Chapter 1.5

Analysis of the Function of the Agouti Gene in Obesity and Diabetes

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In this chapter, I will discuss the *agouti* gene and dominant mutations in that gene that lead to agouti-induced obesity, and recent work with transgenic mice to elucidate the role of agouti in obesity.

Agouti was cloned in 1992 by the lab of Rick Woychik at Oak Ridge National Laboratory, making it the first of many recently cloned mouse obesity genes (1). Sequence analysis predicted that mouse agouti is a secreted protein of 131 amino acids. The mature protein has a basic central region (lys57-arg85), a proline-rich domain (pro86-pro91) and a C-terminal region (cys 92-cys 131) containing 10 cysteine residues which form 5 disulfide bonds. The human homologue of *agouti* has also been cloned by the Woychik lab and maps to human chromosome 20q11.2 (2). Human agouti is 132 amino acids long and is 85% similar to the mouse agouti protein and is normally expressed in adipose tissue.

Agouti naturally functions to regulate coat color in mice (3). Understanding of the mechanisms of agouti action within the hair follicle may provide insight into the mechanisms of agouti-induced obesity. Within the hair follicle, α-melanocyte stimulating hormone (αMSH) binds to it's receptor (MC1-R), which is coupled to heterotrimeric guanine nucleotide-binding proteins that activate adenlyate cyclase (4). The resulting increase in intracellular cAMP levels leads to the activation of the rate limiting enzyme in melanogenesis, tyrosinase (5, 6). Tyrosinase converts tyrosine to dopa and then to

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dopaquinone, which is cyclized and oxidized to form eumelanin (dark-brown to black pigment) (7). Agouti decreases the overall rate of melanogenesis by antagonizing the binding of αMSH to MC1-R (8), which by unknown mechanisms leads to increased incorporation of sulphydryl compounds into dopaquinone to form cysteinyldopas and ultimately yellow pigments (pheomelanin) (7). The relative amounts of eumelanin and pheomelanin produced by the melaoncyte determines the color of growing hair.

Several dominant *agouti* mutations produce structural changes in the *agouti* gene that cause wild-type *agouti* to be expressed in a ubiquitous manner, suggesting that ubiquitous expression of *agouti* may somehow induce these mice to beocome obese (9). However, since each of the dominant mutant alleles contain a major structural change in the *agouti* locus, it was unclear whether ubiquitous expression of *agouti per se* causes the pleiotropic effects or whether there is an additional gene located in the vicinity of *agouti* that is up- or down-regulated. Therefore, in order to demonstrate that *agouti* was solely responsible for the obesity, hyperinsulinemia and hyperglycemia, transgenic mice were developed that ubiquitously expressed the wild-type *agouti* cDNA under control of the ß-actin and phosphoglycerate kinase-1 promoters (10). These transgenic mice have yellow fur, a faster rate of weight gain and higher final body weight than their non-transgenic littermates. Additionally, the transgenic animals develop hyperinsulinemia and hyperglycemia. These results demonstrate conclusively that ectopic expression of *agouti* is solely responsible for the mutant traits in these animals.

Having established that ubiquitous expression of wild-type *agouti* induces obesity and characteristics of NIDDM, we began a systematic approach to identify target organs of agouti action by expressing *agouti* in tissues known to be involved in the development of obesity. To determine if liver or adipose tissue are major effector organs for *agouti*-induced obesity and diabetes, the wild-type *agouti* cDNA was placed under the transcriptional control of the albumin promoter for liver-specific expression (alb-a), or the aP2 promoter for adipose tissue-specific expression (aP2-a). Several alb-a transgenic lines were generated and two (alb83, alb86) were examined in greater detail. They were chosen because alb83 had comparable levels of *agouti* expression in the liver as did the obese ß-actin promoter-agouti transgenic line (BAP20), and the amount of *agouti* mRNA in liver of the other line, alb86, was approximately 4-fold greater than in the alb83 mice (data not shown). Figure 1 illustrates that *agouti* gene expression was specific for the liver in alb86 mice and is about 5-fold greater than the level of agouti gene expression in liver of the BAP20 line.

