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MINIMAL COOPERATION BETWEEN MUTANT H-RAS AND C-MYC OR TGF α IN THE REGULATION OF MOUSE HEPATOCYTE GROWTH OR TRANSFORMATION IN VIVO

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Abstract

Background—Liver carcinogenesis is associated with multiple genetic changes in affected cells, including alterations in the Hras signaling 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 (TGF α).

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 TGF α . 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 coexpression of c-myc or TGF α . 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 TGF α 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 compared to 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 TGF α growth signaling pathways in mouse liver. This finding emphasizes the tissue-specific character of molecular growth regulation.

Keywords

hepatocellular carcinoma; hepatocyte transplantation; Hras; C-myc; TGF α

Introduction

Hras is the most frequently mutated oncogene in spontaneous and induced liver tumors of mice (1–4), and Hras mutation is thought to be one mechanism of liver cancer initiation. To define further 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 tumor 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 over-expressed in HCC from mice and humans (9). Overexpression of transforming growth factor alpha (TGF α) 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 modeling 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 TGF α 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-TGF α 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 (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 TGF α 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 by 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 pattern 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 10ul via intrasplenic injection within 6 hours 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 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 is expressed as a ratio between median cross-sectional areas of experimental (oncogene plus hPAP) versus 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 analog incorporated into the DNA of cells in the synthesis phase of cellular replication. Cells undergoing replication were detected by immunohistochemistry as described (17). BrdU index was calculated 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 TGF α 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 TGF α transgenic mice, the Dox water was replaced by water treated with 50 mM zinc sulfate to induce hepatocyte expression of TGF α 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 lethargy and a prominently palpable caudal spine. Nontransgenic, AL-myc, and MT-TGF α mice did not reach endstage, but were euthanized at ages comparable to Hras/myc and Hras/TGF α 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 TGF α (Table 1). As expected, the liver weight as a percentage of body weight was elevated significantly in all Hras-expressing groups compared to other mice, but was not different among Hras, Hras/myc, and Hras/TGF α 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 labeling and apoptotic indices of hepatocytes expressing Hras alone and Hras in combination with the other genetic alterations were increased significantly compared to hepatocytes of age-matched control mice (Table 1). There were slight differences in BrdU labeling indices of hepatocytes expressing Hras alone versus Hras in combination with other genetic alterations. Apoptosis was increased significantly in tTA/Hras/AL-myc and tTA/Hras/MT-TGF α mouse livers compared to Hras alone.

Microscopically, the endstage livers from mice expressing Hras alone displayed a moderately heterogeneous hepatocyte population consisting of large and small hepatocytes (Figure 1A,B). Most Hras/myc-endstage livers appeared similar (Figure 1C). Liver from Hras/TGF α mice at endstage consisted of hepatocytes with variable cellular and nuclear sizes and cytoplasmic vacuoles (Figure 1D). Only 3 of 11 Hras/TGF α livers displayed trabecular HCC (Table 1; Figure 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, so instead we measured comparative carcinogenicity using the more precise hepatocyte transplant assay described below.

Hepatocyte-directed expression of c-myc or TGF α 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 TGF α using CHeGA (see Methods). Previous work identified two distinct phases of growth in transplant recipient mouse liver (7). Through week 4 post-transplantation there is a growth permissive environment or “growth phase”, while 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) (Figure 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 TGF α 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 (Figure 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 labeling index compared to controls (Table 2). The addition of TGF α to H-ras actually decreased BrdU labeling 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/TGF α hepatocytes had similar BrdU labeling 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 3 times the interquartile range above the 75th percentile within their distribution. These foci resemble preneoplastic 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 to controls (Figure 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/TGF α mice, but endstage disease actually was delayed in Hras/myc mice compared to Hras only (Table 3). Mice in all groups displayed preneoplastic and/or neoplastic lesions (Table 3 and Figure 1 F-I). Liver sections were stained for expression of the hPAP transgene, which verified that all lesions originated from transplanted hepatocytes (Figure 1 I, J). Thus, neither c-myc nor TGF α complement mutant Hras expression in this assay of hepatocarcinogenesis.

