Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

BASIC STUDIES

Minimal cooperation between mutant Hras and c-myc or TGFα in the regulation of mouse hepatocyte growth or transformation *in vivo*

Timothy J. Stein*, Margaret Bowden and Eric P. Sandgren

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

Keywords
c-myc – hepatocellular carcinoma – hepatocyte transplantation – Hras – TGFα

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.

Received 7 April 2011
Accepted 22 June 2011

DOI:10.1111/j.1478-3231.2011.02596.x

Abstract

Background: Liver carcinogenesis is associated with multiple genetic changes in affected cells, including alterations in the Hras signalling pathway. *Aim*: To define the biological contributions of Hras to mouse hepatocarcinogenesis, we quantified *in vivo* interactions between mutant Hras and other genetic alterations frequently associated with liver cancer, including overexpression of the transcription factor c-myc and the epidermal growth factor receptor ligand transforming growth factor alpha (TGFα). *Methods*: To accomplish this aim, we initiated expression of an activated Hras in hepatocytes of adult mice with or without simultaneous overexpression of either c-myc or TGFα. Potential interactions also were assessed through the use of the comparative hepatocyte growth assay, a hepatocyte transplantation assay that measures effects of altered gene expression on hepatocyte growth *in vivo*. *Results*: Hras expression caused diffuse liver enlargement (hepatomegaly), and this phenotype was not changed by co-expression of c-myc or TGFα. Using the transplant system, we found that expression of mutant Hras alone was sufficient to induce hepatocyte focus growth in a quiescent liver. Paradoxically, adding expression of TGFα or c-myc reversed this Hras effect. Finally, the frequencies of transplant foci with the preneoplastic feature of extreme growth potential and of liver neoplasms were increased for Hras and both combinations when compared with control hepatocytes, but did not differ among oncogene-expressing groups. *Conclusions*: Hras-associated hepatocyte growth deregulation is not complemented by activation of c-myc or TGFα growth signalling pathways in mouse liver. This finding emphasizes the tissue-specific character of molecular growth regulation.

Hras is the most frequently mutated oncogene in spontaneous and induced liver tumours of mice (1–4), and Hras mutation is thought to be one mechanism of liver cancer initiation. To further define the contribution of activated ras family gene mutations in the process of hepatocarcinogenesis, Figueiredo and colleagues developed a tetracycline-responsive system (5, 6) to create transgenic mice with liver-targeted expression of inducible mutant Hras or mutant Kras (Figueiredo, Stein, Sandgren, manuscript submitted). Hepatic expression of mutant Hras or Kras initiated within either fetal or adult mice resulted in hepatomegaly. The effect of mutant ras on hepatocyte growth homeostasis was assessed in FVB strain mice using the comparative hepatocyte growth assay (CHeGA) (7), an assay that measures growth of transplanted hepatocytes in a diseased recipient mouse liver. Hepatocyte expression of mutant Hras did not increase the rate of growth of transplant foci within a growth-permissive environment. However, it was sufficient to induce continued focus growth in a growth-restrictive environment. The presence of activated Hras also was associated with an increase in the frequency of extreme outlier foci, which are hepatocyte foci displaying the extreme growth potential typical of the preneoplastic phenotype. Surprisingly, mutant Kras was unable to induce any change in transplanted hepatocyte growth. These findings indicated that mutant Hras-induced growth effects were cell autonomous in hepatocyte foci, and identified a further role for this gene in liver

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

© 2011 John Wiley & Sons A/S

tumour promotion and progression in addition to its known role during initiation.

Like other cancers, liver cancer is believed to be a multistep disease (8). Other genetic alterations are associated with hepatocellular carcinoma (HCC) in mice, in addition to Hras activation. The transcription factor c-myc is amplified and/or overexpressed in HCC from mice and humans (9). Overexpression of transforming growth factor alpha (TGFα) has been implicated in hepatocarcinogenesis, and it is a potent hepatocyte mitogen (10). Current dogma suggests that molecular changes cooperate during neoplastic cooperation.

In this report, we describe the effects on hepatocyte growth homeostasis of Hras mutation in combination with other genetic alterations commonly found in HCC. We have employed classical transgenic modelling methodologies, targeting genetic alterations to mouse hepatocytes and characterizing gross and microscopic lesions arising in these mice. One limitation of classical transgenic models is the diffuse transgene expression in the organ of interest. Cancer is a disease that arises when single or small collections of cells gain a selective growth advantage. Using a hepatocyte transplantation system, we are able to assess the effect of genetic alterations on hepatocyte growth homeostasis in a way that more faithfully reproduces the natural disease. CHeGA allows us to quantify the growth characteristics, in both growth-permissive and growth-restrictive environments, of separated foci of cells that arise from individual hepatocytes transplanted into a diseased liver. This assay also permits us to measure the frequency with which single or combined genetic changes affect the likelihood that foci will acquire the potential for continuous growth in an otherwise quiescent liver. Surprisingly, we determined that, with respect to hepatocyte growth deregulation, c-myc and TGFα do not appear to complement mutant Hras.

