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Differential Expression of Skin Cancer and Hair-Follicle Cycle Regulated Genes in Tumor Susceptible K14-Agouti Mice

Yesim Aydin Son
University of Tennessee - Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Yesim Aydin Son entitled "Differential Expression of Skin Cancer and Hair-Follicle Cycle Regulated Genes in Tumor Susceptible K14-Agouti Mice." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

Edward J. Michaud, Major Professor

We have read this dissertation and recommend its acceptance:

Cymbeline T. Culiat, Mitchel J. Doktycz, Mary Ann Handel, Jay R. Snoddy

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

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Graduate Studies

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DIFFERENTIAL EXPRESSION OF SKIN CANCER AND HAIR-FOLLICLE CYCLE REGULATED GENES IN TUMOR SUSCEPTIBLE K14-AGOUTI MICE

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Yeşim AYDIN SON
August, 2006

DEDICATION

This dissertation is dedicated to my family,
my father, DR. YUSUF AYDIN,
and my husband, DR. ÇAĞDAŞ D. SON,
for their endless love and support.

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ABSTRACT

The mouse agouti protein is transiently expressed in the skin and signals through the melanocortin 1 receptor to switch pigment production of hair-follicle melanocytes from black to yellow. Ubiquitous over-expression of agouti protein in mice carrying the spontaneous dominant mutations A^{y} and A^{vy} causes a pleiotropic syndrome characterized by solid yellow hair color, obesity, diabetes, and increased susceptibility to carcinogenesis in a wide variety of tissues, including the skin. Over-expression of agouti in the skin of keratin 14 (K14)-Agouti transgenic mice promotes skin carcinogenesis, even in the absence of obesity and diabetes. In this study cDNA microarray and qRT-PCR analyses are used to identify molecular changes in the skin of K14-Agouti mice associated with the promotion stage of carcinogenesis. Histological analysis of the skin revealed that there were no differences in gross morphology or in timing of hair-follicle stages between transgenic and control mice. However, cDNA microarray analysis identified 181 genes with significantly altered expression levels in the skin of transgenic mice. Additionally, qRT-PCR analysis demonstrated that the levels and temporal patterns of expression of 10 genes previously associated with skin and/or other epithelial cancers were significantly altered in the skin of K14-Agouti transgenic mice. Agouti-induced over-expression of these proto-oncogenes in the skin of K14-Agouti mice is proposed to be associated with the increased susceptibility to skin carcinogenesis. A hypothetical model is presented to explain the mechanism of action of the agouti protein as a tumor promoter in skin carcinogenesis. Additionally, strategies for future follow-on experiments to further investigate the role of agouti in tumor promotion and to test aspects of the proposed hypothetical model are discussed.

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CHAPTER I

BACKGROUND AND SIGNIFICANCE

Agouti is a paracrine-acting signaling molecule that is normally expressed only in the skin of mice, where it regulates coat color pigmentation. Ubiquitous over-expression of wild-type agouti protein in mice carrying spontaneous dominant agouti mutations results in solid yellow hair color, obesity, diabetes, and increased susceptibility to carcinogenesis in a wide variety of tissues, including the skin. In transgenic mice in which the agouti protein is over-expressed in the skin under the regulatory control of the keratin 14 promoter (K14-Agouti mice), body weight and blood glucose levels are normal, but transgenic mice are significantly more susceptible to skin cancer and it was shown that agouti acts as a tumor promoter in two-stage skin carcinogenesis experiments. The goal of this study was to determine the molecular level changes associated with the promotion stage of skin carcinogenesis in the uninitiated skin of K14-Agouti mice. Since tumor promotion is a reversible and rate-limiting step in carcinogenesis, understanding the molecular changes associated with tumor promotion may lead to strategies for the treatment and prevention of cancer. Microarray and qRT-PCR experiments were performed to determine the expression profiles of genes in the skin of K14-Agouti and control mice at different ages and at different stages of the hair-follicle cycle. Genes known to be associated with skin cancer and other epithelial cancers are shown to be up-

regulated and to have altered temporal expression profiles in the skin of K14-Agouti transgenic mice. It is proposed that these gene expression changes underlie the increased susceptibility of K14-Agouti mice to skin carcinogenesis. A testable model that synthesizes the data obtained in this study is presented to explain the mechanism of action of the agouti protein as a tumor promoter in skin carcinogenesis.

THE MOUSE NONAGOUTI LOCUS

The nonagouti locus (*a*), which is located on mouse chromosome 2, regulates the pigmentation pattern in the hairs of mice. The transcript of the nonagouti locus (referred to as the agouti gene hereafter) is normally expressed only in the skin and encodes a 131 amino acid (aa) signal peptide. The agouti protein acts in a paracrine fashion in the microenvironment of hair follicles [1, 2]. In wild-type mice, the agouti protein is secreted from cells of the dermal papillae during the mid-portion of the hair-growth cycle [3, 4]. Agouti protein competes with alpha-melanocortin stimulating hormone (α-MSH) for binding to the melanocortin 1 receptor (MC1R) on the surface of melanocytes [5]. In response to agouti signaling through MC1R, melanocytes switch from eumelanin (black/brown) pigment production to phaeomelanin (yellow/red) pigment production. As a result of this switch mechanism, individual hairs of wild-type mice have an agouti pigmentation pattern, which consists of a subapical yellow band in an otherwise black hair.

An interest in the coat colors of mice can be traced back thousands of years in China to the first domestication of "fancy mice" that had spontaneous mutations in various coat-color genes. The mouse fancy gained in popularity

during the 1700s and 1800s in Japan and Europe, when mice with a number of different coat colors were bred as pets. At the beginning of the 20th century, after the rediscovery of Mendelian genetics in plants, the next question was if the law of inheritance also applied to the other organisms. Mendelian ratios for the inheritance of different mouse coat colors were demonstrated by Cuenot in 1902, who then discovered the first embryonic lethal mutation in mammals through segregation studies in mice with a completely yellow coat. These mice are now known to carry a dominant regulatory mutation (lethal yellow, A^y) in the agouti gene. During this period, the first case of multiple alleles at the agouti gene was also described by Cuenot [6, 7].

To date, 85 different alleles of the nonagouti locus have been identified, as reported in the Mouse Genome Informatics (MGI) database (http://www.informatics.jax.org). Recessive mutant alleles of the agouti gene are associated with the underproduction of phaeomelanin, which results in an increase in the presence of black pigmentation. Dominant mutations in the agouti gene affect the regulation of wild-type agouti expression, causing high levels of expression in all tissues of the body. Ectopic over-expression of the wild-type gene product in all tissues results in yellow-haired mice that are obese, diabetic and have increased susceptibility to hyperplasia, and spontaneous and chemically induced tumors ([8, 9], reviewed in [10-19]). Tumor susceptibility has been observed in a wide variety of tissues including the skin [20, 21], liver [22-28], lung [23, 27], mammary gland [22, 23, 29-31] and urinary bladder [32]. Cloning of the agouti gene and identification of its protein product led to significant advances in characterization of the molecular mechanisms of wild-type mouse coat pigmentation, obesity and diabetes [1, 2, 13, 33]. An historical perspective on the agouti gene can be found in the review by Wolff in 2003 [12].