In order to verify that functional protein was being produced in the liver of the alb-a mice, functional agouti protein was assayed by its ability to inhibit binding of α-MSH to

the MCR-1 receptor. Murine melanoma B16F10 cells were cultured and used in the [^{125}I]-α-MSH binding assay. Cells were incubated for 2 hr at room temperature with about 0.1 nM [^{125}I]-α-MSH plus increasing amounts of partially purified control serum, liver homogenate, liver homogenate spiked with recombinant agouti, or liver homogenate from transgenic mice. Functional agouti protein was present in the alb86 liver, as indicated by antagonism of [^{125}I]-NDP-α-MSH binding (Figure 2), but there was no detectable agouti protein in the serum of the alb-a mice.

Examination of body weight revealed no significant increase in either males or females when comparing transgenic to non-transgenic mice in the alb86 and alb83 transgenic lines (Figure 3). Circulating levels of insulin and glucose were also not affected by *agouti* expression in liver (Table 1). These results demonstrated that the production of active agouti protein solely in the liver does not produce the neccessary metabolic changes to induce weight gain.

Examination of *agouti* mRNA levels in several tissues from 3 aP2-a lines (aP212, aP273 and aP274) is shown in Figure 4. All 3 lines exhibited comparable levels of *agouti* mRNA in white and brown adipose tissue, and much greater levels of *agouti* mRNA in white adipose tissue than the BAP20 line. Detectable levels of *agouti* mRNA were also found in other tissues of the aP2-a mice, albeit at much lower levels than in adipose tissue. *Agouti* mRNA levels were consistently higher in most tissues of the aP274 line than in the other 2 lines, and was especially prominent in muscle. To determine if the observed expression in tissues other than white and brown adipose tissue was due to the presence of adipocytes, the adipose tissue-specific gene, *ob*, was used as a marker (Figure 4). There was no detectable adipose tissue contamination in liver or brain, and only a small amount in muscle, but the level of expression in muscle was similar in the three lines. These results prove that the observed high level of *agouti* expression in the muscle of aP274 mice is genuine and unique to this line

When examining the weight curves for the various aP2-a lines, it was evident that the aP274 line was becoming obese and the other lines were not. Also there was no significant differences in body weight between the remaining aP2a lines. Therefore the body weights of four other aP2-a Tg/- mice were pooled. The body weights from all wild-type littermates were also pooled. Figure 5 compares the body weight of nontransgenic mice with the average of four aP2 lines, the aP274 line and the BAP20 line. The average weight of aP274 mice first became significantly greater ($p<0.05$) than littermate weights by 12 weeks of age in males and females (Figure 5). The other aP2-a lines did not have a significantly greater body weight than their control littermates (Figure 5). The most

consistent explanation for obesity in the aP274 line, but not the other aP2-a lines, is the ectopic expression of *agouti* in tissues other than fat.

Free fed blood glucose concentrations are normal in all of the aP2-a mice, but plasma insulin concentrations are about 2-fold greater in the aP274 mice when compared to control littermates (Table 1). Glucose tolerance test also reveals impaired glucose clearance in the aP274 mice, but not in the other aP2-a lines (Figure 6).

Many mouse models for obesity have hyperphagia, presumably at the level of the hypothalamus, as a primary cause of obesity with increased food efficiency being secondary, but the reverse seems to be true for agouti-induced obesity. Two week food intake was not elevated in the aP274 and BAP20 transgenic mice when compared with control mice (Figure 7). However, weight gain over that same two week period was significantly increased in both the aP274 mice and the BAP20 mice. A possible explaination for the increased in food efficiency is a reduction in body core temperature in the aP274 and BAP20 mice. There is an 8/10 of a degree decrease in body core temperature in the BAP20 mice when compared to controls and a 6/10 of a degree decrease in body core temperature in the aP274 line.

Our recent studies indicate that the regulation of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) by agouti contributes to *agouti*-induced obesity. We reported that agouti mutant mice exhibit elevations in both steady state $[Ca^{2+}]_i$ and Ca^{2+} influx in both myocytes and adipocytes, and that these increases are highly correlated with both the degree of ectopic agouti expression and body weight (11). In addition, the recombinant agouti gene product directly stimulates Ca^{2+} channel-mediated Ca^{2+} influx (11). Agouti's ability to increase $[Ca^{2+}]_i$ in all cell types examined to date suggests that agouti has a physiological role of regulating $[Ca^{2+}]_i$ and alters the normal signal transduction pathways that result in obesity.

In summary, with these transgenic lines, we've been able to recapitulate obesity, hyperinsulinemia, and hyperglycemia with the ubiquitous expression of agouti. We have also determined that agouti expression in either liver or adipose tissue alone does not cause obesity, and that there's a dose-dependent effect of agouti on body weight, food efficiency, body temperature, and insulin and glucose levels. We are currently generating transgenic lines that express *agouti* in skeletal muscle, pancreas, and the central nervous system.