Materials and methods

Animals

Mice were maintained according to The Guide for the Care and Use of Laboratory Animals in an AAALAC-accredited facility. 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-uPA line 350-2, TgN (MupPlau) 1Eps; hsMT-LacZ line 379-4, TgN (MtlnLacZ)4Eps; R26-hPAP line 808-6, TgN(R26ALPP)5Eps; MT-TGFα line 641-3, TgN(Mt1Tgfa)149Bri; AL-c-myc line 741-3, TgN(Alb1Myc)82Bri; tetOCMV-Hras line 1562-1, TgN (tetOpHRAS^{G12V})19EPS; LAP-tTA, TgN(tTALap)5Uh.

Mice bearing oncogene-expressing transgenes have been described (11, 12; Figueiredo, Stein, Sandgren, manuscript submitted). Briefly, inducible Hras expression was targeted to hepatocytes using the liver-enriched activator protein (13) gene promoter-tetracycline transactivator activator (tTA) transgene (described in Results). Constitutive c-myc expression was targeted using the albumin (AL) enhancer/promoter, which initiates expression in fetal liver as it begins to differentiate from gut endoderm. Constitutive TGFα expression was targeted using the metallothionein (14) enhancer/promoter. MT is expressed in fetal hepatoblasts and adult hepatocytes, as well as additional epithelial cell lineages. MT expression can be increased in hepatocytes by administration of zinc to drinking water.

Mice carrying transgene constructs used to differentially mark donor hepatocytes have been described (15, 16). To generate experimental tri- and quad-transgenic mice, breeding was set up between appropriate lines. All experimental mice were of the FVB6F1 strain. All transgenic mice were identified using PCR analysis of tail DNA.

Lesions were classified on fixed and stained tissue sections. Altered hepatocyte foci (AHF) were identified as small, well-circumscribed patches of parenchyma with atypical hepatocyte morphology. Hepatocytes in AHF typically were smaller than normal adjacent hepatocytes, more basophilic and slightly compressing surrounding normal parenchyma. Hepatocellular adenomas were identified as hepatocyte masses greater than one millimeter in diameter with a solid pattern of hepatocyte organization and lack of bile duct epithelial cells. Hepatocellular carcinomas (HCC) were identified as hepatocyte masses greater than one millimeter in diameter with a trabecular patter of hepatocyte organization and lack of bile duct epithelial cells.

Comparative hepatocyte growth assay (CHeGA)

The comparative hepatocyte growth assay (CHeGA) is a transplantation-based assay used to quantify the effect of oncogene expression on hepatocyte growth homeostasis *in vivo* (7, 17). In this assay, two differentially marked populations of hepatocytes are isolated from two donor transgenic mice via a two-step EDTA/Collagenase procedure. One population of hepatocytes contains the LacZ marker transgene encoding β-galactosidase. The second population expresses the hPAP marker and one or more oncogene-encoding transgenes. Hepatocyte populations are combined in a 1:1 ratio of viable cells, and surgically transplanted into recipient mice in 10 μl via intrasplenic injection within 6 h of isolation. Recipient mice are 3- to 4-weeks of age with uPA-mediated liver disease. Transplanted hepatocytes travel from the spleen to the liver via the portal circulation and proliferate within hepatic parenchyma as foci through clonal expansion. By 4 weeks post-transplant, the hepatic parenchyma has been replaced by a mixture of donor hepatocytes and endogenous hepatocytes that no longer express the

© 2011 John Wiley & Sons A/S

uPA transgene, and the liver becomes quiescent. The effect of growth regulatory gene alterations on hepatocyte growth is evaluated by comparing the clonal progeny of the two types of donor hepatocytes within each recipient at 4 and 12 or more weeks post-transplant using enzyme histochemistry to identify β-galactosidase and hPAP. The surface cross-sectional area of about 50 foci of the hPAP donor type and at least 15 foci of the LacZ donor type is determined for each recipient mouse. For each donor group to be evaluated, at least two separate donor cell preparations and multiple recipients are evaluated at each time-point post-transplant. Data are expressed as a ratio between median cross-sectional areas of experimental (oncogene plus hPAP) vs. normal (lacZ) donor hepatocyte foci within each mouse to control for interanimal variation in absolute focus size (focus ratio distribution).

