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Liver-specific expression of the *agouti* gene in transgenic mice promotes liver carcinogenesis in the absence of obesity and diabetes

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Abstract

Background: The agouti protein is a paracrine factor that is normally present in the skin of many species of mammals. Agouti regulates the switch between black and yellow hair pigmentation by signalling through the melanocortin 1 receptor (Mc1r) on melanocytes. *Lethal yellow* (A^y) and *viable yellow* (A^{vy}) are dominant regulatory mutations in the mouse *agouti* gene that cause the wild-type protein to be produced at abnormally high levels throughout the body. Mice harboring these mutations exhibit a pleiotropic syndrome characterized by yellow coat color, obesity, hyperglycemia, hyperinsulinemia, and increased susceptibility to hyperplasia and carcinogenesis in numerous tissues, including the liver. The goal of this research was to determine if ectopic expression of the *agouti* gene in the liver alone is sufficient to recapitulate any aspect of this syndrome. For this purpose, we generated lines of transgenic mice expressing high levels of agouti in the liver under the regulatory control of the albumin promoter. Expression levels of the agouti transgene in the liver were quantified by Northern blot analysis. Functional agouti protein in the liver of transgenic mice was assayed by its ability to inhibit binding of the α-melanocyte stimulating hormone (αMSH) to the Mc1r. Body weight, plasma insulin and blood glucose levels were analyzed in control and transgenic mice. Control and transgenic male mice were given a single intraperitoneal injection (10 mg/kg) of the hepatocellular carcinogen, diethylnitrosamine (DEN), at 15 days of age. Mice were euthanized at 36 or 40 weeks after DEN injection and the number of tumors per liver and total liver weights were recorded.

Results: The albumin-agouti transgene was expressed at high levels in the livers of mice and produced a functional agouti protein. Albumin-agouti transgenic mice had normal body weights and normal levels of blood glucose and plasma insulin, but responded to chemical initiation of the liver with an increased number of liver tumors compared to non-transgenic control mice.

Conclusions: The data demonstrate that liver-specific expression of the *agouti* gene is not sufficient to induce obesity or diabetes, but, in the absence of these factors, agouti continues to promote hepatocellular carcinogenesis.

Background

The wild-type agouti coat color exhibited by many mammals consists of individual hairs that are black with a subterminal band of yellow [1]. The mouse *agouti* gene product is a secreted paracrine factor that regulates the alternate production of black and yellow pigments produced by hair-bulb melanocytes [2-4]. Binding of αMSH to the Mc1r on the surface of hair-bulb melanocytes results in the production of black pigment that is deposited in the growing hair. The *agouti* gene is transiently expressed in the skin during the mid-portion of the hair growth cycle. At this time, the agouti protein binds to the Mc1r, thereby excluding αMSH binding and causing a switch from black to yellow pigment production by melanocytes, which results in the appearance of the sub-terminal yellow band in the otherwise black hair [5-10].

Recessive mutations in the *agouti* gene affect only the coat color of mice, causing either a partial or complete loss of yellow pigment in the hair [11,12]. The dominant *agouti* mutations, *lethal yellow* (A^y) and *viable yellow* (A^{vy}), affect coat color by causing an increase in the amount of yellow pigment in the hair. Additionally, these dominant mutations cause mice to develop type II diabetes (peripheral insulin resistance, pancreatic islet hypertrophy and hyperplasia, hyperinsulinemia, and hyperglycemia), obesity (hyperphagia and increased adipose mass), increased somatic growth (increased fat-free dry mass and slightly longer bones), and increased susceptibility to hyperplasia and carcinogenesis in numerous tissues [reviewed in refs. [13-22]]. This syndrome is manifested in *lethal yellow* and *viable yellow* mice because they carry regulatory mutations in the *agouti* gene that cause the normal protein to be produced at abnormally high levels throughout the body [23-26].

In addition to its normal role of regulating pigmentation through Mc1r, agouti can also antagonize αMSH binding to other melanocortin receptor family members [5,27-31]. The ability of agouti to antagonize binding of αMSH to the Mc4r is of particular relevance, as Mc4r is expressed in the brain and mice lacking functional Mc4r are hyperinsulinemic, hyperphagic, and obese [32]. Mutations in human MC4R were also identified in dominantly inherited forms of human obesity [33,34]. These results suggest that the hyperinsulinemia, hyperphagia, and obesity in *lethal yellow* and *viable yellow* mice is caused primarily by agouti-induced antagonism of Mc4r in the hypothalamus, a center of autonomic regulatory control in the brain [35]. In addition to a role in the central nervous system, *agouti* expression in adipose tissue also appears to contribute to the obesity syndrome. Transgenic mice with adipocyte-specific *agouti* expression were shown to have significantly increased fat mass compared to control mice, which was accompanied by an increase in the protein levels of three transcription factors (Pparg, peroxisome proliferator activated receptor gamma; Stat1, signal transducer and activator of transcription 1; and Stat3) in their adipose tissue [36]. These three transcription factors were also upregulated in mature 3T3-L1 adipocytes in culture following treatment with recombinant agouti protein [36]. Additionally, recombinant agouti protein causes an increase in fatty acid synthase expression and activity, and the accumulation of triglycerides in cultured adipocytes [37]. Together, these results suggest that the obesity-related factors of the dominant *agouti* syndrome are mediated by *agouti* expression in both the brain and peripheral tissue(s).