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QUESTIONS AND ANSWERS

Question: There seems to be some discrepancies in your results. There are elevated levels of insulin and glucose in the ubiquitous expressing agouti mice, but liver specific or adipose specific transgenic mouse do not cause obesity, but do not have elevated levels of insulin and glucose metabolism ? And the other question is, can the lack of obesity in liver specific or adipose specific transgenic mouse be explained by low levels of plasma *agouti* ?

Dr. Mynatt: In answer to your second question first, all evidence to date from parabiosis and skin graft experiments indicates that agouti is not a circulating protein. Also the aP274 line has no detectable levels of circulating agouti, and it becomes obese. This is consistent with the paracrine hypothesis of *agouti*'s function in coat color.

To answer the the first question----while liver and adipose tissue are major players in gluconeogenesis and utilization, they do not soley dictate plasma glucose concentrations. I think a clue may be again with the aP274 mice, which have higher levels of *agouti* expression in skeletal muscle, and that in turn may be leading to insulin resistance.

Question: I was intrigued by your observation that the core temperatures of these animals was 8/10 of a degree lower than normal. If you were to raise these animals at a higher temperature, would they have the same improved feed efficiency? Secondly, how do these animals tolerate cold exposure?

Dr. Mynatt: We haven't directly tested cold exposure in the transgenic mice, but it has been shown that the viable yellow and lethal yellow mice have decreased thermogenic capacity and cannot tolerate cold exposure as well as their litter mates. As far as housing the mice at an elevated temperature, we have not tried the experiment, but I would speculate that food efficiency would increase in all mice since they do not have to expend as much energy to maintain body temperature.

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Table 1 Circulating Glucose and Insulin Concentrations in A gouti Transgenic Mice

	Insulin (μU/ml)	Glucose (mg/dl)
wild-type	31 ± 2 (46)	120 ± 7 (20)
alb83	28 ± 3 (6)	115 ± 12 (5)
alb86	32 ± 3 (11)	112 ± 7 (5)
aP212	31 ± 12 (4)	ND
ap273	26 ± 5 (6)	ND
aP274	50 ± 7 * (6)	122 ± 11 (5)
BAP20	235 ± 49* * (10)	250 ± 37 ** (5)

Blood samples were collected between 9 and 11 am from ad libitum fed male mice. Alb 83 and alb 86 refer to transgenic lines in which the albumin promoter was used to express agouti cDNA. AP212, aP273 and aP274 refer to transgenic lines in which the aP2 promoter was used to express agouti cDNA . BAP 20 refers to a transgenic line in which the ß-actin promoter was used to express agouti cDNA. See materials and methods for details on blood. Data are presented as mean ± SEM. Numbers in parenthesis are number of mice per group. There was no significant difference between wild-type mice from the different lines so they were pooled. * indicates that the value is significantly different ($p \geq 0.05$) from wildtype mice. ** indicates that the value is significantly different ($p \geq 0.01$) from wildtype mice. ND=not determined.

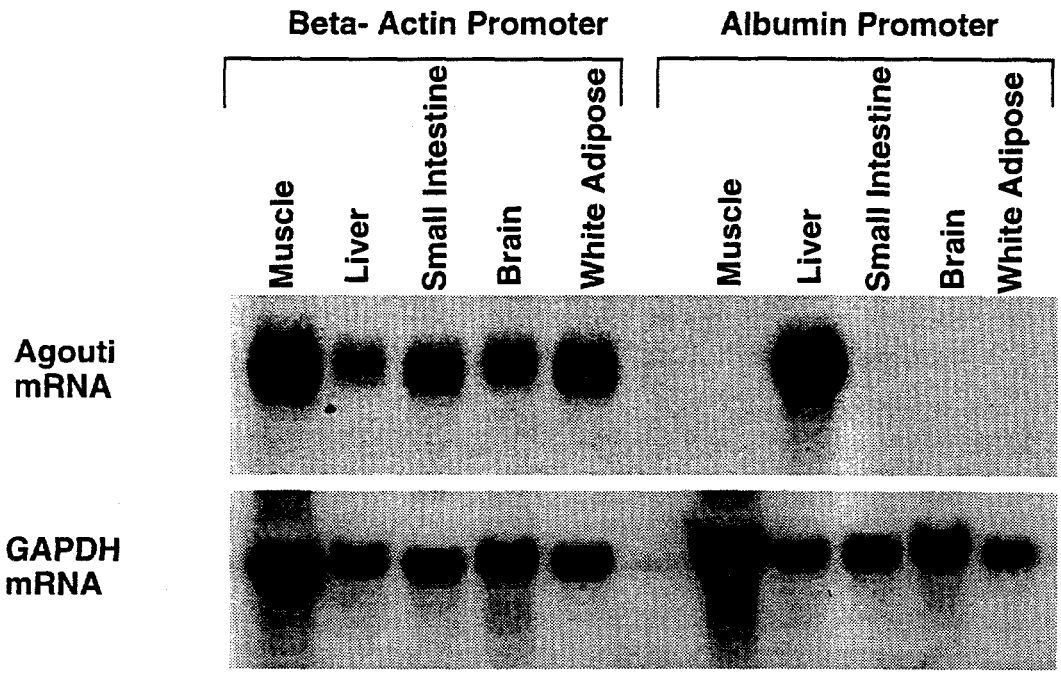
Beta- Actin Promoter
Albumin Promoter
Muscle
Liver
Small Intestine
Brain
White Adipose
Muscle
Liver
Small Intestine
Brain
White Adipose
Agouti mRNA
GAPDH mRNA