Quantification of hepatocyte proliferation and apoptosis

One hour prior to euthanasia, all mice were administered 200 mg/kg bromodeoxyuridine (BrdU) IP, a nucleotide analogue incorporated into the DNA of cells in the synthesis phase of cellular replication. Cells undergoing replication were detected using immunohistochemistry as described (17). BrdU index outliers were identified as the percent of all hepatocyte nuclei that incorporated BrdU. Apoptotic hepatocytes were identified using morphological criteria, specifically chromatin condensation and nuclear fragmentation, eosinophilic cytoplasm and cell shrinkage. Apoptotic index was calculated as the percent of all hepatocyte nuclei examined displaying evidence of apoptosis.

Statistical analyses

Data were compared using one- or two-sided Wilcoxon rank sum comparisons. Transplant focus was calculated using the method of Tukey (18). Sample sizes for transplant studies were selected based on earlier studies (7, 17).

Results

Hepatocyte-directed expression of either c-myc or TGFα does not complement mutant Hras expression during growth deregulation of mouse liver

Mutant Hras was expressed in liver using the liver-activated protein tetracycline transactivator (LAP-tTA) transgene and a target transgene carrying tet-operator cytomegalovirus minimal promoter fused to a mutant Hras coding sequence (tetOCMV-Hras). In bitransgenic mice, binding of tTA to the minimal promoter induces expression of mutant Hras. Doxycycline (Dox) abrogates tTA binding to the tetO sequence. For these studies, all mice were maintained on dox-treated drinking water to repress mutant Hras transgene expression until 6 weeks of age. For TGFα transgenic mice, the Dox water was replaced by water treated with 50 mM zinc sulphate to induce hepatocyte expression of TGFα transgene from the heavy metal responsive MT-promoter. All other transgenic mice had Dox water replaced with regular drinking water. Animals were euthanized when they showed signs of disease, identified as lethargic and a prominently palpable caudal spine. Nontransgenic, AL-myc and MT-TGFα mice did not reach endstage, but were euthanized at ages comparable to Hras/myc and Hras/TGFα mice for liver weight comparisons. There was no significant difference in the time to endstage among transgenic mice expressing activated Hras alone and activated Hras in combination with myc or TGFα (Table 1). As expected, the liver weight as a percentage of body weight was elevated significantly in all Hras-expressing groups compared with other mice, but was not different among Hras, Hras/myc and Hras/TGFα groups (Table 1). Grossly, at endstage, all livers from mice expressing Hras alone or in combination with other genetic alterations were uniformly enlarged (diffuse hepatomegaly). The BrdU labelling and apoptotic indices of hepatocytes expressing Hras alone and Hras in combination with the other genetic alterations were increased significantly compared with hepatocytes of age-matched control mice (Table 1). There were slight differences in BrdU labelling indices of hepatocytes expressing Hras alone vs. Hras in combination with other genetic alterations. Apoptosis was increased significantly in tTA/Hras/AL-myc and tTA/Hras/MT-TGFα mouse livers compared with Hras alone.

Microscopically, the endstage livers from mice expressing Hras alone displayed a moderately heterogeneous hepatocyte population consisting of large and small hepatocytes (Fig. 1A,B). Most Hras/myc-endstage livers appeared similar (Fig. 1C). Liver from Hras/TGFα mice at endstage consisted of hepatocytes with variable cellular and nuclear sizes and cytoplasmic vacuoles (Fig. 1D). Only 3 of 11 Hras/TGFα livers displayed trabecular HCC (Table 1; Fig. 1E). However, accurate comparison of the hepatocarcinogenic effects of these oncogenes was not possible because of the development of diffuse hyperplasia as the cause of death in all groups, and so instead, we measured comparative carcinogenicity using the more precise hepatocyte transplant assay described below.

Hepatocyte-directed expression of c-myc or TGFα can inhibit the effects of mutant Hras on transplanted hepatocyte growth

We compared the effect on transplanted hepatocyte growth of mutant Hras alone and Hras in combination with c-myc or TGFα using CHeGA (see Materials and methods). Previous work identified two distinct phases of growth in transplant recipient mouse liver (7). Through week 4 post-transplantation, there is a

© 2011 John Wiley & Sons A/S

Table 1. Effect on liver growth of hepatocyte-specific expression of mutant H-ras alone or in combination with other genetic alterations

Transgene(s)	Age, weeks	Li/body wt. (%)	BrdU (%)	Apoptosis(%)	Incidence of HCC
Control	17.2 ± 2.5 (6)	5.4 ± 0.5 (6)	0.10 ± 0.1 (8)	0.02 ± 0.04 (6)	-
AL-myc	19.3 ± 1.5 (9)	6.1 ± 0.2 (9)**	0.28 ± 0.2 (8)*	0.10 ± 0.12 (5)	-
MT-TGFα	17.9 ± 1.2 (12)	9.3 ± 0.5 (12)***	0.30 ± 0.3 (11)	0.03 ± 0.04 (5)	-
tTA/Hras	17.4 ± 3.3 (16)	17.0 ± 3.0 (16)***	0.57 ± 0.5 (5)*	0.19 ± 0.16 (6)*	0/10
tTA/Hras/AL-myc	19.3 ± 1.8 (8)	17.6 ± 3.7 (8)***	0.83 ± 0.3 (5)***	1.10 ± 0.38 (7)**‡	0/8
tTA/Hras/MT-TGFα	19.7 ± 6.3 (10)	17.3 ± 3.9 (10)***	1.16 ± 0.7 (11)***†	0.57 ± 0.29 (9)***‡	3/11

Data presented as mean ± SD (*n*). Significantly different than non-transgenic controls using one-sided Wilcoxon rank sum test: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

†Different ($P = 0.06$) than tTA/Hras.