Dominant mutations in the *agouti* gene also cause an increase in the susceptibility to hyperplasia and carcinogenesis in the liver [38-45], skin [46,47], lung [44,48-50], mammary gland [38,39,51-54], and urinary bladder [55]. It is likely that agouti-mediated antagonism of melanocortin receptors is mainly responsible for the obesity and diabetes of *lethal yellow* and *viable yellow* mice, but it is not known if melanocortin receptors are involved in their increased susceptibility to cancer. Whereas the obesity-related factors may contribute to the increased predisposition to carcinogenesis, there is some evidence to support the hypothesis that ectopic expression of the *agouti* gene *per se* may promote carcinogenesis in the liver and lung, even in the absence of hyperinsulinemia and obesity [44].

The liver is a primary site of insulin-mediated glucose disposal and lipogenesis in the mouse. Based on this fact, and on the previous reports of increased susceptibility to hepatic carcinogenesis in dominant agouti mutant mice, we were interested in determining if *agouti* expression in the liver alone would be sufficient to induce any of the phenotypes observed in *lethal yellow* or *viable yellow* mice. For this purpose, we generated lines of transgenic mice in which the wild-type murine *agouti* cDNA was expressed only in the liver at levels similar to or greater than those observed in *lethal yellow* or *viable yellow* mice. Transgenic and control mice were compared with respect to body weights, blood glucose levels, plasma insulin levels, and tumorigenic responses to chemical initiation in the liver.

Results

The albumin promoter directs expression of the wild-type agouti cDNA to the liver in transgenic mice

The albumin promoter was used to direct liver-specific expression [56] of the wild-type murine *agouti* cDNA in transgenic mice. Three lines of transgenic mice were established and two of these lines were characterized in detail: FVB/N-*Tg(Alb1-a)86R* and FVB/N-*Tg(Alb1-a)83R* (hereafter referred to as alb-agouti 86 and alb-agouti 83, respectively). As expected, *agouti* expression was detected only in the liver of the transgenic mice after an *agouti* cDNA probe

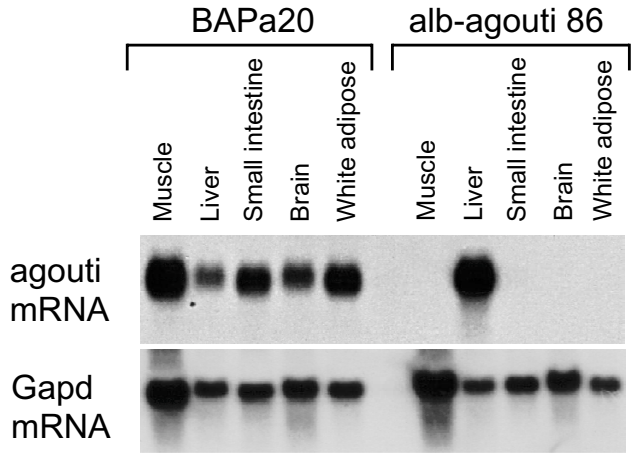


Figure 1
Northern blot analysis of *agouti* expression in BAPa20 and alb-agouti 86 transgenic mice. A Northern blot containing ~2.5 μg of poly $(A)^+$ RNA per lane was hybridized with a radiolabeled *agouti* cDNA probe. The blot was stripped and rehybridized with a radiolabeled glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) cDNA probe to control for mRNA loading and quality. The *agouti* gene is expressed only in the skin of wild-type mice [2]. Therefore, the *agouti* mRNA detected in these BAPa20 and alb-agouti 86 tissues is transgene-specific.