Molecular Characterization of the Agouti Gene

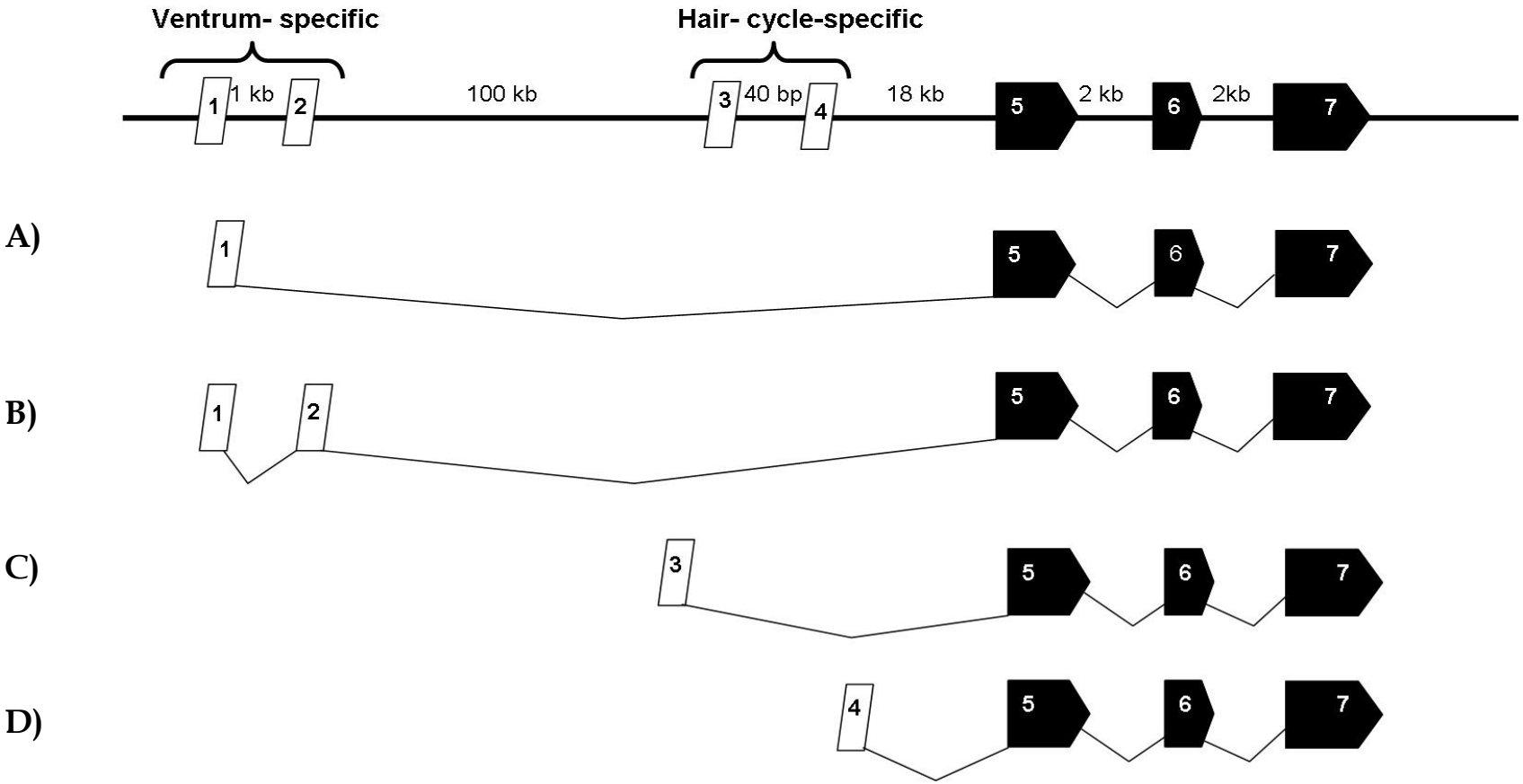
Agouti Gene Structure

In 1992, the agouti gene was cloned by Bultman et al. utilizing the radiation induced inversion mutation, *Is(17; In2)1Gso*. The limb deformity *(Fmn1,* previously known as *ld)* and nonagouti (*a*) loci are normally separated by 22 cM on mouse chromosome 2. In mice carrying the *Is(17;In2)1Gso* mutation, an interstitial inversion in chromosome 2 with breakpoints in *a* and *Fmn1* resulted in the juxtaposition of *Fmn1* and *a* [34]. Probes for the previously cloned *Fmn1* gene gave molecular access to the *a* locus, and the cloning and characterization of the agouti gene and protein structure [1].

Agouti is a seven exon gene, which spans over a 165-kb distance on mouse chromosome 2 (Fig. 1) [35]. The first four exons of the agouti gene are part of the 5′ untranslated region (UTR). Exons 5, 6 and 7 contain the protein coding sequences, which are 170 bp, 65 bp, and 385 bp long, respectively [1].

Alternative isoforms of agouti mRNAs contain different combinations of non-coding exons spliced to the same three coding exons (Fig. 1). Agouti gene expression is controlled by ventral-specific, and hair-cycle-specific promoters, which independently regulate the production of solid yellow hairs on the ventrum, or banded agouti hairs on the dorsum and ventrum, respectively. Two ventral-specific transcripts contain either exon 1, or exons 1 and 2, spliced to the three coding exons. These ventral-specific transcripts are expressed throughout the hair-follicle cycle in the ventrum and produce solid yellow hair coloration. Two hair-cycle-specific transcripts contain either exon 3 or exon 4 spliced to the

Figure 1. Mouse agouti gene structure and alternative transcripts. The agouti gene structure is shown in the top panel, not drawn to scale. The four non-coding exons (1-4) are represented by parallelograms, and the three coding exons (5-7) are shown as block arrows. The length of each intron is indicated. Two ventral-specific transcripts contain either exon 1 (A) or exons 1 and 2 (B) spliced to the three coding exons, and they both specify solid yellow belly hairs in wild-type agouti mice. Two hair-cycle-specific transcripts contain either exon 3 (C) or exon 4 (D) spliced to the three coding exons, and they both specify the banded hairs on the dorsum and ventrum of wild-type agouti mice. Figure adapted from published literature [1, 36-38].


Ventrum- specific
Hair- cycle-specific
1
1 kb
2
100 kb
3
40 bp
4
18 kb
5
2 kb
6
2kb
7
A)
B)
C)
D)

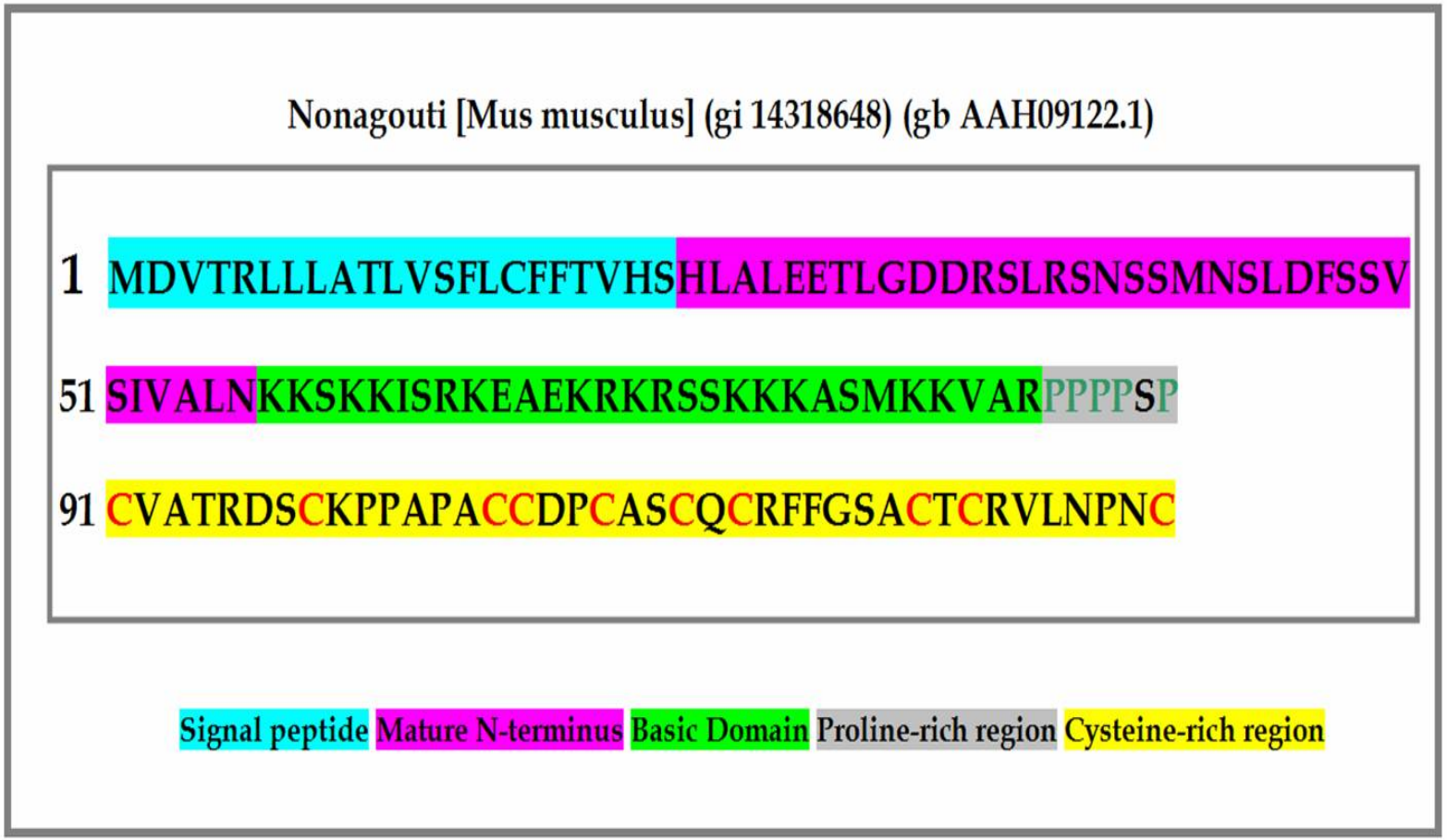
three coding exons. These hair-cycle-specific transcripts are expressed between days 2-7 of the hair-follicle cycle in both dorsal and ventral skin and produce the sub-terminal band of yellow pigmentation in dorsal and ventral hairs [1, 37]. The regulatory regions of the agouti gene and the manner in which these different agouti promoters are activated are still unknown [12].