‡Significantly different ($P < 0.01$) than tTA/Hras.

HCC, hepatocellular carcinoma.

growth-permissive environment or 'growth phase', whereas weeks 4 through 12 is a 'quiescent phase'. Transplanted normal hepatocytes clonally expand during the growth phase, creating multiple foci throughout the liver that can be measured, then growth stops during the quiescent phase. Consistent with previous studies (7, 17), there was no difference in the mean cross-sectional surface area between hPAP marker-only hepatocyte populations at any time post-transplant (focus ratio distribution ≈ 1) (Fig. 2A). As in the FVB strain (Figueiredo, Stein, Sandgren, manuscript submitted), Hras in the FVB6F1 strain induced continuous focus growth. At 12 weeks, Hras focus size was significantly larger than at 4 weeks, indicating focus growth in a quiescent hepatic environment. To confirm that growth was progressive, we also calculated focus ratio distributions at 16 weeks (5.8 ± 1.3, $n = 5$) and 20 weeks (8.7 ± 2.1, $n = 7$) post-transplant. As predicted, each value was significantly higher than the 12 week value ($P < 0.01$).

As reported earlier (7), expression of TGFα or c-myc was associated with increased growth in the growth-permissive phase (assessed at 4 weeks), and increased growth also was observed in the present study when either oncogene was combined with mutant Hras (Fig. 2A). Unexpectedly, neither could enhance the rate of Hras-induced focus growth in quiescent liver, and instead appeared to eliminate the progressive growth (the larger focus size in the Hras/myc foci at 12 weeks was not statistically different than the 4 week value). Thus, the combinations induced a more normal pattern of growth than Hras alone.

Measures of proliferation in transplanted hepatocyte foci were consistent with focus growth characteristics. In livers collected at 2 weeks post-transplant during the growth-permissive phase, Hras and Hras/myc focus hepatocytes had a significantly increased BrdU labelling index compared with controls (Table 2). The addition of TGFα to H-ras actually decreased BrdU labelling during the growth-permissive (2 weeks) phase. At the 8 week timepoint, a quiescent or growth-restrictive environment as at 12 weeks, transplant foci originating from Hras, Hras/myc and Hras/TGFα hepatocytes had similar BrdU labelling indices, each higher than controls (Table 2).

Finally, the method of Tukey (18) was utilized to identify 'extreme outlier' (EO) foci in our data. Foci in an individual recipient liver were considered extreme outliers when their cross-sectional surface area was three times the interquartile range above the 75th percentile within their distribution. These foci resemble pre-neoplastic foci and therefore provide a quantitative measure of neoplastic progression (7). The EO percentage for each animal at the 12 week timepoint was averaged to give the EO frequency for each donor genotype. Mutant Hras alone plus both combinations significantly increased the frequency of extreme outliers at 12 weeks post-transplant compared with controls (Fig. 2B), but there were no significant differences among the various combinations, indicating they did not interact to change outlier frequency.

Transplant recipients develop liver neoplasia

A subset of transplant recipients from each combination was maintained until they developed signs of disease, as defined by lethargy and loss of muscle mass over the caudal spine. There was no statistically significant difference in the time to endstage disease development between Hras and Hras/TGFα mice, but endstage disease actually was delayed in Hras/myc mice compared with Hras only (Table 3). Mice in all groups displayed preneoplastic and/or neoplastic lesions (Table 3 and Fig. 1 F–I). Liver sections were stained for expression of the hPAP transgene, which verified that all lesions originated from transplanted hepatocytes (Fig. 1 I, J). Thus, neither c-myc nor TGFα complement mutant Hras expression in this assay of hepatocarcinogenesis.