Figure 1

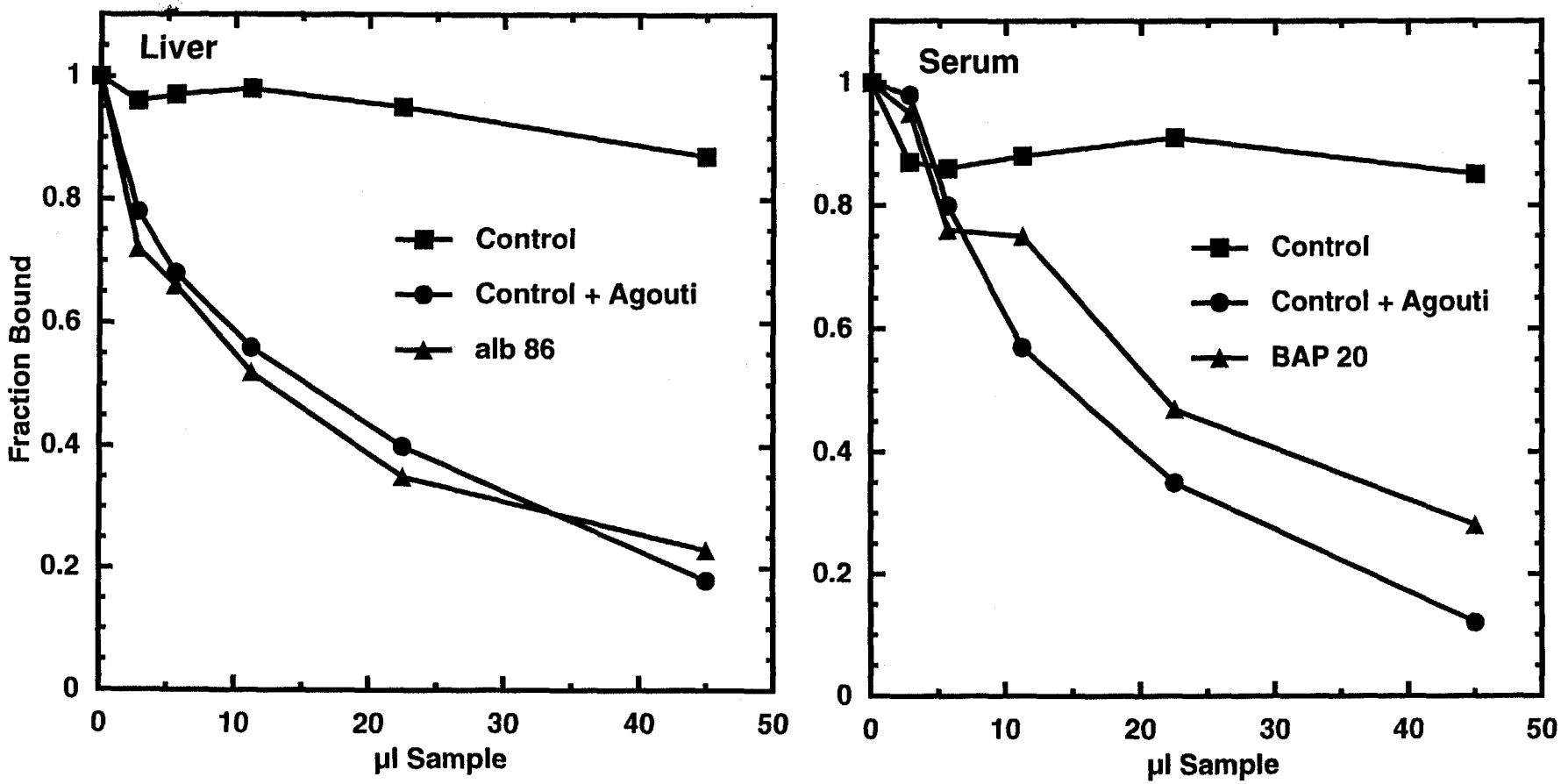
Liver
Fraction Bound
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
µl Sample
Control
Control + Agouti
alb 86
Serum
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
µl Sample
Control
Control + Agouti
BAP 20