Agouti Protein Structure and Action

The mouse agouti protein is a 131-aa signaling peptide with a molecular weight of 14,341 Daltons. The agouti protein contains five domains; a signal sequence, mature N-terminus, basic domain, proline-rich region, and a cysteine-rich carboxyl terminus (Fig. 2).

The hydrophobic N-terminus of the agouti protein contains a 22-aa putative signal sequence, which is essential for *in vivo* agouti signaling [39]. The mature N-terminus is 34-aa long and carries a conserved arginine. Glycosylation of this arginine is required for full biological activity of agouti protein *in vivo* [40]. The central basic domain (29 aa) has a high proportion of arginine and lysine residues, and is important for the efficiency of agouti signaling [40, 41]. A polyproline stretch lies between the basic domain and the cysteine-rich C terminus. *In vitro* studies showed that the contribution of the proline-rich region to agouti protein activity is minimal, and suggested that it might be important in forming the tertiary structure of the protein by acting as a flexible hinge between the C-terminus and the N-terminus of the protein [40, 42]. At the C-terminus of the agouti protein are 10 cysteine residues within the last 40 amino acids, which form 5 disulfide bonds. This cysteine–rich domain is required for melanocortin receptor binding, and can antagonize the binding of α-MSH to the melanocortin receptors *in vitro* [43, 44].

Figure 2. Agouti protein sequence and domain structure. The nonagouti protein has five different domains. The signal peptide sequence consists of the first 22 amino acids, highlighted in aqua color. The next 34 amino acids, presented in magenta color, form the mature N-terminus. The basic domain, which is highlighted with green, contains 29 amino acids. The 6 amino acids highlighted in grey constitute the proline-rich region. The yellow color-coded area is the cysteine-rich region that has 10 cysteine residues out of a total 40 amino acids. Proline residues and cysteine residues are shown in green and red color, respectively. Figure adapted from published literature [1, 2].


Nonagouti [Mus musculus] (gi 14318648) (gb AAH09122.1)
1 MDVTRLLLATLVSFLCFFTVHSHLALEETLGDDRSLRSNSSMNSLDFSSV
51 SIVALNKKSKKISRKEAEKRKRSSKKKASMKKVARPPPPSP
91 CVATRDSCKPPAPACCDPCASCQCRFFGSACTCRVLNPNC
Signal peptide
Mature N-terminus
Basic Domain
Proline-rich region
Cysteine-rich region

Parallel to the changes in gene expression, agouti protein is highly expressed during the early stage of the hair-follicle cycle, as shown by immunohistochemistry studies [4]. Agouti protein is localized to the outer root sheath of the hair follicle and functions in the microenvironment of the hair follicle [4, 45] in a paracrine fashion [3].

Agouti's paracrine action was first noted by parabiosis experiments. Parabiotic union, where two animals are sharing blood circulation, has been used to study the interrelationships of the endocrine glands between the agouti mutants, lethal yellow (A^y/a), and their nonagouti (a/a) littermates. Comparison of the rate of body-weight gain between parabiotic pairs of A^y/a and a/a mice throughout the experiment showed that there were no effects of parabiosis on the rate of weight gain of either animal. This observation suggested that agouti protein acted in a localized manner to induce obesity, and not by entering into the general circulation [46].

Skin transplantation experiments by Silvers and Russell [45, 47-50] indicated that the primary site of action of the agouti protein is the follicular microenvironment, and that the agouti protein acts in a cell non-autonomous manner. Transplantation of non-pigmented but genetically yellow embryonic skin to newborn mice with black hair color resulted in intense yellow pigmentation of hair around the edges of the skin graft, demonstrating that host melanocytes migrate into the graft follicles and produce pigment according to the follicular genotype of the graft, independent from their own genotype. In other words, paracrine action of agouti protein expressed from follicular cells of graft tissue was regulating the melanogenesis in the host melanocytes.

Further research with keratin-agouti-SV40 (KAS) transgenic mice designed to understand the regulation of pigmentation patterning also supported the findings of the skin transplantation experiments, showing paracrine action of agouti protein [3]. In KAS transgenic mice, agouti expression is directed to basal cells of the epidermis, which are the non-pigmented cells at the bottom layer of the epidermis that give rise to the epithelial keratinocytes. Stripes of yellow pigmentation are observed in KAS transgenic mice, which corresponded to patches of basal epidermal cells expressing agouti. This study demonstrated the limited radius of agouti protein action, and supported the previous studies suggesting paracrine action of agouti protein.

Orthologs of the mouse agouti gene have been isolated in different species, including *B. taurus* (cattle) [51], *C. familiaris* (dog) [52], *H. sapiens* (human) [53], *R. norvegicus* (rat) [54], and *S. scrofa* (pig) [55]. The agouti proteins are highly conserved in these species. The rat and mouse agouti proteins are 93% identical at the amino acid level. The pig, dog, human and cattle proteins are 81%, 80%, 78% and 77% identical to the mouse agouti protein, respectively.

Agouti Mutants

Agouti mutant mice exhibit a wide range of coat color phenotypes as a result of different mutations that impact agouti regulation or protein structure. All the different alleles of the nonagouti locus represent an intricate dominance hierarchy of coat color [36, 50, 56, 57]. Hair pigmentation changes from solid yellow, as observed in mice carrying the most dominant alleles, to solid black in mice with the most recessive mutations.

At the top of the dominance hierarchy is the lethal yellow (A^{y}) allele. Lethal yellow mice have a regulatory mutation that causes constitutive over-expression of the wild-type agouti gene product in all tissues in heterozygous animals, including the skin, which results in full yellow coat coloration over all of the body. Black-and-tan (a^{t}) and nonagouti (a) are two examples of recessive mutations at the nonagouti locus. An insertion in the nonagouti locus in black-and-tan (a^{t}/a^{t}) mice has a negative impact on expression of the hair-cycle-specific transcripts that make banded hairs, but does not affect expression of the ventral-specific transcripts that make solid yellow hairs. Therefore, black-and-tan mice have completely black hair on the dorsum and completely yellow hair on the ventrum [37]. Nonagouti (a) has a pigmentation pattern that is very close to being a null mutation. Nonagouti mice are predominantly black, except for small amounts of phaeomelanin around the pinnae, nipples, and perineum [50]. In mice carrying the nonagouti allele, an insertion in the locus blocks most all expression of the hair-cycle-specific and ventral-specific transcripts [1]. At the bottom of the hierarchical series are mice carrying the extreme nonagouti (a^{e}) allele, which have a completely black coat color resulting from a complete loss of agouti function [58].