Discussion

The high frequency of Hras mutations in spontaneous and induced mouse liver tumours suggests that altera-

© 2011 John Wiley & Sons A/S

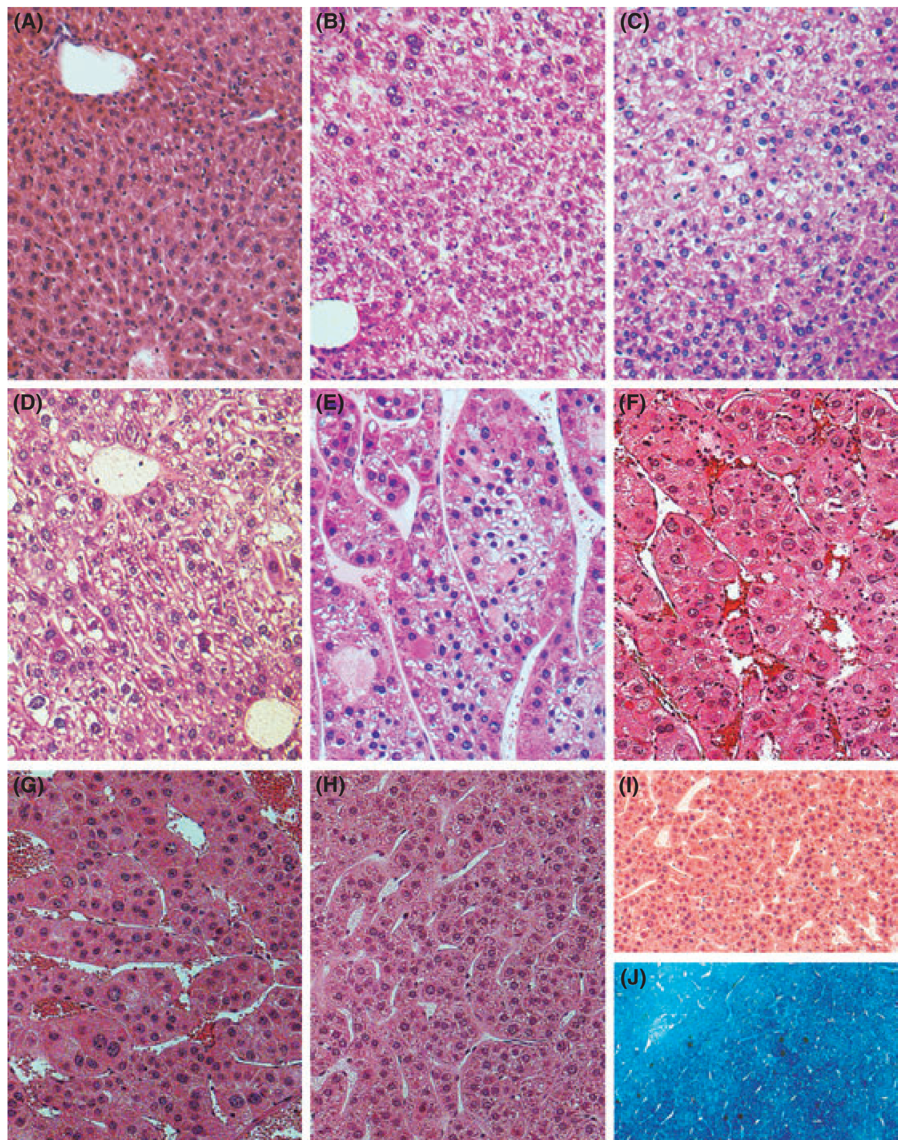

Fig. 1. A–E. Microscopic appearance of liver in transgenic mice. (A) Non-transgenic control liver; (B) Hras liver; (C) Hras/myc liver; (D) Hras/TGFα liver; (E) trabecular HCC in an Hras/TGFα liver. F–J. Microscopic appearance of liver in MUP-uPA mice receiving transplanted hepatocytes expressing mutant Hras alone or in combination with other genetic alterations. Well-differentiated trabecular HCC in livers from mice receiving (F) Hras hepatocytes, (G) Hras/c-myc hepatocytes and (H) Hras/TGFα hepatocytes. (I) Hras/TGFα recipient liver showing an adenoma; (J) BCIP-stained Hras/TGFα-induced adenoma, showing dark colouration indicating expression of the hPAP marker transgene. A-I, H&E; J, hPAP histochemistry. A–H, 200x; I, J, 100x.

tions in this ras signalling pathway play an important role in mouse hepatocarcinogenesis. The frequency of Hras mutations in human liver tumours is relatively low. However, Calvisi and colleagues found Ras and Jak/Stat pathways to be activated to a greater extent in all human HCC when compared with surrounding non-neoplastic and normal liver tissue via selective suppression of one or more Ras GTPase activating proteins (19, 20). These results indicate that activation of Ras signalling pathways is important in both rodent and human hepatocarcinogenesis, though mechanisms of pathway activation may differ between species.