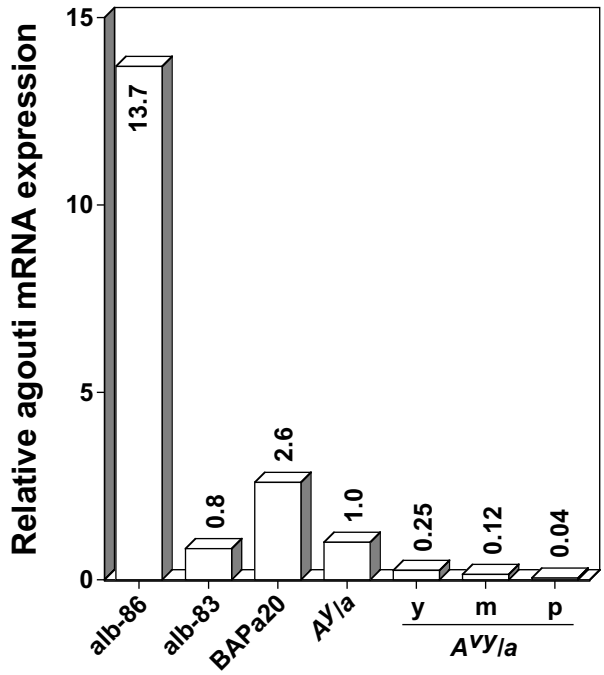


Figure 2
Estimate of *agouti* expression levels in the livers of alb-agouti 86, alb-agouti 83, BAPa20, *lethal yellow* (A^y/a) and *viable yellow* (A^{vy}/a) mice. A Northern blot of poly $(A)^+$ RNA, isolated from the liver of a single mouse for each of the genotypes indicated, was hybridized with a radiolabeled *agouti* cDNA probe, then stripped and rehybridized with a radiolabeled *Gapd* cDNA probe to control for the amount of mRNA loaded for each sample. Levels of *agouti* mRNA relative to *Gapd* mRNA levels were quantified for each sample by densitometric analysis (see Methods). The level of *agouti* mRNA expression in *lethal yellow* mice was assigned the value of 1.0, and the expression levels in *viable yellow* mice and transgenic mice were normalized relative to this value. The *viable yellow* mice used in this assay had coat colors that were completely yellow (y), mottled yellow and agouti (m), or pseudoagouti (p).

was hybridized to Northern blots containing ~2.5 μg of poly $(A)^+$ RNA from adult muscle, liver, small intestine, brain and white adipose tissue. The expression level of *agouti* in the liver of an alb-agouti 86 mouse is compared to that of a BAPa20 mouse (FVB/N-*TgN(BAPa)20Rpw*) in Figure 1. BAPa20 is a line of transgenic mice in which the wild-type *agouti* cDNA is under the regulatory control of the human β-actin promoter and enhancer. It was previously demonstrated that BAPa20 mice express the *agouti* gene in a ubiquitous manner and, consequently, become hyperinsulinemic and obese [57]. As seen in Figure 1, alb-agouti 86 mice express the agouti cDNA in the liver at a higher level than do BAPa20 mice.

The expression levels of *agouti* in the livers of alb-agouti 86 and 83 transgenic mice was next estimated by densitometry and compared to the levels of *agouti* expressed in the livers of BAPa20, *lethal yellow* (A^y/a), and *viable yellow* (A^{vy}/a) mice (Fig. 2). *Viable yellow* mice have coat colors ranging from completely yellow (y) to mottled yellow and agouti (m) to pseudoagouti (p), and the amount of yellow pigment in the coat is correlated with the level of *agouti* expression throughout the body and the severity of the obesity, diabetes, and neoplasia that they display [16]. The alb-agouti 86 mice express *agouti* in the liver at ~13.7 times the level in *lethal yellow* liver, and at a level that is also substantially greater than in BAPa20 liver and the livers of all three phenotypic classes of *viable yellow* mice. In contrast, the alb-agouti 83 mice express *agouti* in the liver at ~0.8 times the level in *lethal yellow* liver, at approximately one third the level in BAPa20 liver, but still at a greater level than in the livers of all three classes of *viable yellow* mice. Thus, under the hypothesis that expression of *agouti* in the liver alone is sufficient to induce the obesity

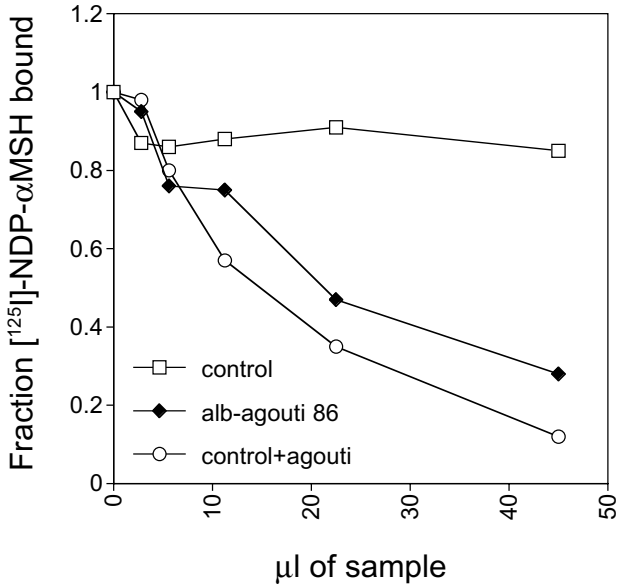


Figure 3
Analysis of functional agouti protein in the liver of alb-agouti 86 transgenic mice. Functional agouti protein was assayed by its ability to inhibit binding of [^{125}I]-NDP-αMSH to the Mc1r in murine melanoma B16F10 cells. The livers from three alb-agouti 86 mice were excised, pooled, homogenized, and enriched for agouti protein. The livers from three non-transgenic control mice were treated in the same manner (see Methods). The B16F10 cells were incubated for 2 hr at room temperature with 0.1 nM [^{125}I]-NDP-αMSH plus increasing amounts of either partially purified liver homogenate from control mice (control), liver homogenate from alb-agouti 86 mice (alb-agouti 86), or liver homogenate from control mice spiked with recombinant agouti protein (control+agouti). Bound radioactive ligand was measured and plotted (y-axis) against the amount of liver homogenates used in the assay (x-axis).

