransgenic mice for both Hras<sup>G12V</sup> lines displayed mean survival ranging from 6.4 to 9.5 weeks after birth. Providing Dox to pregnant and lactating mothers of bitransgenic mice blocked ras expression. Following removal of Dox at birth, average mouse survival for H or Kras lines ranged from 5 to 9 weeks. Removal of Dox from adults (6 weeks of age) permitted average survival for an additional 13–15 weeks after initiation of ras expression regardless of the age at transgene initiation. Length of survival typically reflected the animal's condition: mice were euthanized when they became lethargic and lost body mass, such that the caudal spine could be palpated easily.

**Table 2.** Clinical chemistry†

Serum parameter	Genotype		
	Control‡	tTA/Hras	tTA/Kras
Alanine amino transferase (ALT) (U/L)	39 ± 9 (9)	48 ± 17 (8)	329 ± 350 (2)*
Total protein (g/dL)	3.9 ± 0.4 (13)	6.5 ± 1.3 (10)***	7.3 ± 1.5 (2)**
Albumin (g/dL)	1.9 ± 0.5 (13)	2.7 ± 0.4 (9)**	2.8 ± 0.9 (2) <sup>P=0.06</sup>
Glucose (mg/dL)	258 ± 48 (14)	254 ± 102 (8)	190 ± 58 (2)
Ammonia (umol/L)	43 ± 17 (7)	132 ± 61 (4)*	46 ± 4 (2)

\* $P \leq 0.05$ ;\*\* $P < 0.01$ ;\*\*\* $P < 0.001$  vs. non-transgenic samples using two-sided Wilcoxon rank sum test.

†Data expressed as mean ± SD (n). Some control and tTA/Hras mice in this study were in the FVB6F1 strain background. All tTA/Hras mice were from line 1562-1. Mutant ras-expressing mice were not receiving Dox, and were approaching end stage disease as noted in Table 1.

‡Controls were non-transgenic and single transgenic mice that did not express mutant H- or Kras.

For all bitransgenic mice removed from Dox, liver weights increased significantly compared with non-transgenic or single transgenic control mouse livers, irrespective of age at Dox removal (Table 1). Grossly, 3 of 13 bitransgenic mice (23%) older than 24 weeks of age, two expressing Hras<sup>G12V</sup> and one expressing Kras<sup>G12D</sup>, displayed large hepatic masses confirmed microscopically to be hepatocellular trabecular or cystic carcinomas. Hepatic architecture remained normal in the enlarged livers, although two types of hepatocytes were identified (data not shown). Periportal hepatocytes were smaller and more uniform in size than pericentral hepatocytes. At the time of euthanasia, most bitransgenic mice also displayed renal tubule dilatation.

Clinical chemical evaluation of mouse serum identified physical hepatocyte damage (increased ALT) in one of two tTA/Kras<sup>G12D</sup> mice, increased blood ammonia in tTA/Hras<sup>G12V</sup> mice, and elevated albumin and total protein in both (Table 2).

We also measured ras expression, and examined the effects of Hras<sup>G12V</sup> on expression of a panel of genes normally active in liver by real time qPCR. For Hras, we measured mutant (human only) and total (mouse plus human) Hras mRNA; we could not develop primers specific for mouse mRNA that did not also cross react with the human mRNA. For Kras, we measured mutant (human only), endogenous wildtype (mouse only) and total Kras mRNA. Compared with non-transgenic control liver, Hras total mRNA increased 2.2-fold and Kras total mRNA increased 2.6-fold (Table 3). Thus, transgene expression for both groups of transgenic mice was close to the physiological range of endogenous ras expression. Kras wildtype mRNA expression also increased, by 1.9-fold, in transgenic mice. Comparing fold increases for wildtype vs. total Kras mRNA indicates that about 27% of total expression in transgenic livers was from the transgene. Hras<sup>G12V</sup> altered expression of nitrogen metabolizing enzymes, and genes reflective of cellular differentiation (Table 4). In particular, alpha-fetoprotein expression was increased 200-fold and Cyp expression was decreased, reflective of a less