Figure 2

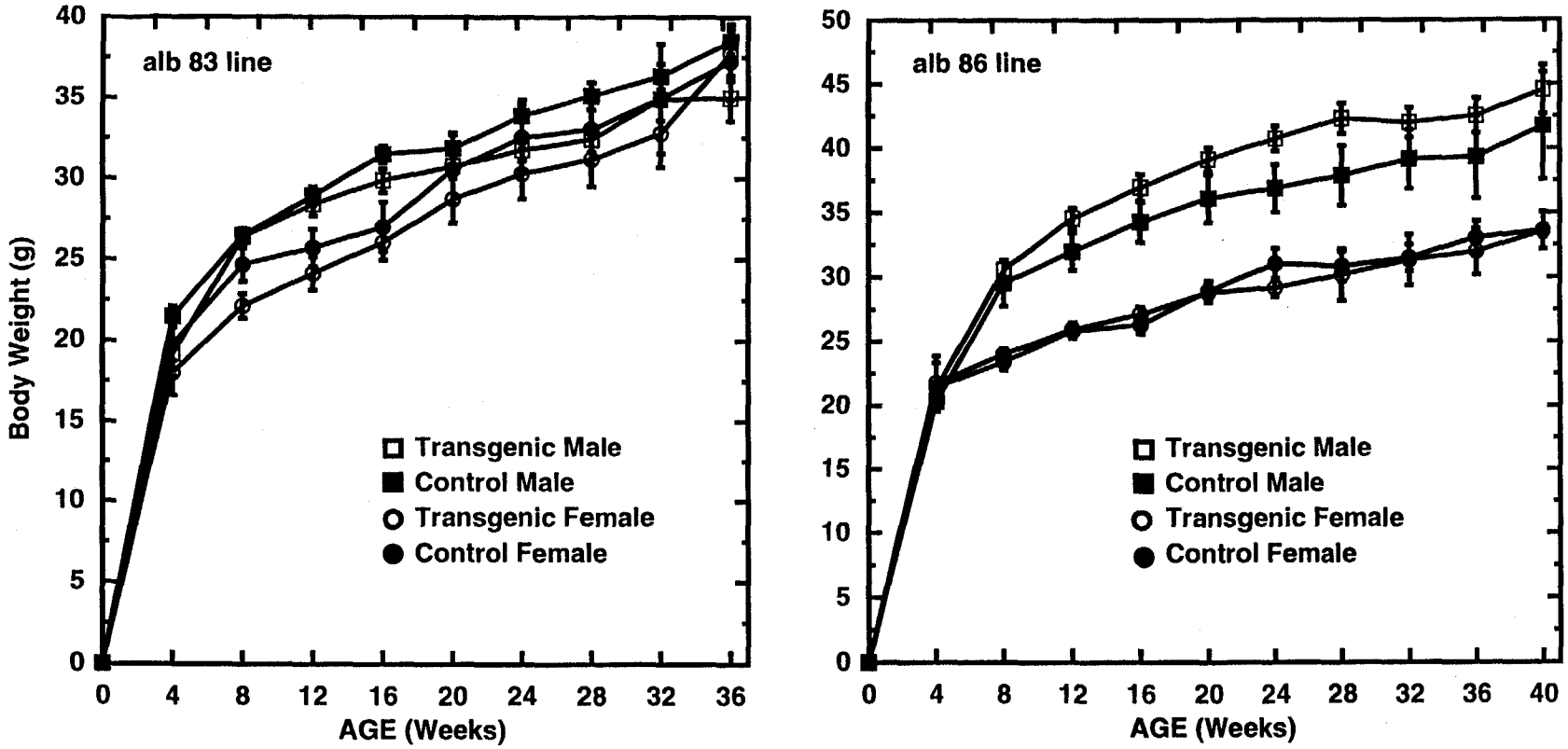
alb 83 line
Body Weight (g)
AGE (Weeks)
Transgenic Male
Control Male
Transgenic Female
Control Female
alb 86 line
AGE (Weeks)
Transgenic Male
Control Male
Transgenic Female
Control Female