and diabetes of *viable yellow* mice, both lines of alb-agouti transgenic mice express the wild-type *agouti* cDNA in the liver at levels that should be adequate to induce these effects.

alb-agouti transgenic mice produce functional agouti protein in the liver

Recombinant murine agouti protein inhibits the binding of [^{125}I]-NDP-αMSH to the Mc1r in B16F10 murine melanoma cells. This assay was used as previously described [58,59] to determine if the alb-agouti transgene produces a functional agouti protein in the liver of the transgenic mice. Prior to the assay, an S Sepharose chromatography procedure was used to enrich for agouti protein in liver homogenates from alb-agouti 86 transgenic mice. Liver homogenates from non-transgenic control mice and liver homogenates from non-transgenic control mice spiked with recombinant agouti protein were treated in the same manner. Relative inhibition of [^{125}I]-NDP-αMSH binding to the Mc1r for each of the three samples is displayed in Figure 3. The alb-agouti 86 sample inhibited [^{125}I]-NDP-αMSH binding in the assay almost as effectively as the control sample spiked with recombinant agouti protein, whereas the control sample from non-transgenic mice did not significantly affect αMSH binding.

alb-agouti transgenic mice have normal body weight, and normal levels of plasma insulin and blood glucose

To determine if expression of the *agouti* gene in the liver alone is sufficient to induce obesity and diabetes, the body weight, and levels of blood glucose and plasma insulin were analyzed in the alb-agouti transgenic mice and compared to those of non-transgenic siblings. There were no significant differences ($p > 0.05$) in body weight between transgenic and control mice fed a diet containing 11% fat by weight (Fig. 4). Levels of plasma insulin and blood glucose did not differ significantly ($p > 0.05$) between the alb-agouti transgenic mice and the non-transgenic controls, whereas both insulin and glucose levels were significantly elevated in BAPa20 mice (positive controls) as expected (Table 1). Therefore, these data indicate that expression of the *agouti* gene in the liver alone is not sufficient to induce obesity or diabetes.

alb-agouti transgenic mice respond to chemical initiation of the liver with an increased number of tumors per liver

To determine if the presence of agouti in the livers of alb-agouti 83 and 86 transgenic mice promotes liver carcinogenesis in the absence of obesity and diabetes, a single intraperitoneal injection of the liver carcinogen, DEN (10 mg/kg body weight), was administered to transgenic and control male mice at 15 days of age. Body weights of all injected mice were recorded from 4–42 weeks of age, and there were no significant differences ($p > 0.05$) between either of the transgenic lines and their non-transgenic littermates at any time point (data not shown). Three mice from each of the transgenic and control groups were euthanized at various times after DEN injection, and it was determined that 36 and 40 weeks post-injection were the most appropriate times for sampling the mice, because tumors at those ages were macroscopically visible and had not yet coalesced (data not shown). Therefore, separate groups of transgenic and control mice (n = 18–20 per group) were euthanized at 36 weeks or 40 weeks post-injection, and their tumor numbers and liver weights were recorded (Fig. 5).

At 36 weeks after DEN injection, the number of tumors per liver was 2.3 ± 0.4 (mean ± one standard error of the