**Table 3.** Relative transgene expression†

qPCR target	Genotype (n)		
	Non-transgenic (4)	tTA/Hras (5)	tTA/Kras (3, 4)
Hras <sup>G12V</sup> (human)	0.013 ± 0.006	1.00 ± 0.37**	–
Hras total	1.00 ± 0.04	2.18 ± 0.27**	–
Kras <sup>G12D</sup> (human)	0.003 ± 0.002 <sup>n=3</sup>	–	1.00 ± 0.07*
Kras wildtype (mouse)	1.00 ± 0.14	–	1.90 ± 0.28*
Kras total	1.00 ± 0.23	–	2.62 ± 0.20*

\* $P < 0.05$ ;\*\* $P = 0.01$  vs. non-transgenic samples using one-sided Wilcoxon rank sum test. Wildtype and total ras mRNAs were normalized to equal 1.00 in non-transgenic control mice. Human transgene mRNA was normalized to equal 1.00 in tTA/Hras line 1562-1 and tTA/Kras mice. These mice were not being administered Dox, so mutant ras transgene expression was induced.

†Data expressed as mean ± SD (n).

well-differentiated liver state. Paradoxically, albumin gene expression also was elevated. Albumin typically is expressed at the highest level in well-differentiated hepatocytes.

#### Interrupting mutant Hras<sup>G12V</sup> expression in adult liver reverses hyperplasia.

To determine whether Hras<sup>G12V</sup>-mediated growth effects were reversible, we removed Dox from 6- to 8-week-old bitransgenic line 1562-1 mice, then readministered Dox beginning 12–13 weeks later. After Dox removal, bitransgenic mouse livers increased in mass as a per cent of body weight (Table 5). However, after 2 weeks of Dox readministration to turn off transgene expression, liver weights decreased significantly to only 1.5-fold above the control values, and remained constant ( $8.0 \pm 1.3\%$  bw at 5 weeks post-Dox readmin-

**Table 4.** Relative hepatic gene expression†

Gene	Genotype (n)	
	Non-transgenic (4)	tTA/Hras (6)
<b>Nitrogen metabolism</b>		
Glutamine synthase (GS)	1.00 ± 0.47	0.23 ± 0.12**
Transporter of glutamate (Glt 1)	1.00 ± 0.65	0.20 ± 0.22*
Ornithine aminotransferase (Oat)	1.00 ± 0.79	0.11 ± 0.12**
Ornithine carbamylase (Otc)	1.00 ± 0.71	0.87 ± 0.26
Argininosuccinate synthase (Ass1)	1.00 ± 0.24	0.36 ± 0.19**
Argininosuccinate lyase (Asl)	1.00 ± 0.61	0.56 ± 0.27
Carbamoylphosphatase synthetase I (Cps)	1.00 ± 0.12	0.76 ± 0.30
Arginase (Arg 1)	1.00 ± 0.52	1.32 ± 0.60*
<b>Carbohydrate and lipid metabolism</b>		
Glucose-6-phosphatase (G6p)	1.00 ± 0.96	0.74 ± 0.33
Ketohexokinase (Khk)	1.00 ± 0.70	1.69 ± 0.55
Acetyl CoA carboxylase $\alpha$ (Acaca)	1.00 ± 0.34	0.80 ± 0.53
<b>Cellular differentiation/turnover</b>		
Albumin (Al)	1.00 ± 0.23	2.56 ± 1.09*
Alphafetoprotein (Afp)	1.00 ± 0.99	208 ± 131**
Cytochrome p450 2e1 (Cyp2e1)	1.00 ± 0.27	0.28 ± 0.18**
Cytochrome p450 7a1 (Cyp7a1)	1.00 ± 0.02	0.30 ± 0.24**
Cyclin D1 (Ccnd1)	1.00 ± 0.71	0.45 ± 0.16
Cyclin B1 (Ccnb1)	1.00 ± 0.21	4.50 ± 3.09*
Bax (Bax)	1.00 ± 0.03	1.93 ± 0.48**

\* $P \leq 0.05$ ;\*\* $P < 0.01$  vs. non-transgenic samples using two-sided Wilcoxon rank sum test.†Data expressed as mean  $\pm$  SD. Hepatic gene mRNAs were normalized to equal 1.00 in non-transgenic control mice. Transgenic line 1562-1 mice were not being administered Dox, so Hras<sup>G12V</sup> expression was induced.

istration,  $n = 10$ ). BrdU labelling index was elevated in mouse liver expressing Hras<sup>G12V</sup>, and returned to normal after Dox readministration. Finally, the apoptotic index was increased significantly by Hras<sup>G12V</sup>, and increased even more when Hras<sup>G12V</sup> expression was turned off by Dox. These findings indicated that mutant ras-induced liver hyperplasia is mostly although not completely reversible once transgene expression is interrupted in adult liver via a mechanism consistent with apoptosis.