Dominant Regulatory Mutations of Agouti

Six dominant regulatory mutations — lethal yellow (A^{y}), viable yellow (A^{vy}), intracisternal A particle yellow (A^{iapy}), intermediate yellow (A^{iy}), sienna yellow (A^{sy}), and hypervariable yellow (A^{hvy}) — all exhibit yellow coat coloration due to continuous action of the agouti protein in the skin.

The lethal yellow (A^{y}) mutation is the result of a 120-170 kb deletion of the coding region of the ubiquitously expressed hnRNP associated with lethal

yellow (*Raly*) gene, which places agouti under the regulatory control of the *Raly* promoter [9, 59]. Under the transcriptional control of the *Raly* promoter, the agouti gene is ubiquitously over-expressed in A^{y} mutants [8]. Animals homozygous for the A^{y} mutation die during embryogenesis due to the deletion of the *Raly* gene [9, 59]. Heterozygotes for the A^{y} mutation have yellow coat color, and exhibit the pleiotropic effects of obesity, diabetes, and cancer as a result of the ectopic over-expression of the wild-type agouti protein [8].

The five non-lethal dominant regulatory mutations — A^{vy}, A^{iapy}, A^{iy}, A^{hvy}, and A^{sy} — are the result of insertions into the non-coding regions of the nonagouti locus. Expect for A^{sy}, these dominant mutations are caused by insertions of an intracisternal A particle (IAP) into the locus, which changes the regulation of agouti expression [60-63]. Regulatory sequences within one of the long terminal repeats of the IAP elements constitutively activate the agouti gene, much like the *Raly* promoter in the A^{y} allele, and results in ubiquitous over-expression of the agouti protein in all cell types throughout the body. A similar situation exists for the A^{sy} mutation, wherein a novel element is inserted to the locus, instead of an IAP.

The pleiotropic effects associated with ubiquitous over-expression of agouti have been called the 'Yellow Obese Mouse Syndrome' [50], where the main characteristics of the syndrome include yellow coat color, obesity, hyperinsulinemia, increase somatic growth, and tumor susceptibility (reviewed in [11, 15, 38, 64]). The full spectrum of the yellow obese mouse syndrome can be observed in mice carrying the two dominant regulatory mutations A^{y} and A^{vy}. Obesity, hyperinsulinemia, and increased somatic growth have also been demonstrated for mice carrying the mutations A^{iy}, A^{sy}, A^{hvy}, and A^{iapy}. It is highly likely that mice carrying these mutations also have an increased susceptibility to

tumorigenesis, but this has not yet been investigated for these mice. In mice carrying dominant agouti mutations, increasing levels of agouti expression are associated with the amount and intensity of phaeomelanin (yellow pigment) in the coat and with the degree of obesity [63, 65]. Additionally, studies with transgenic mice that ubiquitously express the normal agouti protein throughout their body demonstrated that ectopic agouti expression is sufficient to recapitulate the obesity, diabetes, and yellow coat color phenotypes observed in mice with these spontaneous dominant agouti mutations [33].

Regulation of Pigmentation by Agouti Signaling

Melanocytes switch from eumelanin to phaeomelanin pigment production depending on the presence of the α-MSH or agouti proteins within their follicular environment. α-MSH and agouti compete for the binding of MC1R on melanocytes with similar levels of affinity (K_d 2.3 nmol/l and K_d 3.7 nmol/l, respectively) [66, 67]. α-MSH activates adenylyl cylase through MC1R, which increases cyclic adenosine monophosphate (cAMP) synthesis. This is followed by an increase in tyrosinase synthesis, which triggers eumelanin pigment production (reviewed in [12, 68]). Agouti is transiently expressed during the early stages of each hair-follicle cycle and regulates the switch from eumelanin to phaeomelanin pigment production. When agouti binds to the MC1R, it displaces or excludes the binding of α-MSH, and thus prevents adenylyl cylase activation and cAMP production [5]. In response to reduced cAMP levels, tyrosinase synthesis decreases and eumelanin pigment production is switched to phaeomelanin production (reviewed in [12, 68, 69]). Agouti signaling has been shown to down-regulate transcription of eumelanogenic genes such as tyrosinase-related protein 1 (*Tyrp1*), dopachrome tautomerase (*Dct*), and

tyrosinase (*Tyr*) [70, 71]. Additionally, up-regulation of three genes, minichromosome maintenance deficient 6 (*Mcm6*), transcription factor 4 (*Tcf4*), and an unknown retinal gene, have been shown during phaeomelanogenesis [72].

Molecular Mechanisms of Obesity and Diabetes in Yellow Mice

Ubiquitous over-expression of agouti is responsible for the pleiotropic effects observed in mice carrying dominant agouti alleles. Confirmation of the association between agouti protein expression, obesity, and diabetes was first demonstrated by ectopic expression of the wild-type agouti gene under the ubiquitously expressed β-actin promoter in transgenic mice [14, 33].

Melanocortin receptors are seven-transmembrane G protein-coupled receptors (GPCR) that respond to α-MSH and adrenal corticotropic hormone (ACTH) stimuli [73]. All five members of the melanocortin receptor family (MC1R through MC5R) differ in their tissue distribution, physiological roles, and ligand affinity (reviewed in [74, 75]). MC1R is expressed in the skin in melanocytes and keratinocytes, and regulates coat color pigmentation. It binds to α-MSH with the highest affinity, which is followed by adrenocorticotropic hormone (ACTH), and β-MSH. MC2R, which is expressed by the adrenal cortex and adipocytes, only responds to ACTH, and induces adrenocortical steroidogenesis [76, 77]. MC3R and MC4R are widely expressed in the brain, and participate in the hypothalamic regulation of food intake and body weight. MC4R loss of function is associated with severe early onset obesity in humans. Both MC3R and MC4R are activated by α-MSH, β-MSH and ACTH, and MC3R

also binds to γ-MSH (reviewed in [78, 79]). MC5R is ubiquitously expressed in peripheral tissues and sebaceous glands, and activated by α-MSH, β-MSH, and ACTH signaling. Recently, MC5R has been shown to act in regulation of exocrine gland function and human sebocyte differentiation [80].

Ectopic expression of agouti in the brain is primarily responsible for the obesity and diabetes phenotypes in lethal yellow (A^{y}) and viable yellow (A^{vy}) mice. Transgenic studies with *Mc3r* and *Mc4r* knockout mice, which exhibit a similar phenotype to "yellow obese mice" with regard to weight gain and diabetes, pointed out the possible interaction between central melanocortin receptors and agouti protein [81, 82]. Pharmacological studies showed that agouti antagonizes MC3R and MC4R in the brain ([83], reviewed in [79, 84]), as it does MC1R in the skin of wild-type mice, resulting in altered feeding behavior and body weight.

Agouti action on adipocytes is also associated with obesity and diabetes, independent of its action through MC3R and MC4R in the hypothalamus. Paracrine/autocrine action of agouti in adipose tissue has been suggested to contribute to the obesity phenotype in yellow mice [13, 85, 86]. Fatty acid binding protein 4, adipocyte (*Fabp4*)-agouti transgenic mice, which express agouti only in adipocytes, had significantly heavier body weights even though the regulation of food intake by the hypothalamus was intact [87]. Transgenic studies with Fabp4-agouti mice demonstrated that agouti regulates adipogenesis, and proliferation and differentiation of preadipocytes through melanocortin receptors in the adipose tissue [88]. These findings suggested that weight gain and adipogenesis are coordinately regulated by melanocortin signaling in the hypothalamus and adipose tissue [87-90].

In humans the agouti gene is normally expressed in adipose tissue [53], and agouti mRNA levels are significantly elevated in adipose tissue of patients with type 2 diabetes [88]. Agouti signaling in adipose tissue has been shown to interact with insulin and induce obesity by up-regulating lipogenesis [90], and agouti synergistically works with insulin to increase fatty acid synthetase (FASN) activity [91]. Agouti has been shown to increase *Fasn* mRNA levels through an agouti responsive element, which is a novel sequence located in the *Fasn* promoter region [91]. Additionally, agouti is known to increase leptin (*Lep*) expression, synthesis, and secretion in adipose tissue, independent of melanocortin receptor antagonism [92].