Figure 3

A

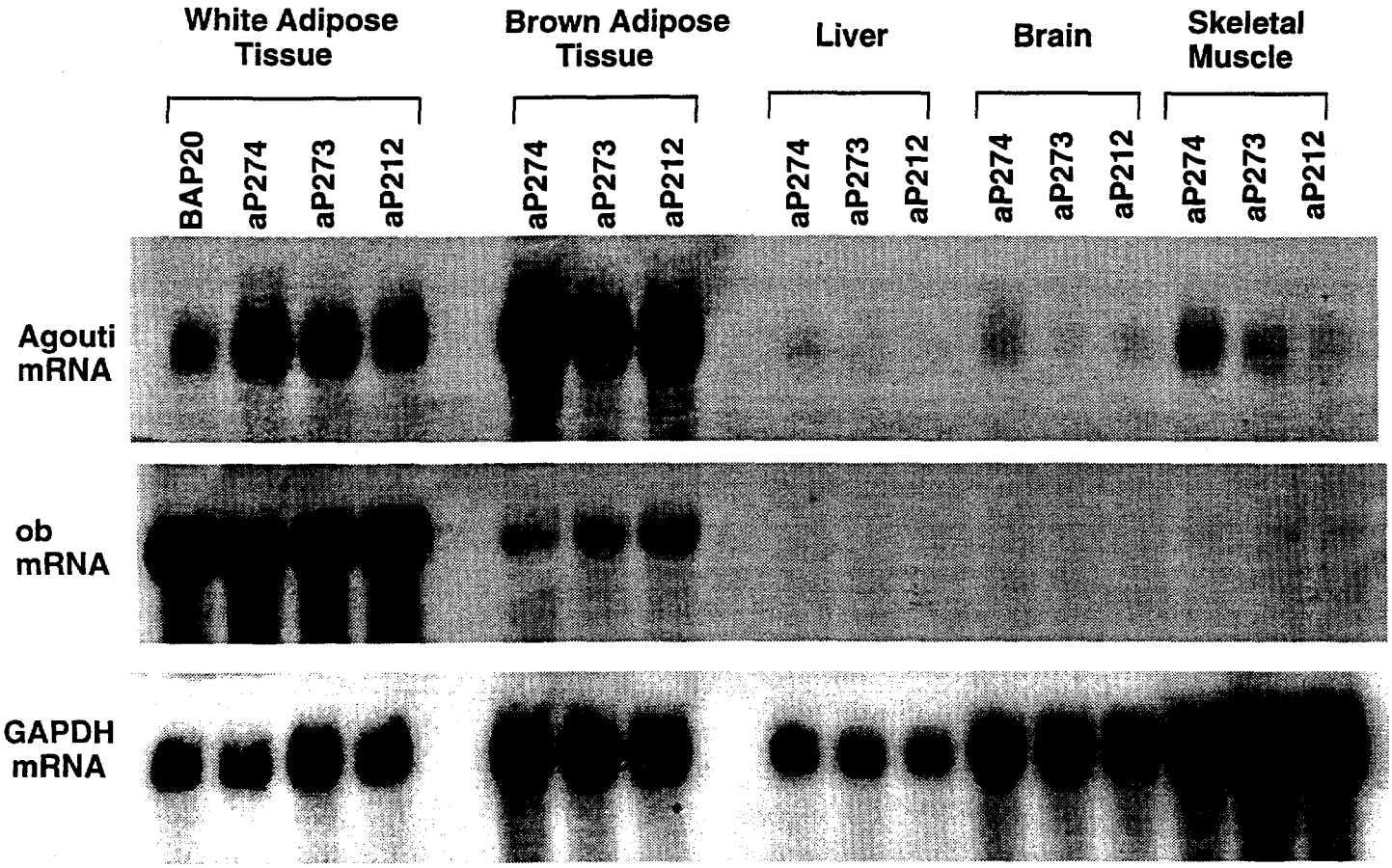
White Adipose Tissue
Brown Adipose Tissue
Liver
Brain
Skeletal Muscle
BAP20
aP274
aP273
aP212
aP274
aP273
aP212
aP274
aP273
aP212
aP274
aP273
aP212
aP274
aP273
aP212
Agouti mRNA
ob mRNA
GAPDH mRNA