#### Mutant Hras<sup>G12V</sup>, but not Kras<sup>G12D</sup> alters transplanted hepatocyte growth and transformation frequency

We used CHEGA to compare growth of mutant ras-expressing and control donor hepatocyte foci following transplantation. For all recipient animals at each time point post-transplant, we measured cross-sectional areas

**Table 5.** Reversibility of mutant Hras-induced liver phenotype in adult line 1562-1 mice†

Genotype (n):	Control (10, 11)	tTA/Hras (4, 5)	tTA/Hras (7, 8)
DOX status:	–	No DOX	14 days DOX
Transgene status:	none	ON	14 days OFF
Li wt/body wt	5.1 ± 1.0	17.8 ± 3.5**	8.0 ± 1.5‡ ***§
BrdU index	0.66 ± 0.68	1.7 ± 1.2*	1.0 ± 0.6
Apoptosis index	0.19 ± 0.17	0.69 ± 0.45**	2.0 ± 1.0***§

\* $P \leq 0.05$ ;\*\* $P < 0.01$ ;\*\*\* $P < 0.001$  vs. control samples using two-sided Wilcoxon rank sum test. Experimental mice were removed from Dox when they were 6- to 8-weeks-old, then 12–13 weeks later were euthanized (No DOX group) or readministered Dox for 2 weeks prior to euthanization (14 days DOX group).†Data expressed as mean  $\pm$  SD.

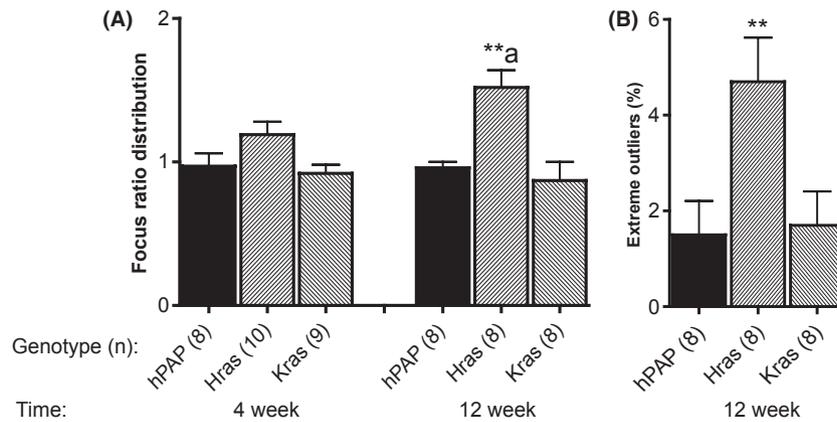
‡One mouse with Li wt/body wt of 21% was excluded from this calculation.

§ $P < 0.01$  vs. tTA/Hras No DOX samples using two-sided Wilcoxon rank sum test.

of 50 foci expressing hPAP and 50 foci expressing LacZ. To address inter-recipient focus growth variability, we normalized hPAP focus size distributions by dividing each hPAP focus cross-sectional area by the median lacZ (control) focus cross-sectional area from the same recipient. The median of this distribution should equal one if the lacZ and hPAP hepatocyte foci grow at the same rate; for normal hPAP-marked hepatocytes, all values were close to one (Fig. 1A).

Following transplantation of normal hepatocytes into 4-week-old MUP-uPA mouse livers, there is a permissive or 'growth phase' through 4 weeks post-transplant, during which transplanted cells expand as clonal foci. This is followed by a 'quiescence phase' that spans weeks 4–12 post-transplant, during which normal transplant foci do not grow further (18, 21). Kras<sup>G12D</sup> expression had no effect on hepatocyte growth at any time post-transplant (Fig. 1A). Hras<sup>G12V</sup> expression did not consistently affect hepatocyte growth in a growth-permissive liver environment, as determined by 4 week focus ratio distribution, but it was sufficient to induce sustained growth in quiescent liver (comparing 4–12 weeks post-transplant; Fig. 1A).