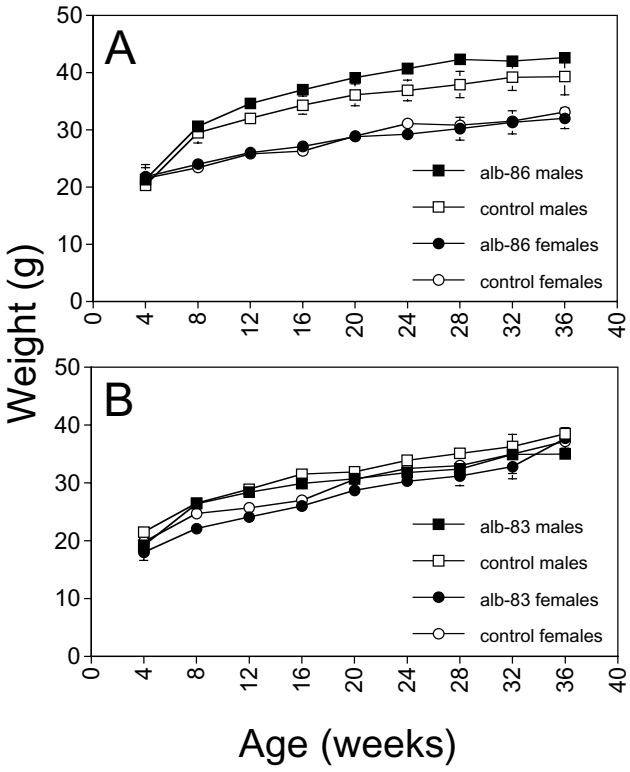


Figure 4
Growth rates of alb-agouti transgenic mice and wild-type littermate controls. Body weights of alb-agouti 86 (A) and alb-agouti 83 (B) mice were recorded once every four weeks from 4–36 weeks of age and compared to littermate control mice. Shown are the means ± one standard error of the mean (for many of the means, the standard errors are too small to see the error bars). Sample sizes range from 6–22 for each mean. The weights of transgenic and control mice did not differ significantly ($p > 0.05$) at any time.

mean) for the controls, 3.5 ± 0.7 for alb-agouti 83, and 4.0 ± 0.9 for alb-agouti 86 (Figure 5A). Both lines of transgenic mice had a greater number of liver tumors than did the controls at 36 weeks, but these differences were not statistically significant (Tukey-Kramer multiple-comparison test at $\alpha = 0.05$). At 40 weeks after DEN injection, the mean number of tumors per liver for the controls, and the alb-agouti 83 and 86 mice was 4.1 ± 1.1, 6.8 ± 1.6, and 10.4 ± 2.5, respectively (Figure 5A). At this time, the mean number of tumors for the alb-agouti 86 mice differed significantly ($p < 0.05$) from the controls, but not from the alb-agouti 83 mice. The mean number of tumors for the alb-agouti 83 mice did not differ significantly from the controls ($p > 0.05$). At both 36 and 40 weeks, the number of tumors per liver for the transgenic mice was positively correlated with the level of *agouti* expression in the liver. Whereas transgenic mice had more tumors per liver than did the control mice, the size distribution of tumors was similar in transgenics and controls (data not shown). The fact that alb-agouti 86 mice have normal body weights and levels of blood glucose and plasma insulin, but responded to DEN with significantly more liver tumors than did the non-transgenic mice demonstrates a more direct effect of agouti in the promotion of liver carcinogenesis.

Total liver weight was also recorded at 36 and 40 weeks after DEN injection. Both lines of transgenic mice had greater average liver weights than did the control mice (Fig 5B). The differences in liver weights between alb-agouti 86 mice and controls were significant ($p < 0.05$) at both 36 weeks (1.95 g ± 0.06 vs. 1.63 g ± 0.07) and 40 weeks (1.97 g ± 0.08 vs. 1.67 g ± 0.03) after DEN injection, whereas the differences between alb-agouti 83 mice and controls were not significant ($p > 0.05$). Unlike the livers from obese *lethal yellow* and *viable yellow* mice, where visual examination reveals an excessive amount of fat deposition in the liver (E. J. M., personal observations), the livers from lean alb-agouti 83 and 86 mice did not appear to be different from control livers in terms of fat content (data not shown). This suggests that the heavier livers in DEN injected alb-agouti transgenic mice resulted from agouti protein-induced hyperplasia and/or greater tumor burdens, not of an increased triglyceride content of the liver.

Discussion

The current investigations were undertaken to determine if *agouti* expression in the liver of transgenic mice could recapitulate any aspects of the dominant pleiotropic syndrome (i.e., obesity, hyperglycemia, hyperinsulinemia, and/or liver cancer) observed in *lethal yellow* and *viable yellow* mice. To address this question, lines of transgenic mice were generated in which the wild-type *agouti* cDNA was ectopically expressed only in the liver under the regulatory control of the albumin promoter. Different lines of transgenic mice were shown to express the *agouti* gene in the liver at levels that were similar to or greater than the levels detected in the livers of mice that express *agouti* ubiquitously and exhibit the pleiotropic syndrome (*lethal yellow*, *viable yellow*, and BAPa20 mice).

Although the liver is a key lipogenic tissue in the mouse and a major site of glucose disposal (conversion to glycogen), the finding that body weight, blood glucose and plasma insulin did not differ significantly between the alb-agouti transgenic and control mice demonstrates that *agouti* expression in the liver alone is insufficient to induce obesity, hyperglycemia or hyperinsulinemia. However, expression of *agouti* in the livers of the transgenic mice did cause an increase in the susceptibility to DEN-induced

Table 1: Circulating glucose and insulin concentrations in transgenic and control mice[a]

Line	Blood glucose (mg/dl)		Plasma insulin (μU/ml)	
	controls	transgenics	controls	transgenics
alb-agouti 83	116 ± 8 (5)	115 ± 7 (5)	32 ± 3 (10)	28 ± 3 (6)
alb-agouti 86	115 ± 12 (5)	112 ± 7 (5)	26 ± 3 (6)	32 ± 3 (11)
BAPa20	122 ± 12 (5)	241 ± 37 (8)*	31 ± 6 (5)	197 ± 33 (9)*