We have reported that expression of mutant Hras resulted in rapid and diffuse liver enlargement, regardless of whether expression was initiated in fetal or adult hepatocytes (Figueiredo, Stein, Sandgren, manuscript submitted). We now have extended these studies to assess potential interactions in the adult liver between mutant Hras and two other genetic alterations commonly found in liver cancer, overexpression of c-myc or TGFα. Unexpectedly, the effect of combinations

© 2011 John Wiley & Sons A/S

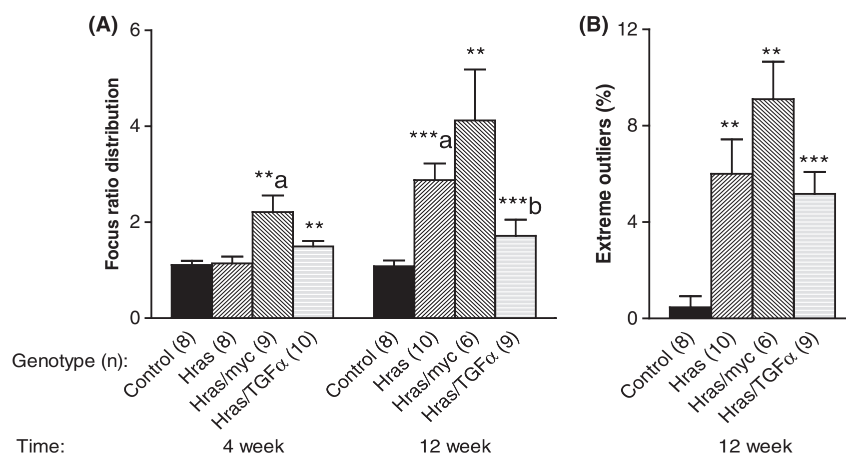

Fig. 2. Focus ratio distribution medians (A) and outlier frequency (B) of transplanted hepatocyte foci. $**P < 0.01$; $***P < 0.001$ vs. hPAP at the same time point using one-sided Wilcoxon rank sum test. [a]Statistically different ($P < 0.05$) than Hras 4-week time point using two-sided Wilcoxon rank sum test. [b]Statistically different ($P < 0.01$) than Hras 12 week time point using two-sided Wilcoxon rank sum test. Hras/myc 12 week vs. 4 week, $P = 0.11$ (one-sided). Hras/TGFα 12 week vs. 4 week, $P = 0.42$ (one-sided).

Table 2. Cell proliferation indices in donor-derived hepatocyte foci

Transgene(s)	Weeks post-transplant (# mice)	BrdU% Mean±SD†
hPAP only	2 (6)	6.9 ± 4.4
	8 (9)	0.6 ± 0.3
tTA/Hras	2 (4)	22.6 ± 7.8**
	8 (4)	3.8 ± 2.2*
tTA/Hras/myc	2 (5)	19.6 ± 8.9*
	8 (5)	7.9 ± 2.3*
tTA/Hras/TGFα	2 (7)	12.0 ± 7.1‡
	8 (6)	4.4 ± 1.4*

†Significantly different than hPAP at the same timepoint using one-sided Wilcoxon rank sum test: $*P < 0.5$; $**P < 0.01$.
‡Statistically different ($P = 0.05$) than Hras 2 week timepoint using two-sided Wilcoxon rank sum test.

did not differ greatly from that of mutant Hras alone. Expression of activated Hras alone and in combination with myc or TGFα resulted in diffuse liver enlargement with a rapid onset. The time to endstage and the endstage liver weight as a percentage of body weight were similar among all groups. The development of diffuse liver enlargement may be the result of either a primary transgene effect or a compensatory enlargement of the liver to replace a functional deficit induced by the expression of activated ras in hepatocytes. Interestingly, apoptosis was increased in livers of Hras/c-myc and Hras/TGFα mice compared with Hras alone, consistent with a mechanism of liver weight regulation by the balance of cell proliferation and cell death.

The transplantation-based CHeGA allowed us to quantify directly the effects of Hras in combination with overexpression of myc or TGFα on hepatocyte growth characteristics. Mutant Hras alone does not increase hepatocyte growth under growth-stimulatory conditions. However, it does induce continued hepatocyte growth in an otherwise quiescent environment in a cell autonomous fashion, an important characteristic of cells involved in neoplastic progression. The ability of Hras to induce cell autonomous growth allows for other genetic alterations to become fixed in progeny cells, thereby favouring neoplastic progression. In this report, we find that hepatocytes with the Hras/c-myc combination display increased focus growth during

Table 3. Endstage disease in transplant recipients

Transgene(s) (# mice)	Weeks post-transplant mean ± sd	Lesions AHF	Adenoma	HCC
tTA/Hras (10)	24.0 ± 3.2	10/10	9/10	3/10
tTA/Hras/AL-myc (7)	45.8 ± 17.1*	6/7	0/7	5/7
tTA/Hras/MT-TGFα (10)	31.8 ± 17.3	10/10	2/10	3/10

*Significant difference ($P < 0.001$) in survival between Hras and Hras/myc using two-sided Wilcoxon rank sum test.
AHF, altered hepatocyte focuskl; HCC, hepatocellular carcinoma.