Discussion

The high frequency of Hras mutations in spontaneous and induced mouse liver tumors suggests that alterations in this ras signaling pathway play an important role in mouse hepatocarcinogenesis. The frequency of Hras mutations in human liver tumors 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 to 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 signaling 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 TGF α . Unexpectedly, the effect of combinations did not differ greatly from mutant Hras alone. Expression of activated Hras alone and in combination with myc or TGF α 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 due to 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 and, to a lesser extent, BrdU labeling index were increased in livers of Hras/c-myc and Hras/TGF α mice compared to 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 growth effects of Hras in combination with overexpression of myc or TGF α 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, as measured by EO frequency. The ability of Hras to induce cell autonomous growth allows for other genetic alterations to become fixed in progeny cells, thereby favoring neoplastic progression. In this report, we find that hepatocytes with the Hras/c-myc combination display increased focus growth during 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/TGF α combination increased focus growth within the permissive environment, again reflecting earlier findings with TGF α 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 TGF α or Hras and c-myc are not additive. In fact, coexpression can have a negative effect on hepatocyte growth when compared to Hras alone. TGF α 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 TGF α signal propagated principally through other pathways, some of which then inhibit hepatocyte growth. This would be consistent with the increase in the BrdU labeling index of transplanted hepatocytes expressing Hras alone compared to Hras/TGF α at 2 weeks post-transplant (Table 2), and the increase in apoptosis in Hras/TGF α mouse hepatocytes compared to Hras alone (Table 1). Lack of cooperation between c-myc and Hras was

unexpected, since these oncogenes strongly complement each other in other in vivo experimental systems. Again, this combination displayed increased apoptosis compared to Hras alone, suggesting that an increased rate of cell death slowed the growth of these foci.

A small fraction (18%) of Hras/TGF α 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 to Hras alone. EO foci likely 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 preneoplastic feature of extreme growth potential (7). Thus, there are no indications of cooperation between Hras and c-myc or TGF α 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 (5 of 7 mice examined), but these mice also were the oldest among groups.

In striking contrast, c-myc and TGF α 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/TGF α 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 TGF α 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 TGF α , 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.

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Figure 1.

A–E. Microscopic appearance of liver in transgenic mice. (A) Non-transgenic control liver; (B) Hras liver; (C) Hras/myc liver; (D) Hras/TGF α liver; (E) trabecular HCC in an Hras/TGF α 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/TGF α hepatocytes. (I) Hras/TGF α recipient liver showing an adenoma; (J) BCIP-stained Hras/TGF α -induced adenoma, showing dark

coloration indicating expression of the hPAP marker transgene. A-I, H&E; J, hPAP histochemistry. A–H, 200x; I, J, 100x.

Figure 2.

Focus ratio distribution medians (A), and outlier frequency (B) of transplanted hepatocyte foci. ^{**} $p < 0.01$; ^{***} $p < 0.001$ versus hPAP at the same time point using one-sided Wilcoxon rank sum test. ^aStatistically different ($p < 0.05$) than Hras 4 week time point using two-sided Wilcoxon rank sum test. ^bStatistically different ($p < 0.01$) than Hras 12 week time point using two-sided Wilcoxon rank sum test. Hras/myc 12 week versus 4 week, $p = 0.11$ (one-sided). Hras/TGF α 12 week versus 4 week, $p = 0.42$ (one-sided).

Table 1

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

Transgene(s)	Age, weeks	Li/body wt. %	BrdU %	Apoptosis %	Incidence of HCC ^b
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)** ^d	0/8
tTA/Hras/MT-TGFα	19.7±6.3 (10)	17.3±3.9 (10)***	1.16±0.7 (11)*** ^c	0.57±0.29 (9)*** ^d	3/11

^aData presented as mean ± SD (n). Significantly different than non-transgenic using one-sided Wilcoxon rank sum test:

* p<0.05;

** p<0.01;

*** p<0.001.

^bHCC, hepatocellular carcinoma.

^cDifferent (p=0.06) than tTA/Hras.

^dSignificantly different (p<0.01) than tTA/Hras.

Table 2

Cell proliferation indices in donor-derived hepatocyte foci.

Transgene(s)	Weeks post-transplant(# mice)	BrdU % Mean±SD ^a
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/TGF α	2(7)	12.0±7.1 ^b
	8(6)	4.4±1.4 [*]

^aSignificantly different than hPAP at the same timepoint using one-sided Wilcoxon rank sum test:^{*}
p<0.5;^{**}
p<0.01.^b Statistically different(p=0.05) than Hras 2 week timepoint using two-sided Wilcoxon rank sum test.

Table 3

Endstage disease in transplant recipients.

Transgene(s)(# mice)	Weeks post-transplant mean±SD	Lesions		
		AHF ^a	Adenoma	HCC ^a
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

^a AHF, altered hepatocyte focus; HCC, hepatocellular carcinoma.

* Significant difference (p<0.001) in survival between Hras and Hras/myc using two-sided Wilcoxon rank sum test.