When examining focus ratio distributions, we observed occasional foci that were much larger than others in the distribution. We used the method of Tukey (26) to quantify the fraction of 'extreme outliers' in each 12 week focus ratio distribution (Fig. 1B). Tukey defined interquartile range (IQR) as the distance between the first and third quartile in a distribution and extreme outliers as values lying  $3 \times$  IQR or more above the third quartile. There was no increase in extreme growth outliers detected in Kras<sup>G12D</sup> foci. However, for Hras<sup>G12V</sup>, 4.7% of foci fell into the outlier class. These



**Fig. 1.** Focus ratio distribution medians (A), and outlier frequency (B) of transplanted hepatocyte foci.  $**P < 0.01$  vs. hPAP at the same time point using one-sided Wilcoxon rank sum test. <sup>a</sup>Statistically different ( $P = 0.015$ ) than Hras<sup>G12V</sup> 4 week time point using one-sided Wilcoxon rank sum test.

foci likely developed from transplanted cells that had accumulated additional genetic or epigenetic alterations that permitted excessive growth (21).

To identify cell turnover characteristics in transplant foci, we determined hepatocyte DNA synthesis (BrdU labelling) and apoptotic indices in foci during the growth (samples collected 2 weeks post-transplant,  $n = 4-6$ ) and quiescent (samples collected 8–12 weeks post-transplant,  $n = 5-6$ ) phases post-transplant. Relative to control foci, Hras<sup>G12V</sup> foci displayed slight, but significant ( $P < 0.05$ ) increases in apoptosis at 2 weeks ( $1.1 \pm 0.2$  vs.  $0.67 \pm 0.10$ ) and BrdU labelling at 8–12 weeks ( $2.1 \pm 0.4$  vs.  $1.1 \pm 0.6$ ), the latter consistent with the continued growth of these foci. No other differences were observed.

## Discussion

We designed these experiments to test two hypotheses. Firstly, we hypothesized that Hras<sup>G12V</sup> and Kras<sup>G12D</sup> would have different effects depending on whether expression was initiated in fetal or adult liver. Previously, in the AL- Hras<sup>G12V</sup> model, we observed that diffuse expression of Hras<sup>G12V</sup> in fetal hepatocytes caused liver hyperplasia leading to perinatal mouse death (13). Mice from lines surviving to adulthood had normal liver size, but developed hepatocellular carcinoma or lung neoplasia at 3–12 months of age (13, 27). In the present model, initiating expression of mutant H- or Kras caused similar progressive hepatomegaly and decreased animal survival regardless of whether expression was initiated in fetal or adult liver. These changes were observed with near-physiological levels of mutant ras expression. Our observations indicate that, following diffuse hepatocyte-targeted expression, the primary effect of either mutant ras is diffuse liver enlargement regardless of animal age, disproving our hypothesis. Diffuse growth could represent a direct effect of mutant ras

expression, or a compensatory increase in liver mass because of alterations in liver function induced by ras expression in hepatocytes.

Rosseland and colleagues identified different effects of Hras and Kras on proliferation and survival of cultured rat hepatocytes (28). Both Hras<sup>G12V</sup> and Kras<sup>G12V</sup> promoted hepatocyte survival (anti-apoptosis), Hras through ERK and PI3K and Kras only through PI3K. However, only Hras<sup>G12V</sup> enhanced ERK-mediated hepatocyte proliferation. These findings would explain our results if the chronic anti-apoptotic signals of either mutant ras render the liver non-responsive to normal signals regulating liver mass, thereby permitting hepatomegaly.