© 2011 John Wiley & Sons A/S

the growth-stimulatory phase, as expected, given the ability of c-myc alone to produce this effect (7), but did not induce significant ($P = 0.11$) continued growth in the quiescent phase. The Hras/TGFα combination increased focus growth within the permissive environment, again reflecting earlier findings with TGFα alone, but did not induce significant ($P = 0.42$) continued growth in the quiescent phase. Lack of progressive growth by hepatocyte foci expressing oncogene combinations is surprising, given the ability of Hras alone to accomplish this effect. In this context, the effects of Hras and TGFα or Hras and c-myc are not additive. In fact, co-expression can have a negative effect on hepatocyte growth when compared with Hras alone. TGFα binds to and activates the epidermal growth factor receptor (EGFR), which, like ras, utilizes the mitogen-activated protein kinase (MAPK) cascade for propagation of its signal. The constitutive signal from mutant Hras may saturate this pathway, leaving the TGFα signal propagated principally through other pathways, some of which then inhibit hepatocyte growth. This would be consistent with the greater BrdU labelling index of transplanted hepatocytes expressing Hras alone compared with Hras/TGFα at 2 weeks post-transplant (Table 2), and the increase in apoptosis in Hras/TGFα mouse hepatocytes compared with Hras alone (Table 1). Lack of cooperation between c-myc and Hras was unexpected, as these oncogenes strongly complement each other in other *in vivo* experimental systems. Again, this combination displayed increased apoptosis compared with Hras alone, suggesting that an increased rate of cell death slowed the growth of these foci.

A small fraction (18%) of Hras/TGFα mice displayed hepatocellular carcinoma at endstage, whereas no carcinomas were observed in the Hras or Hras/c-myc groups. However, the potent growth stimulus of diffuse activated ras expression in this system precluded us from rigorously assessing carcinogenic interactions between oncogenes in these mice. Instead, carcinogenicity was compared in a more quantitative way using hepatocyte transplant recipient mice. The frequency of extreme outlier formation in CHeGA was not different when combinations were compared with Hras alone. EO foci probably represent the clonal expansion of donor hepatocytes that had accumulated additional genetic alterations, either pre-transplantation or in the earliest stages of liver repopulation, thereby conferring upon them the pre-neoplastic feature of extreme growth potential (7). Thus, there are no indications of cooperation between Hras and c-myc or TGFα in this measure of carcinogenesis. Finally, endstage transplant recipients, in which we could measure potential carcinogenic interactions because the whole liver was not diffusely affected by transgene expression, also failed to demonstrate oncogene cooperativity in progression to malignancy (Table 3). At endstage, Hras/c-myc transgenic mice displayed the highest incidence of HCC (five of seven mice examined), but these mice also were the oldest among groups.

In striking contrast, c-myc and TGFα in this experimental system have additive to synergistic effects when combined: they complement each other to increase growth in permissive (though not quiescent) liver, and together, but not alone, increase focus outlier frequency (7). We and others also have demonstrated potent c-myc/TGFα cooperation in several mouse models of HCC (21–23). Similarly, Hras and mutant β–catenin interact to enhance hepatocarcinogenesis (24).

In summary, combining transgenic and hepatocyte transplant methodologies allows us to more precisely define mechanistic contributions of oncogenes to hepatocarcinogenesis. Overexpression of c-myc and TGFα and mutation of Hras are common features of mouse HCC. Mutant Hras, when expressed in foci of hepatocytes, induces cell autonomous hepatocyte growth in quiescent liver and increases growth outlier frequency (Figueiredo, Stein, Sandgren, manuscript submitted). C-myc, and to a lesser extent TGFα, increase the rate of hepatocyte growth under permissive conditions, and together, though not separately, increase growth outlier frequency (7). However, our findings indicate that mutant Hras appears not to cooperate with those other oncogenes, demonstrating that the precise combination of genetic lesions rather than their total number defines the course of liver cancer progression.

Acknowledgements

We thank Adam Jochem for technical assistance. This work was supported by the National Institutes of Health (RO1-DK49787, RO1-ES07671 and PO1-CA022484 to EPS and KO1-RR020033 and 1UL1RR025011 to TJS).

References

1. Maronpot RR, Fox T, Malarkey DE, Goldsworthy TL. Mutations in the ras proto-oncogene: clues to etiology and molecular pathogenesis of mouse liver tumors. *Toxicology* 1995; **101**: 125–56.
2. Reynolds SH, Stowers SJ, Maronpot RR, Anderson MW, Aaronson SA. Detection and identification of activated oncogenes in spontaneously occurring benign and malignant hepatocellular tumors of the B6C3F1 mouse. *Proc Natl Acad Sci USA* 1986; **83**: 33–7.
3. Reynolds SH, Stowers SJ, Patterson RM, Maronpot RR, Aaronson SA, Anderson MW. Activated oncogenes in B6C3F1 mouse liver tumors: implications for risk assessment. *Science* 1987; **237**: 1309–16.
4. Wiseman RW, Stowers SJ, Miller EC, Anderson MW, Miller JA. Activating mutations of the c-Ha-ras protooncogene in chemically induced hepatomas of the male B6C3 F1 mouse. *Proc Natl Acad Sci USA* 1986; **83**: 5825–9.