[a]Data are presented as the mean ± one standard error of the mean. Numbers in parentheses indicate sample sizes. Asterisks denote significant differences between transgenic and control mice ($p < 0.05$).

liver carcinogenesis. The total tumor burden in the livers of the exposed mice was estimated by considering both the numbers of tumors visible on the surface of the livers and the total weights of the livers. Thus, whereas the alb-agouti 86 transgenic mice had significantly more liver tumors than control mice at 40, but not 36, weeks after injection, the transgenic livers were significantly heavier than the control livers at both time points (Fig. 5). Taken together, these results indicate that the agouti protein stimulated a significant increase in liver hyperplasia and/or tumor burden of the alb-agouti 86 transgenic mice at the earlier time point (36 weeks) as well.

The present observation that agouti has a primary role in promoting liver carcinogenesis, independent of obesity and diabetes, is in agreement with previous findings on *viable yellow* mice [44]. Viable yellow mice express the *agouti* gene in a ubiquitous manner, but exhibit a wide range in the level of expression of the gene and the associated phenotypes. At one end of the spectrum are individuals with very high levels of ubiquitous *agouti* expression; these mice have completely yellow coats, and they are obese and hyperinsulinemic. At the other end of the spectrum are mice with very low levels of ubiquitous *agouti* expression; these mice have coat colors that appear almost normal (called pseudoagouti), and they have normal body weights and levels of circulating insulin. Mice in the middle of the spectrum exhibit a moderate level of ubiquitous *agouti* expression, have coats that are a patchwork of yellow and agouti hairs (i.e., mottled), and are likely to become obese and hyperinsulinemic. Wolff and colleagues [44] fed *viable yellow* mice and control mice a diet supplemented with lindane (γ-hexachlorocyclohexane) for 24 months, then examined the mice for the development of tumors. They found that both the yellow mice (obese and hyperinsulinemic) and the pseudoagouti mice (lean and normoinsulinemic) had a higher prevalence of chemically initiated liver and lung tumors (i.e., a greater number of mice with tumors) than did control mice. Tumor prevalence, however, was highest in the yellow mice. Thus, a key discovery of these experiments, and of particular relevance to this study, was that a very low level of ubiquitous *agouti* expression did not cause obesity or hyperinsulinemia, but was sufficient to promote carcinogenesis in some tissues. The higher prevalence of liver tumors in pseudoagouti mice than in control mice suggested that the tumor promoting effect of agouti was a direct consequence of *agouti* expression in the liver. However, because pseudoagouti mice express the *agouti* gene in a ubiquitous manner, albeit at a low level, it remained a possibility that this low-level ubiquitous expression of *agouti* may have elicited some other physiological response that was in turn responsible for the tumor phenotype. The fact that a low level of constitutive *agouti* expression in the skin of pseudoagouti mice is sufficient to cause a subtle alteration in coat color [60] lends credence to this possibility. The alb-agouti transgenic mice presented here have *agouti* expressed only in the liver and, although they are lean and normoinsulinemic, are predisposed to an increased number of liver tumors. In fact, the number of tumors per liver in the transgenic mice was correlated with their level of *agouti* expression in the liver. These results demonstrate that *agouti* expression in the liver is sufficient to promote liver carcinogenesis, independent of any other apparent agouti-mediated physiological effects.

The molecular mechanism underpinning the role of the agouti protein in promoting liver carcinogenesis is currently unknown. Whether agouti promotes carcinogenesis by antagonizing a melanocortin receptor in the liver, or by acting in a melanocortin-independent manner remains to be determined. In this regard, it is interesting to note that the only melanocortin receptor currently known to be expressed in a widespread manner, including in the liver, is Mc5r [61-63], but agouti protein appears to have little to no effect on antagonizing the binding of αMSH to Mc5r [5,64]. Whether agouti antagonizes the interaction of a different ligand with the Mc5r is not known. The data presented here set the stage for future studies aimed at elucidating the mechanism of action of the *agouti* gene in the promotion of hepatocellular hyperplasia and neoplasia.

Conclusions

In summary, we have demonstrated that liver-specific expression of the *agouti* gene in transgenic mice was insuf-