Agouti protein has been shown to increase intracellular calcium levels in certain cells *in vitro*, including adipocytes [89, 93, 94]. In both mouse and human adipocytes, agouti regulates lipogenesis and lipolysis partially through an intracellular calcium dependent mechanism [85, 95, 96]. Agouti treatment increases *Fasn* activity and mRNA levels, and increases triglyceride accumulation in 3T3-L1 adipocytes, which is inhibited by Ca^{2+} channel blockade [95]. Treatment of yellow obese A^{vy}/a mice with the calcium channel blocker, Nifedipine, resulted in significant decrease in fat pad weights and FASN activity [96].

In adipocytes, in addition to the regulation of *Fasn* and *Lep* gene expression, agouti also directly regulates expression of signal transducer and activator of transcription 1 (*Stat1)* and *Stat3*, and peroxisome proliferator activated receptor gamma (*Pparg*) [97]. Increased expression levels of *Stat1*, *Stat3*, and *Pparg* was first observed in Fabp4-agouti transgenic mice, and similar increases were observed in 3T3-L1 adipocyte cultures treated with agouti protein [97]. Agouti has been suggested to regulate *Pparg* expression through

melanocortin signaling, as treatment of 3T3-L1 adipocytes with α-MSH has been shown to block the effects of agouti on *Pparg* expression [87]. However, the molecular mechanisms that underlie the regulation of *Stat1* and *Stat3* by agouti are still under investigation.

AGOUTI SIGNALING IN TUMOR PROMOTION

The association between the agouti gene and susceptibility to tumor formation has been recognized since the identification of yellow mice (reviewed in [10-19]). Recently, transgenic experiments confirmed the role of agouti as a tumor promoter, and demonstrated that it promotes tumorigenesis in the absence of obesity and diabetes [98, 99]. In order to understand the context and significance of current knowledge of agouti signaling during tumor promotion, background information on the following processes will be presented: two-stage skin carcinogenesis model, tumor susceptibly in mouse skin, and the increased susceptibility of mouse skin to tumorigenesis during the anagen stage of the hair-follicle cycle.

Two-Stage Model of Skin Carcinogenesis

Skin cancer is the most common form of cancer in humans. Every year over one million new patients are diagnosed with skin cancer [100]. Skin acts as a protective barrier against different environmental conditions, such as ultraviolet radiation (UV), injury, infectious agents, and chemicals, which are known risk factors for skin cancer. Although the main risk factor of skin cancer is the exposure to artificial or natural UV, others factors like human papillomavirus infections or exposure to chemicals like arsenic can cause development of cancers

in the skin. Additionally, certain hereditary conditions can increase the tendency to develop skin cancer [101, 102]. Depending on the underlying cause, different cell types in the epidermal layer of the skin are affected and the origin of the tumor cells determines the characteristics of the skin cancer. The most common form of skin cancer is basal cell carcinoma (BCC), which originates from the round basal cells located in the lower layer of the epidermis. Melanomas are the leading cause of death due to skin cancer, and originate from pigment producing cells, melanocytes. Squamous cells at the upper epidermal layer give rise to squamous cell carcinomas (SCC). Additionally, precancerous conditions such as follicular papillomas, actinic keratosis, and Bowen's disease can turn into squamous cell carcinomas [103].

Skin cancer can be recapitulated in mice in a two-stage carcinogenesis model. These experiments on mouse skin have shown that transformation of normal cells requires at least two separate events, initiation and promotion, which are then followed by tumor progression [104-109] (Fig. 3).

The first step in tumorigenesis is initiation, which is a single, irreversible, mutagenic event, and precedes tumor promotion. The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA), is frequently used as an initiating agent in skin carcinogenesis experiments. A single application of DMBA to the skin causes mutations in the Harvey rat sarcoma virus oncogene 1 (*Hras1*) gene, which is frequently observed in papillomas and SCC [110]. After initiation with DMBA, promotion of the skin with physical, chemical, or biological agents is required for clonal expansion of the initiated cell that carries

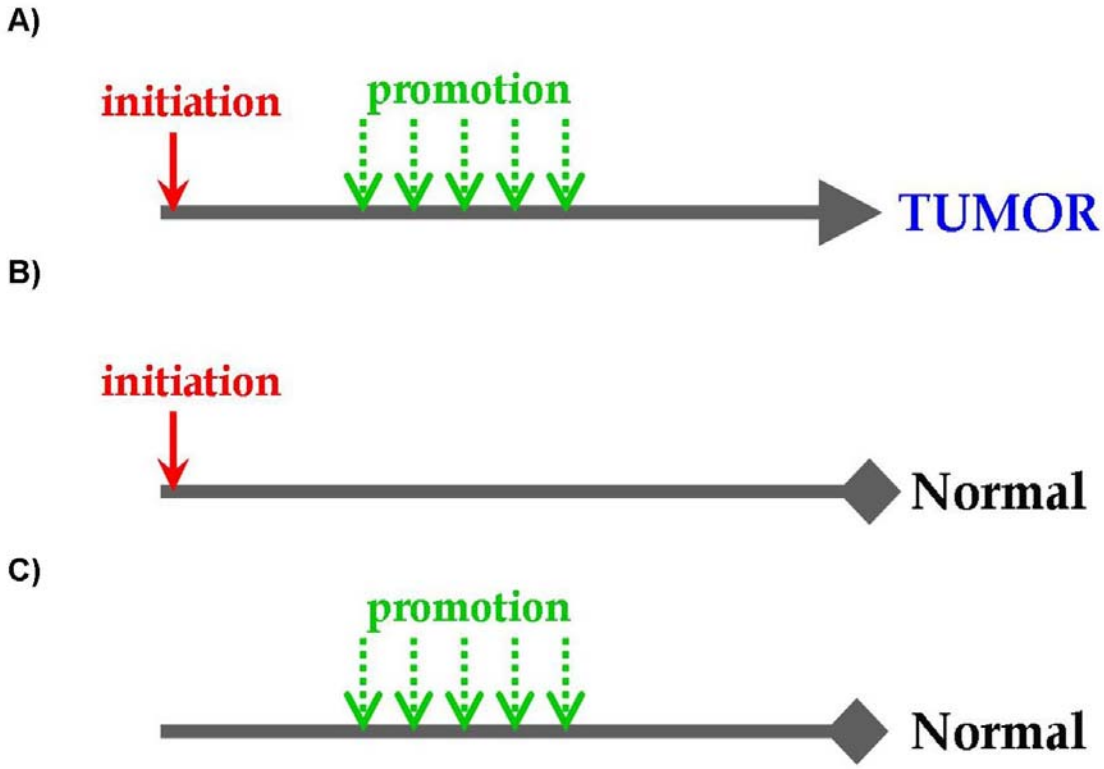


Figure 3. Two-stage model of carcinogenesis. Possible outcomes in a two-stage carcinogenesis experiment. (A) Tumors develop following a one-time initiation event and repeated exposure of initiated cells to a tumor promoter. Either tumor initiation (B) or tumor promotion (C) alone is inadequate to induce the development of tumors.

a non-lethal mutation. Tumor promoters may be external or internal stimuli. Repeated exposure of the skin to promoting agents in short intervals results in altered gene expression and affects cell growth and differentiation of the initiated cells. During tumor promotion, a continuous mitogenic stimulus provides a growth advantage to the initiated cells by either increasing resistance to differentiation or apoptosis or increasing proliferation rates [111]. Promotion is an essential step for tumor development, which is rate-limiting and reversible; thus, therapeutic interventions focused on the prevention and regression of tumor promotion are very likely to be effective [109, 112-114].