We indirectly evaluated liver function in bitransgenic mice by examining mouse serum clinical chemistry and hepatic gene expression. That increased ALT was found in only one tTA/Kras<sup>G12D</sup> mouse indicates that physical hepatocyte injury is not a general cause of the liver hyperplasia. However, Hras<sup>G12V</sup> generally decreased expression of hepatic genes involved in nitrogen metabolism and hepatocyte differentiation, and was accompanied by a 3-fold elevation in serum ammonia. Changes in the differentiation-specific genes *Afp*, *Cyp2e1* and *Cyp7a1* resembled changes observed in mice with hepatomegaly secondary to diffuse expression of the Wnt pathway protein  $\beta$ -catenin (22). The 200-fold increase in *Afp* expression and the increased albumin expression and serum concentration could account for the increased serum total protein. Proapoptotic *Bax* gene expression also was increased by Hras<sup>G12V</sup> (this study) and  $\beta$ -catenin (22). Neither changed expression of *cyclinD1*, but Hras<sup>G12V</sup> significantly increased *cyclinB1* expression. Interestingly, changes in nitrogen metabolizing genes were opposite to those identified in  $\beta$ -catenin mice, demonstrating that the patterns of gene expression changes are regulated differently by the different oncogenes. Overall, the changes observed follow-

ing Hras<sup>G12V</sup> expression suggest induction of a periportal hepatocyte metabolic phenotype, whereas  $\beta$ -catenin induces a pericentral hepatocyte metabolic phenotype (22, 29).

Finally, we demonstrated that the Hras<sup>G12V</sup>-induced growth phenotype is almost fully reversible via apoptosis, indicating that maintenance of increased liver weight requires the continuous presence of mutant ras, and consistent with the reported association of mutant ras expression with hepatocyte survival (28). Loss of Hras<sup>G12V</sup> anti-apoptotic activity promotes liver mass normalization via hepatocyte death. Regarding the association of ras expression with neoplasia, only a minority of ras-expressing animals (23%) developed hepatocellular neoplasms. Incomplete penetrance likely reflects animal mortality secondary to hepatomegaly.

Compare these results with those of Schuhmacher and colleagues (30) and Chen and colleagues (31). Their models activated expression of Hras<sup>G12V</sup> in all tissues beginning in fetal development from an endogenous Hras allele. Schuhmacher *et al.* (30) developed an Hras locus knock-in that created a polycistronic mRNA with Hras<sup>G12V</sup> coding sequence-IRES- $\beta$ -galactosidase coding sequence. They identified no liver phenotype, and could not identify any downstream evidence of active Hras<sup>G12V</sup> signalling, which they attributed to negative feedback.

Chen *et al.* (31) described different results. They identified a severe phenotype in mice expressing an otherwise unmodified Hras<sup>G12V</sup> allele from the endogenous Hras locus. These mice had 80% perinatal lethality and, in survivors, a highly penetrant neoplastic phenotype in skin, stomach and endothelium. Cause of death of neonates was not determined, and, in particular, the liver was not described. Liver in surviving mice was not altered in size, but displayed elevated pMEK and pERK. They did not quantify Hras<sup>G12V</sup> expression in liver. They addressed the stark difference between their model and Schuhmacher's by suggesting that the IRES- $\beta$ -galactosidase cassette interfered with expression of Hras<sup>G12V</sup> such that a threshold for tumorigenesis was not reached.

Neither of those studies definitively addressed the hepatic consequences of physiological levels of Hras<sup>G12V</sup> expression. Schuhmacher did not precisely quantify Hras<sup>G12V</sup> expression or identify the pattern of expression in liver, and, as noted by Chen, the Hras<sup>G12V</sup> expression may be altered by the targeting strategy, which resulted in a bicistronic mRNA. Chen did not quantify or determine the pattern of hepatic Hras<sup>G12V</sup> expression. They also determined the phenotype in only the 20% minority of mice that survived beyond 14 days of age. Something must have been different about this subset of mice for the animals to have been selected for survival. A likely candidate would be a different pattern or level of expression/effects in survivors than in the majority that did not survive. By using the transgenic approach in our studies, we restricted expression to hepatocytes, thus eliminating the possibility that secondary effects on another organ may influence liver

phenotype, or that primary effects on other organs may overshadow a liver phenotype. We also could turn on transgene expression at a time of our choosing, rather than being limited to examining the effects of altered genes throughout development. To address the concern that transgene expression could be at a non-physiological level, thereby inducing diffuse hepatic toxicity, we measured transgene expression using RT-PCR. We identified near-physiological levels of transgene expression. Measurement of serum ALT also indicated that liver toxicity was not a common feature of these mice.