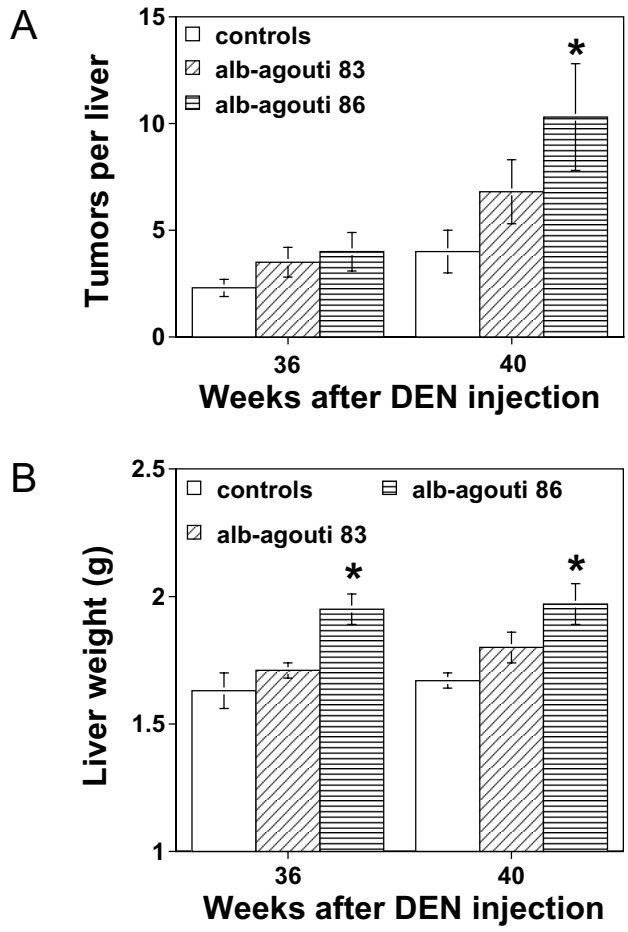


Figure 5
Mean number of tumors per liver and total liver weights in control and alb-agouti transgenic mice following chemical initiation with DEN. Control, alb-agouti 83, and alb-agouti 86 male mice were each given a single intraperitoneal injection of DEN (10 mg/kg) at 15 days of age. Mice were euthanized at either 36 or 40 weeks after DEN injection and the mean number of tumors per liver (A) and mean total liver weights (B) were determined for each group. Shown are the means ± one standard error of the mean. Sample sizes range from 18–20 for each group. Asterisks indicate significant difference from the controls ($p < 0.05$).

ficient to alter body weight, blood glucose level, or plasma insulin level, but it did promote DEN-initiated liver carcinogenesis. Because alb-agouti transgenic mice developed more liver tumors than did the control mice, the expression of the *agouti* gene in the liver alone, in the absence of obesity and diabetes, is sufficient to promote the development of liver tumors in mice. These data suggest that the increased susceptibility of *lethal yellow* and *viable yellow* mice to carcinogenesis in a variety of tissues is mediated, at least in part, by the tumor promoting effect of *agouti* expression in the target tissue, rather than being just a secondary consequence of the obesity-related factors.

Methods

Agouti expression construct

An *agouti* expression construct (plasmid AlbPE-a) containing the murine albumin promoter and enhancer, the wild-type murine *agouti* cDNA, and the simian virus 40 (SV40) polyadenylation sequences was generated as follows. A *Cla*I fragment containing the *agouti* cDNA and SV40 polyadenylation sequences was isolated from plasmid clone BAPa [57]. The *Cla*I ends were filled in with Klenow and *Sal*I linkers were ligated to the fragment [65]. The *Sal*I fragment was then cloned into the *Sal*I site of plasmid NB0.3 alb [56], downstream of the albumin promoter and enhancer to generate the plasmid AlbPE-a. This expression construct was verified by DNA sequencing. The AlbPE-a plasmid was digested with *Sac*I and *Kpn*I to excise the 3.5-kb expression cassette from vector sequences for microinjection into fertilized mouse eggs.

Transgenic mice

The pronuclei of fertilized eggs from a random-bred closed-colony stock of FVB/N mice were microinjected with the AlbPE-a expression cassette, along with a tyrosinase minigene expression cassette [66] (at a total DNA concentration of 3 ng/μl in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5), to generate lines of transgenic mice as described [67].

The tyrosinase expression cassette [66] produces pigmentation in the hair of albino FVB/N mice. Cointegration of the AlbPE-a and tyrosinase expression cassettes permits visual identification of the alb-agouti transgenic mice by their coat color. Genomic DNA was obtained by tail biopsy and all mice were genotyped for inheritance of the AlbPE-a transgene by probing Southern blots of *Bam*HI-digested DNA with an *agouti* cDNA probe, as described [2]. Transgenic founder mice were mated to wild-type FVB/N mice to establish independent transgenic lines, and mice were maintained hemizygous for the transgene by mating transgene carriers to FVB/N mice and genotyping the offspring by Southern blot analysis. For those lines in which the AlbPE-a transgene was shown to cosegregate with coat color, the mice were thereafter genotyped by coat color with confirmation by Southern blotting a few mice at each generation. All experiments involving mice in this study were conducted under approved Institutional Animal Care and Use Committee protocols.