© 2011 John Wiley & Sons A/S

5. Furth PA, St Onge L, Boger H, Gruss P, Gossen M, Kistner A, *et al.* Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter. *Proc Natl Acad Sci USA* 1994; **91**: 9302–6.
6. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 1992; **89**: 5547–51.
7. Figueiredo ML, Wentworth KM, Sandgren EP. Quantifying growth and transformation frequency of oncogene-expressing mouse hepatocytes in vivo. *Hepatology* 2010; **52**: 634–43.
8. Zender L, Villanueva A, Tovar V, Sia D, Chiang DY, Llovet JM. Cancer gene discovery in hepatocellular carcinoma. *J Hepatol* 2010; **52**: 921–9.
9. Kaposi-Novak P, Libbrecht L, Woo HG, Lee YH, Sears NC, Coulouarn C, *et al.* Central role of c-Myc during malignant conversion in human hepatocarcinogenesis. *Cancer Res* 2009; **69**: 2775–82.
10. Breuhahn K, Longerich T, Schirmacher P. Dysregulation of growth factor signaling in human hepatocellular carcinoma. *Oncogene* 2006; **25**: 3787–800.
11. Sandgren EP, Luetteke NC, Palmiter RD, Brinster RL, Lee DC. Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. *Cell* 1990; **61**: 1121–35.
12. Sandgren EP, Quaife CJ, Pinkert CA, Palmiter RD, Brinster RL. Oncogene-induced liver neoplasia in transgenic mice. *Oncogene* 1989; **4**: 715–24.
13. Baumgartner D, Laplante-O'neill PM, Sutherland DE, Najarian JS. Effects of intrasplenic injection of hepatocytes, hepatocyte fragments and hepatocyte culture supernatants on D-galactosamine-induced liver failure in rats. *Eur Surg Res* 1983; **15**: 129–35.
14. Limtrakul P, Lipigorngoson S, Namwong O, Apisariyakul A, Dunn FW. Inhibitory effect of dietary curcumin on skin carcinogenesis in mice. *Cancer Lett* 1997; **116**: 197–203.
15. Kisseberth WC, Brettingen NT, Lohse JK, Sandgren EP. Ubiquitous expression of marker transgenes in mice and rats. *Dev Biol* 1999; **214**: 128–38.
16. Rhim JA, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 1994; **263**: 1149–52.
17. Weglarz TC, Degen JL, Sandgren EP. Hepatocyte transplantation into diseased mouse liver. *Kinetics of parenchymal repopulation and identification of the proliferative capacity of tetraploid and octaploid hepatocytes. Am J Pathol* 2000; **157**: 1963–74.
18. Tukey J. *Exploratory Data Analysis*. 2nd ed. Reading, MA: Addison-Wesley; 1977.
19. Calvisi DF, Ladu S, Conner EA, Seo D, Hsieh JT, Factor VM, *et al.* Inactivation of Ras GTPase-activating proteins promotes unrestrained activity of wild-type Ras in human liver cancer. *J Hepatol* 2011; **54**: 311–9.
20. Calvisi DF, Ladu S, Gorden A, Farina M, Conner EA, Lee JS, *et al.* Ubiquitous activation of Ras and Jak/Stat pathways in human HCC. *Gastroenterol* 2006; **130**: 1117–28.
21. Calvisi DF, Thorgeirsson SS. Molecular mechanisms of hepatocarcinogenesis in transgenic mouse models of liver cancer. *Toxicol Pathol* 2005; **33**: 181–4.
22. Cheung RS, Brooling JT, Johnson MM, Riehle KJ, Campbell JS, Fausto N. Interactions between MYC and transforming growth factor alpha alter the growth and tumorigenicity of liver progenitor cells. *Carcinogenesis* 2007; **28**: 2624–31.
23. Sandgren EP, Luetteke NC, Qiu TH, Palmiter RD, Brinster RL, Lee DC. Transforming growth factor alpha dramatically enhances oncogene-induced carcinogenesis in transgenic mouse pancreas and liver. *Mol Cell Biol* 1993; **13**: 320–30.
24. Harada N, Oshima H, Katoh M, Tamai Y, Oshima M, Taketo MM. Hepatocarcinogenesis in mice with beta-catenin and Ha-ras gene mutations. *Cancer Res* 2004; **64**: 48–54.

© 2011 John Wiley & Sons A/S