Figure 4

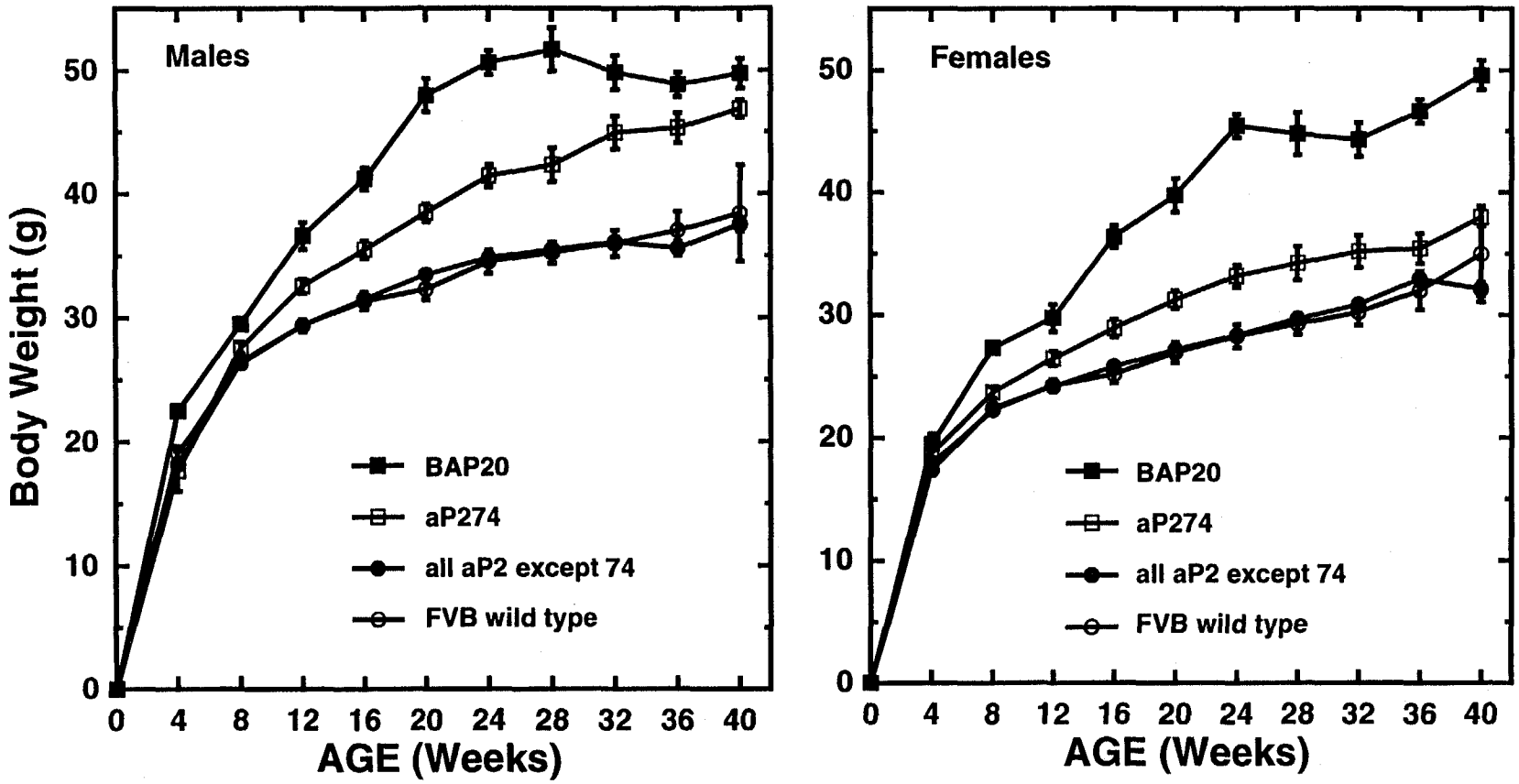
Males
Body Weight (g)
AGE (Weeks)
BAP20
aP274
all aP2 except 74
FVB wild type
Females
AGE (Weeks)
BAP20
aP274
all aP2 except 74
FVB wild type