Northern blot analysis

Isolation of total RNA and poly $(A)^+$ RNA, preparation of Northern blots, radiolabeling of hybridization probes, and hybridization conditions were as described [68]. In order to quantify levels of agouti expression in the livers of various strains of mice (Fig. 2), a Northern blot containing ~2.5 μg of poly $(A)^+$ liver RNA per lane was first hybridized with an *agouti* cDNA probe. The hybridization signals were detected with a FUJIX BAS 1000 phosphorimager and quantified with MacBAS software (Fuji Medical Systems). The blot was then stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) probe and the *Gapd* transcript levels were quantified as described above to control for the amount of RNA loaded for each sample. For each mRNA sample, the level of *agouti* expression was reported as the ratio of *agouti* over *Gapd* mRNA signals. The level of *agouti* mRNA expression in *lethal yellow* mice was assigned the value of 1.0, and the expression levels in *viable yellow* mice and transgenic mice were normalized relative to this value.

Assay for functional agouti protein

Non-transgenic control mice and transgenic mice were euthanized by cervical dislocation and the livers were quickly excised, washed in ice-cold phosphate buffered saline, pH 7.4 (PBS), homogenized, and stored at -80°C. Three livers from each genotype were pooled prior to homogenization. Liver homogenates were enriched for agouti protein as follows. Liver homogenates (2 ml of 20 mg/ml protein) from non-transgenic control mice (negative control), from non-transgenic control mice spiked with 33 nM recombinant agouti protein (positive control), and from alb-agouti 86 mice, each containing 10 mM phosphoramidon, were incubated on a Nutator (4°C for 1 hr) with 2 ml of S Sepharose cation exchange resin equilibrated in PBS. The resin was washed with 0.5 M NaCl in 20 mM HEPES, pH 7.5 and eluted with 1.0 M NaCl, in 20 mM HEPES, pH 7.5. The eluants were desalted on a PD-10 column (Pharmacia) equilibrated in PBS.

The presence of functional agouti protein in the livers of the transgenic mice was assayed by measuring the ability of enriched liver homogenates to inhibit the binding of αMSH to the Mc1r. Murine melanoma B16F10 cells were cultured and used in the [^{125}I]-NDP-αMSH binding assay as previously described [58,59]. B16F10 cells were incubated for 2 hr at room temperature with 0.1 nM [^{125}I]-NDP-αMSH plus increasing amounts of each of the enriched samples described above. Cells were washed twice with ice-cold PBS to remove free ligand before the addition of 125 μl of scintillation cocktail. Bound radioactive ligand was measured using a Wallac 1650 Microbeta counter.

Body weight, plasma insulin, and blood glucose analyses

Transgenic and control mice were fed a diet containing 11% fat by weight (Rodent laboratory diet 5015, PMI Feeds), weaned at three weeks of age, and weighed every four weeks from 4–36 weeks of age. Blood samples were collected by retro-orbital sinus puncture from anesthetized, nonfasted, 40–50 week old male mice between 9–11 a.m. Plasma insulin levels were measured in duplicate by radioimmunoassay according to the manufacturer's recommendations (ICN Biomedicals) with porcine insulin as a standard. Glucose concentrations were measured with the One-Touch glucose determination system (Johnson & Johnson).

Mice used in the carcinogenesis studies were fed a diet containing 4.5% fat by weight (Rodent laboratory diet 5001, PMI Feeds), weaned at three weeks of age, and weighed every two weeks from 4–42 weeks of age.

Liver tumor analysis

Diethylnitrosamine (DEN) was purchased from Sigma Chemical Co. A single intraperitoneal injection of DEN (10 mg/kg) was administered to fifteen-day-old transgenic and control male mice. Groups of animals were weighed, euthanized by cervical dislocation at 36 or 40 weeks after injection, and necropsied. The livers were removed, weighed, and examined for visible lesions, which were counted and measured (diameter).

Statistical analyses

All statistical analyses were performed with the JMP computer software package (SAS Institute Inc.).

List of abbreviations

alb, albumin promoter; alb-agouti mice, transgenic mice expressing the wild-type agouti cDNA in the liver under the regulatory control of the albumin promoter; αMSH, α-melanocyte stimulating hormone; A^{vy}, *viable yellow* allele of the *agouti* gene; A^{y}, *lethal yellow* allele of the *agouti* gene; DEN, diethylnitrosamine; Gapd, glyceraldehyde-3-phosphate dehydrogenase; Mc1r, melanocortin 1 receptor; Mc4r, melanocortin 4 receptor; Pparg, peroxisome proliferator activated receptor alpha; Stat1, signal transducer and activator of transcription 1; Stat3, signal transducer and activator of transcription 3; SV40, simian virus 40.

Authors' contributions

All authors participated in the study design. MLK and RPW generated the transgene construct and the transgenic mice. AIK, MLK, and RLM performed the Northern blot analyses. RLM, LLK, and WOW performed the assay for functional agouti protein. MLK and RLM weighed mice on the 11%-fat-by-weight diet. RLM performed the plasma insulin and blood glucose measurements. AIK and EJM

weighed mice on the 4%-fat-by-weight diet and performed the tumorigenesis studies. EJM drafted the manuscript, and all authors provided comments, critique and suggestions for its improvement. All authors read and approved the final manuscript.

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