The two-stage skin carcinogenesis model has allowed investigators to explain the complex biological events behind carcinogenesis in simple terms, and has been an essential tool to study the tumorigenesis process in mice and humans. Discoveries about tumor initiation and promotion made in two-stage skin carcinogenesis experiments are also relevant to other tissues [115]. These experiments are widely used to elucidate the role of chemical carcinogens or oncogenes, which can be established according to the experimental conditions required for a carcinogen to drive tumor development (Fig. 3). Tumor initiators are expected to give rise to skin tumors after a single application of the agent to the skin of mice, when followed by multiple applications of a tumor promoter to the skin, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Likewise, agents can be tested for their ability to act as tumor promoters by first initiating the skin with DMBA, followed by repeated applications of the agent in question to the skin and then monitoring the skin of mice for the development of skin tumors over time.

Much of our information about tumor development in mice and humans was based on research using the two-stage skin carcinogenesis model. Following

advancements in the understanding of skin carcinogenesis, this model has also been used to study tumor development in other tissues, such as the liver, lung and breast. Recapitulating these different types of tumors allowed investigators to understand biological changes associated with different stages of tumor development.

Tumor Susceptibility in Mouse Skin

Due to the differences in their genetic background, the degree of susceptibility to tumorigenesis varies between inbred mouse strains [105, 116]. Inherited traits that cause initiation or promotion of the normal cells increase the susceptibility to tumorigenesis, as cells have already gone through one of the two essential steps required for tumor development (Fig. 3).

The v-Ha-ras transgenic TG.AC mouse line is one of the examples that demonstrate the relationship between inherited factors and susceptibility to carcinogenesis. In this transgenic model, increased susceptibility to skin carcinogenesis is observed as they carry an activated allele of the *Hras1* oncogene, which is a frequently observed mutation in initiated skin cells [117]. SENCAR, sensitivity to cancer, is another well-known mouse model that was bred for their increased susceptibility to tumor development. SENCAR mice show high susceptibility to initiation and promotion in two-stage carcinogenesis experiments, as they require lower doses of carcinogens, and develop higher numbers of tumors in a shorter time compared to other inbred mice [107, 118]. Although the molecular basis of tumor susceptibility in SENCAR mice is unknown, involvement of multiple genes has been suggested as different strains of SENCAR mice show variability in susceptibility to tumorigenesis [112, 119]. The tumor promotion stage has been observed as the key event that determines

the degree of tumor susceptibility in different inbred mouse strains [116]. Carcinogenesis studies with SENCAR mice have supported previous observations, indicating the correlation between the promotion stage and degree of tumor susceptibility [112].

Inbred mouse strains that demonstrate different degrees of tumor susceptibility have been utilized for quantitative trait analysis, and currently 17 quantitative trait loci (QTL) have been localized for tumor susceptibility in skin: skin tumor susceptibility (*Skts1* through *Skts13*) (reviewed in [120]), and promotion susceptibility (*Psl1* through *Psl4)* loci [121, 122]. Identification of new genetic mechanisms that impact tumor susceptibility will be facilitated by cloning and characterizing the genes associated with these QTLs.

Hair-Follicle Cycle and Tumor Susceptibility

The hair follicle (HF) is a regenerating structure that resides in the dermal layer of the skin (Fig. 4). All mature hair follicles proceed through cyclical changes throughout the life of mammals, which can be categorized into four phases; growth (anagen), regression (catagen), relative resting (telogen), and shedding (exogen). Hair follicles exhibit distinct morphological characteristics at each stage, which is helpful for identification and dissection of anagen, catagen, and telogen stages [123].

The most recognizable structure of the HF is the hair bulb, which is an onion shaped structure formed by relatively undifferentiated matrix cells and HF melanocytes. The dermal papilla (DP) consists of differentiated mesenchymal cells, and resides adjacent to the hair bulb at the base of the HF, which is later

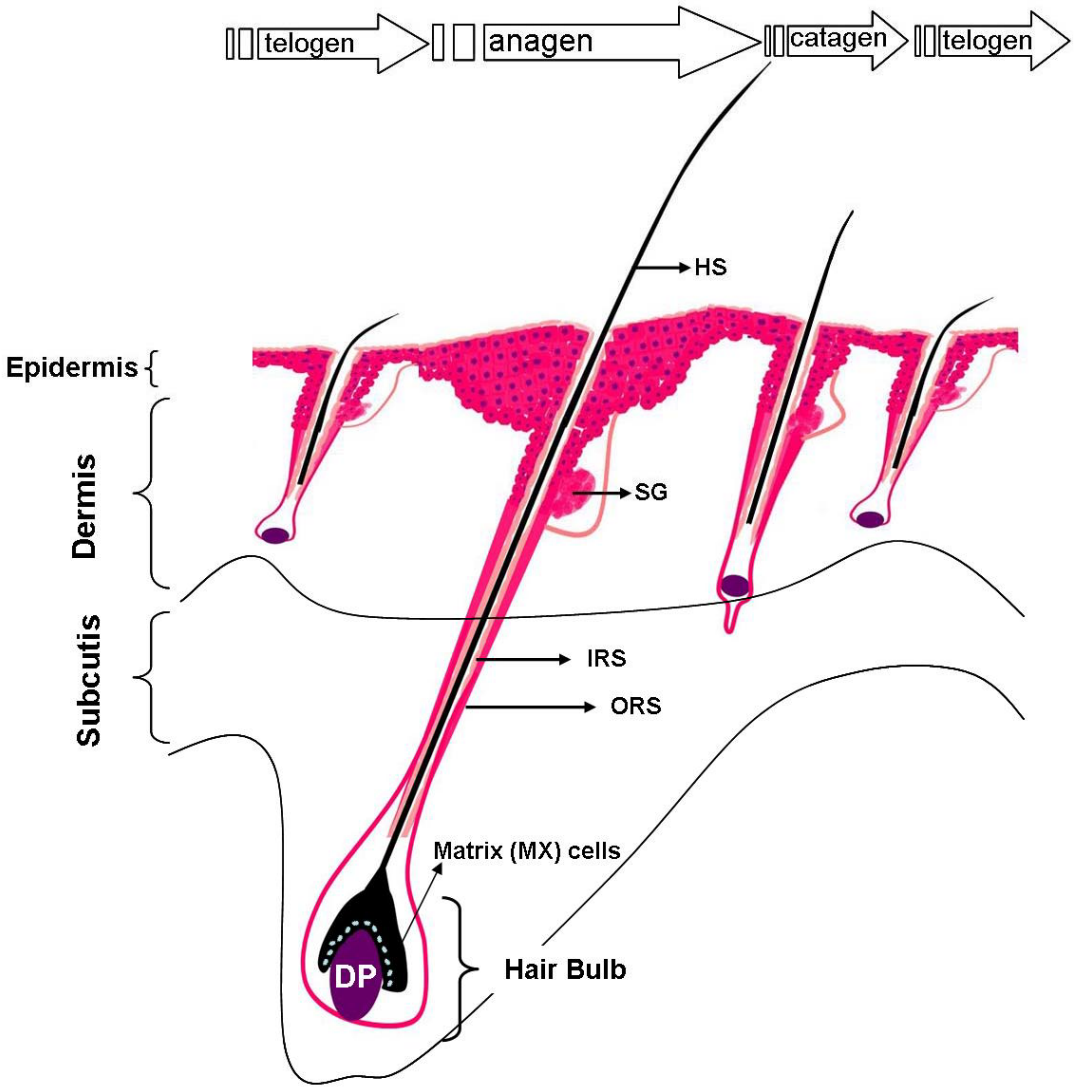


Figure 4. Structure of the hair follicle and the hair-follicle cycle stages. Morphological changes associated with different hair-follicle cycle stages are presented. The stages of the hair-follicle cycle are anagen (growth), catagen (regression), and telogen (resting). The structures of an anagen hair follicle are shown: hair shaft (HS), sebaceous gland (SG), inner root sheath (IRS), outer root sheath (ORS), matrix (MX), and dermal papilla (DP). The hair follicle exhibits distinctive morphology associated with each stage (see text for details). Figure adapted from published literature [123].

surrounded by the hair bulb during the anagen stage of the hair-follicle cycle. DP cells orchestrate hair follicle formation during embryogenesis, and the hair bulb continues to be an important structure during hair-follicle cycling and in hair formation in postnatal life. Matrix cells (MX) of the hair bulb differentiate into the hair shaft (HS) and inner root sheath (IRS), which surrounds the HS. The outer root sheath (ORS) consists of HF keratinocytes, which form the outermost layer of the HF, merging the basal layer of the epidermis and the HF. The bulge region is a specialized part of the ORS located at the insertion site of the arrector pili muscle (APM), which contains stem cells that give rise to both the HF and interfollicular keratinocytes.