Figure 5

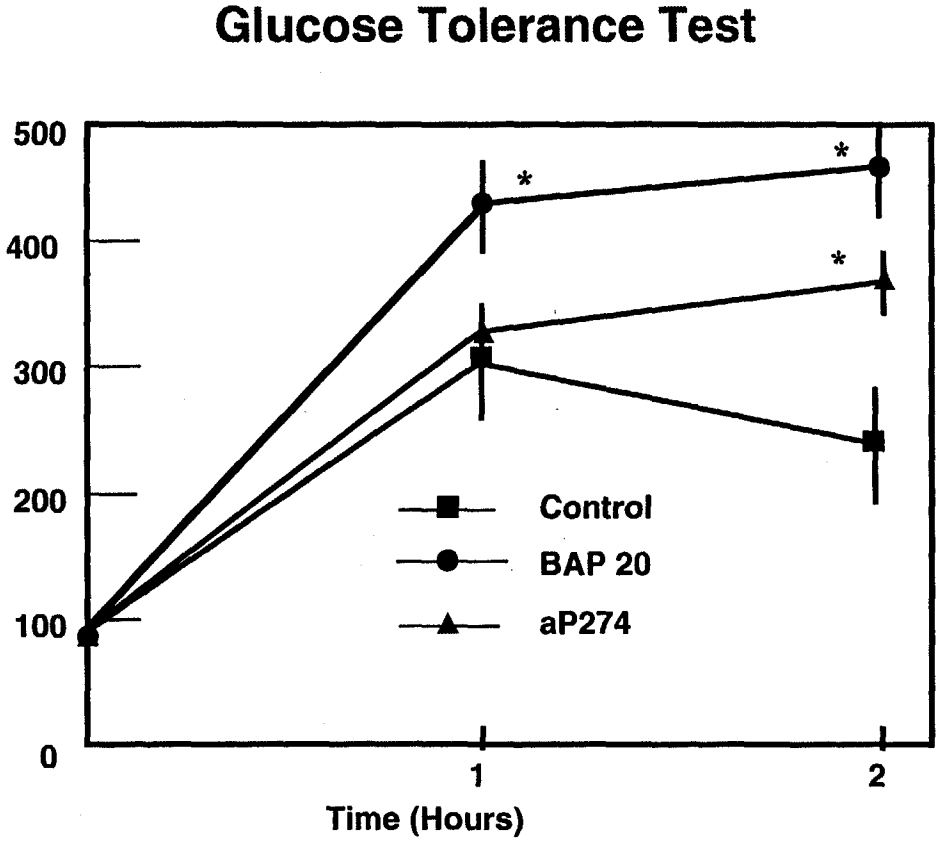
Glucose Tolerance Test
500
400
300
200
100
0
1
2
Time (Hours)
*
*
*
Control
BAP 20
aP274

Figure 6

Agouti Increases Food Efficiency

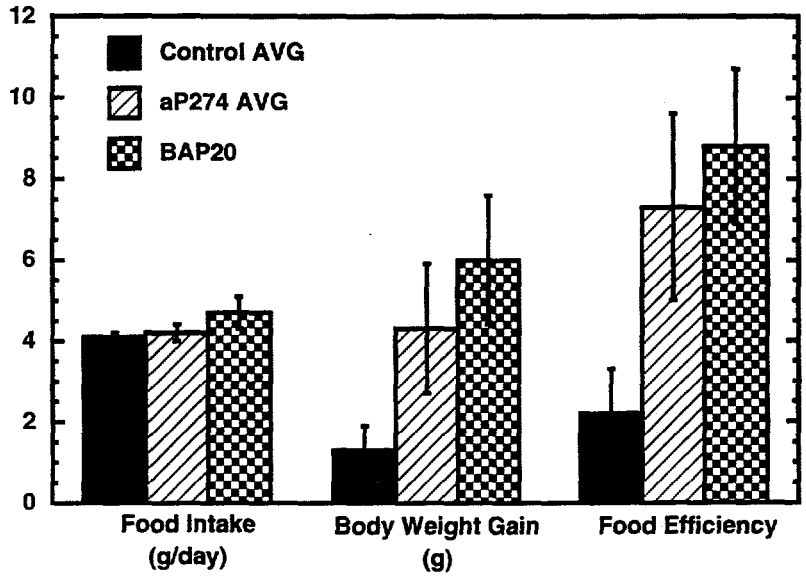

Figure 7