Next, we questioned whether different ras family members could have different biological effects on hepatocarcinogenesis; recall that, when expressed diffusely in mouse liver in our model, their effects appeared to be the same. To examine this question in more detail, we employed our hepatocyte transplantation assay. Following transplantation, normal hepatocytes expand as foci of cells up to 4 weeks post-transplant (growth phase), whereas no significant additional expansion occurs between 4 and 12 weeks following transplantation (quiescence phase). Neither Hras<sup>G12V</sup> nor Kras<sup>G12D</sup> induced consistently increased growth during the growth-stimulatory phase compared with normal cells (assessed at 4 weeks). However, Hras<sup>G12V</sup> but not Kras<sup>G12D</sup> induced continuous increased growth during the quiescent phase (weeks 4 through 12 post-transplant). We included in donor cells, a regulated marker transgene (tetO-hPAP) that, like the ras transgenes, is tTA-responsive. All hepatocyte foci displayed the blue stain, indicating that tTA activity was present in both Hras<sup>G12V</sup> and Kras<sup>G12D</sup> foci, ruling out failure of expression of Kras<sup>G12D</sup> as a cause of the different focus growth characteristics. These findings conform to expectations based on the report of Rosseiland and colleagues: whereas both mutant Hras and Kras have anti-apoptotic properties, only Hras<sup>G12V</sup> can enhance hepatocyte proliferation via the ERK pathway (28).

The expansion of focus size in quiescent liver identifies hepatic growth as a cell autonomous effect of Hras<sup>G12V</sup>. This finding also confirms that Hras<sup>G12V</sup> expression in our model is not directly hepatotoxic. Drinkwater and colleagues correlated increased carcinogen sensitivity and more rapid growth of altered hepatic foci with increased frequency of Hras mutations in different mouse strains following administration of the hepatocarcinogen diethylnitrosamine (32). Our data indicate that Hras<sup>G12V</sup> expression is sufficient for this growth effect in FVB strain mice, identifying one important mechanistic feature of Hras<sup>G12V</sup>-mediated transformation that is not shared by Kras<sup>G12D</sup>. When Harada and colleagues initiated focal hepatocyte expression of Hras<sup>G12V</sup> in adult liver using retrovirally delivered cre recombinase, altered foci and neoplasms were not observed (12). In that system, Hras<sup>G12V</sup> expression is likely activated independently in single hepatocytes, whereas our transplant system involves mutant Hras<sup>G12V</sup> expression in foci of 2000–3000 hepatocytes

(18, 21). Thus, Hras<sup>G12V</sup> expression appears to be insufficient to induce cell autonomous growth in single initiated hepatocytes. Apparently, additional replication of Hras<sup>G12V</sup>-expressing cells into foci is necessary to permit Hras<sup>G12V</sup>-mediated focus-autonomous growth.

We identified a second important difference between effects of mutant H- and Kras. Transplant foci expressing Hras<sup>G12V</sup> displayed a significantly increased outlier fraction (4.7%). Kras<sup>G12D</sup> and mutant  $\beta$ -catenin did not have this effect (22). This finding indicates that Hras<sup>G12V</sup> is associated with a higher frequency of cells and foci with extreme growth characteristics. These large foci resemble preneoplastic foci, which have accumulated additional genetic and/or epigenetic changes (21). Thus, in this context, Hras<sup>G12V</sup> is associated with enhanced neoplastic progression. A differential rate of conversion of foci to neoplasms may explain in part the more frequent identification of Hras than Kras mutations in spontaneous and induced mouse liver tumours. Our data identifies mechanistic roles for Hras<sup>G12V</sup> mutation in the stages of promotion and progression. Kras<sup>G12D</sup> does not support these mechanistic effects in mouse liver.

In summary, diffuse hepatic expression of either Hras<sup>G12V</sup> or Kras<sup>G12D</sup>, initiated in fetal or adult liver, results in hepatomegaly and reduced mouse survival. However, when expressed in focal collections of hepatocytes, their effects are markedly different. Only Hras<sup>G12V</sup> increases the likelihood of progression to cancer, likely via the mechanism of induction of cell-autonomous hepatocyte focus growth in an otherwise quiescent liver, and increased incidence of outlier focus development. These results confirm *in vivo* that mutant Hras and mutant Kras can have distinct growth-related effects on hepatocytes, and may explain the preponderance of Hras mutations vs. Kras mutations in mouse liver neoplasia.

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