Anagen is the stage of active growth, when the hair follicle proliferates and differentiates to produce a new hair shaft. The first set of changes marking the start of anagen is the enlargement and repositioning of the DP inside the hair bulb. After receiving initiation signals from the hair matrix, DP cells signal back to the MX, instructing the matrix cells to differentiate and proliferate to form a new hair shaft. During anagen, melanin pigment is synthesized by hair follicle melanocytes. The hair follicle, which is located in the dermis, elongates and crosses the dermis-subcutis border, growing deep into the subcutis. Additionally, epidermal and dermal layers of the skin thicken to accommodate the growing hair follicle [124-127].

Apoptotic events control the regression of the hair follicle throughout the catagen stage, which is a transitional stage between anagen and telogen. During catagen, the DP condenses and resumes its ball-shape, while the hair bulb retracts back toward the dermis, leaving the DP outside of the bulb. As the HF gets shorter, all the HF structures move back into the dermis leaving behind a membrane trail. At the end of catagen, the hair follicle reaches its smallest size

before entering into the next resting period [124-127]. By the start of telogen, molecular pathways that have regulated the changes during anagen and catagen turn back to their basal activity levels. Throughout telogen, the hair follicle preserves its size and shape, and the hair shaft stays attached to the follicle until it is shed or pushed out by the new hair produced during the following anagen stage [124-127].

Initiation of the hair-follicle cycle, progression through each of the stages, and the transitions from one stage to the next requires the involvement of different signaling pathways communicating mostly between matrix cells of the hair bulb and the DP. These molecular controls and changes associated with each stage of the hair-follicle cycle are less clear than the associated morphological changes. Many signaling pathways that are important in embryonic development, such as hedgehog, patched, Wnt, dishevelled, armadillo, engrailed, and Notch are critical for normal hair follicle morphogenesis [124], and also regulate postnatal hair follicle growth and cycling processes [127]. For instance, transient expression of sonic hedgehog signaling (*Shh*) is required for the embryonic development of the hair follicle, and it also acts as a biological switch initiating the anagen stage in cycling postnatal follicles [128]. Additionally, estrogens, thyroid hormones, glucocorticoids, retinoids, and growth hormones modulate hair growth [124], and many genes, including *a*, *Pdgfa*, *Stat3*, and *Prl* act as molecular mediators of hair follicle growth [126]. Integration of bone morphogenetic protein (*Bmp*) and Wnt signaling pathways has been suggested to regulate hair follicle stem cell differentiation and proliferation [129], along with other recently identified regulators, such as telomerase reverse transcriptase (*Tert*) [130, 131] and RAS-related C3 botulinum substrate 1 (*Rac1*) [132]. The molecular mechanisms that control hair follicle morphogenesis and cycling have been shown to be involved in the regulation of

embryonic and postnatal development of other tissues, such as teeth, feathers, and mammary glands [133], and are suggested to have a role in other developmental systems [127]. Details of the molecular mechanisms underlying the hair-follicle cycle and its regulation have been reviewed by Stenn and Paus [126, 134].

The first two hair-growth cycles (catagen, telogen, anagen) after birth are completed precisely in 12 weeks. The first catagen stage takes place during the first two weeks of life, followed by the first telogen and anagen stages, which are completed in three and a half weeks after birth. The second postnatal hair-follicle cycle takes twice as much time to complete, and occurs during the second and third months after birth. All postnatal hair follicles go through the first and second hair-follicle cycles in a synchronized manner. After the second anagen stage this synchronization is lost and each hair follicle starts to cycle independent from one another, so in one skin section follicles in all different hair-follicle cycle stages can be observed [125]. In adult mice, the hair-follicle cycle can also be synchronized under defined experimental conditions. If hair shafts are removed by hot wax application during the second telogen stage, at around 7 to 8 weeks of life, the second anagen stage will be initiated at the same time for all the hair follicles in the epilated area. As all the hair follicles are induced to initiate anagen at the same time immediately following epilation, they progress synchronously through the next anagen, catagen and telogen stages. Synchronization of the hair-follicle cycle allows one to study histological and molecular changes during each specific stage of the hair cycle [135].

Besides hair shaft formation, the hair-follicle cycle is important at the cellular level as it impacts different molecular events, such as cell-cell

communication, tissue regeneration, aging, and carcinogenesis (reviewed in [126]).

Different cell types of the hair follicle give rise to morphologically distinct tumors, and an association between different types of tumors and specific stages of the hair-follicle cycle has been known for many decades [136-138]. A systematic study was conducted in which the histology of various types of hair-follicle tumors was correlated with the stage of the hair-follicle cycle when tumors arose [131]. This study demonstrated that tumors that arise in anagen originate from the hair MX, ORS and IRS of the hair follicle, which form the hair shaft of the mature anagen follicle [139]. Additionally, it has been demonstrated that a majority (88%) of hair-follicle tumors develop during the anagen phase from the fully differentiated follicular cells [139]. Skin papillomas have been shown to originate from hair follicle structures in v-Ha-ras transgenic TG.AC mice after initiation of the anagen stage [140]. Two-stage carcinogenesis experiments demonstrated that the sensitivity of the skin to tumor formation is greatest during the anagen stage of the hair-follicle cycle [137, 141]. Both Swiss and SENCAR mice developed higher numbers of tumors at the end of the 40-week period following initiation by DMBA during the early anagen stage of the hair-follicle cycle.

These observations have been supported by the demonstration of the transient proliferation of follicular epithelial stem cells during early anagen [142]. All stem cells are especially susceptible to tumorigenesis due to their potential for proliferation and their long-term presence in tissues, which allow for the accumulation of multiple oncogenic events in one cell. Follicular epithelial stem cells located in the bulge region of the ORS give rise to both the hair shaft and epidermis (reviewed in [143, 144]), where the skin papillomas and other anagen

tumors have been shown to originate. It has been suggested that stimulation of follicular epithelial stem cells by the initiation of the anagen stage is responsible for increased susceptibility to tumorigenesis in mouse skin during the anagen stage of the hair-follicle cycle [142].

Agouti is a Tumor Promoter

Even though many aspects of the molecular mechanisms of agouti-induced obesity and diabetes have been explained, virtually nothing is known about the molecular basis of agouti-induced tumor susceptibility in yellow mice.

The dominant agouti mutations A^{y} and A^{vy} influence tumor development by shortening the latency period (time from initial exposure to first detection) of tumor formation [28, 38] and increasing the prevalence of tumors (number of animals effected) [15, 26, 27, 98, 99]. While these typical effects of A^{y} and A^{vy} mutations are observed in many tissues, several studies have revealed contradictory findings. First, there was no change in the latency period of chemically induced thymic lymphomas observed in mice carrying either the A^{y} or A^{vy} mutations [145]. Additionally, a decreased prevalence of spontaneous teratomas [146] and mammary tumors have been reported in mice carrying A^{y}. Despite the decrease in number of animals with mammary tumors, the latency period needed for development of mammary tumors in A^{y} mice was still shorter [147]. There are also contradictory findings on the *in vitro* transformation of fibroblasts from A^{vy} mice. A decreased prevalence of spontaneous and chemically-induced transformation of primary fibroblasts from an A^{vy}/a mouse was observed [148], whereas established cell lines from A^{vy}/a fibroblasts showed

an increased prevalence during spontaneous focus formation experiments compared to *a/a* control cell lines [149].

To date, many different mechanisms have been proposed to explain the role of agouti in increasing tumor susceptibility in mice, including increased proliferation of transformed cells, increased DNA and protein synthesis, increased mitosis in sensitive tissues, and metabolic changes in precancerous cells [15, 16, 38], but the role of agouti in tumor susceptibility remains largely unknown.

The first demonstration that agouti may have a role in tumor promotion independent of its effects on obesity and diabetes came from a study in A^{vy}/a mice. Depending on the methylation level of regulatory elements of the IAP insertion in the A^{vy} allele, A^{vy}/a mice show different levels of agouti expression and different degrees of obesity and diabetes. High levels of IAP methylation result in extremely low levels of ubiquitous agouti expression in A^{vy}/a mice. These mice have normal body weights and normal blood glucose levels, but they have a subtle alteration in coat color (slight increase in size of sub-terminal yellow band) that is called pseudoagouti. Low levels of IAP methylation result in high levels of ubiquitous agouti expression in A^{vy}/a mice. These mice are obese, diabetic, and have a solid yellow coat color. Obese yellow A^{vy}/a mice, lean pseudoagouti A^{vy}/a mice, and lean black nonagouti *a/a* control mice were fed a diet containing lindane (gamma-hexachlorocyclohexane) for 24 months [27]. In response to lindane feeding, lean pseudoagouti A^{vy}/a mice had a higher prevalence of lung tumors, and hepatocellular adenoma and carcinoma than lean *a/a* control mice, whereas the obese yellow A^{vy}/a mice had the highest prevelance in all cases. It is unclear if the higher number of obese yellow A^{vy}/a mice with tumors compared to lean pseudoagouti A^{vy}/a mice was caused by metabolic

effects associated with obesity and diabetes, or to higher levels of ubiquitous agouti expression in the yellow mice. Importantly, the greater tumorigenic response of lean pseudoagouti A^{vy}/a mice compared to lean nonagouti *a/a* mice demonstrated that obesity and tumor susceptibility are independent effects of ectopic agouti expression [27].

A transgenic study in which the albumin promoter was used to direct the expression of the wild-type agouti cDNA to the liver demonstrated that agouti is a tumor promoter in the liver independent of its effects on obesity and diabetes. Albumin (*Alb1*)-agouti transgenic mice, in which agouti protein is ectopically expressed only in the liver, have normal body weight and normal levels of plasma insulin and blood glucose [98]. Diethylnitrosamine (DEN) is a mutagenic agent frequently used for the initiation of liver cells. A single intraperitoneal injection of DEN in alb-agouti transgenic mice and non-transgenic control mice resulted in an increased number of tumors that developed in the livers of the transgenic mice compared to controls. This single initiation event with a hepatocellular carcinogen was sufficient to lead to a significantly greater number of liver tumors in transgenic mice following continuous exposure of the initiated liver cells to high levels of agouti signaling. These findings demonstrated that agouti acts as a tumor promoter in a two-stage carcinogenesis experiment, and its tumor promoting action is independent from the obesity and diabetes phenotypes [98, 99].

A second transgenic study had the goal of extending these findings and defining more precisely the role of agouti in carcinogenesis. For this purpose, transgenic mice expressing the agouti gene in the skin under the regulatory control of the keratin complex 1, acidic, gene 14 (*Krt1-14*, abbreviated as K14) promoter were used in classical two-stage skin carcinogenesis experiments. K14-

Agouti mice over-express the agouti gene in the skin in a constitutive manner, resulting in continuous antagonism of MC1R throughout the hair-follicle cycle and in mice with a solid yellow coat color. The K14-Agouti transgene is expressed in the basal layer of the epidermis and outer root sheath of the hair follicle. Similar to the liver-specific transgenic mice, K14-Agouti transgenic mice are not obese or diabetic, again demonstrating that agouti acts in a paracrine fashion within a few cell distances from its point of secretion, and not by entering into the general circulation [150] (Fig. 5).

K14-Agouti transgenic mice were used in a classical two-stage skin carcinogenesis experiment designed to determine if agouti acts as a tumor promoter in the skin independent of obesity and diabetes [99]. Two groups of transgenic and control mice were treated with either the tumor initiator DMBA, or the tumor promoter TPA, and the number of skin papillomas that developed in transgenic and control mice was determined for 52 weeks. In the first group, where the skin of transgenic and control mice was only initiated by a single topical application of DMBA, the number of mice with skin papillomas and the number of papillomas per mouse were significantly greater in the transgenic mice. These data showed that a high level of agouti protein was able to promote the development of skin tumors following initiation, independent from the obesity and diabetes phenotypes. In the second group, both transgenic and control mice were only promoted with repeated administration of the tumor promoter TPA, without any initiation. Neither the K14-Agouti transgenic mice nor the controls developed any papillomas during the 52 week period, indicating that agouti was not acting as an initiator, and that tumors do not arise spontaneously in K14-Agouti mice in the absence of initiation.

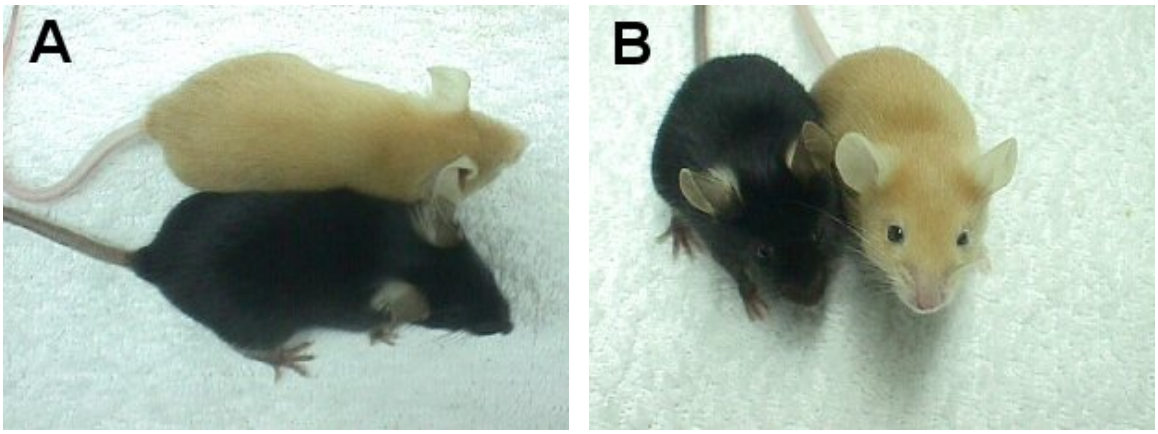


Figure 5. K14-Agouti transgenic and non-transgenic control mice. (A, B) Constitutive agouti signaling in the skin of K14-Agouti transgenic mice causes the mice to have a solid yellow coat color compared to their nonagouti (black) littermate controls. Transgenic mice are not obese or diabetic.

In the same study, a third group of K14-Agouti transgenic and control mice were initiated with DMBA, which was then followed by TPA promotion. As expected, the control mice developed papillomas under these experimental conditions. In transgenic mice, K14-Agouti expression acted synergistically with the chemical tumor promoter, TPA, to increase the cumulative prevalence, multiplicity, and malignant conversion rate of tumors, and to decrease the latent period of tumor formation compared to control mice [99]. These two-stage carcinogenesis experiments with both alb-Agouti and K14-Agouti transgenic mice showed that agouti acts as a tumor promoter in the liver and skin, respectively, in the absence of the obesity and diabetes phenotypes.

Although it has been clearly established that the agouti protein acts as a tumor promoter, the mechanism of action of agouti protein in tumor promotion is still unknown. We speculated that the constant presence of high levels of agouti protein (a paracrine signaling molecule) in the skin of K14-Agouti mice is the promoting force in these mice. As discussed above, the paracrine/autocrine action of agouti in the hypothalamus (in spontaneous dominant agouti mutants) and adipose tissue (in Fabp4-Agouti transgenic mice) was previously shown to be responsible for the obesity and diabetes phenotypes, rather than any systemic action of agouti protein. Additionally, agouti is known to directly regulate the expression of several genes functioning in pigmentation, lipogenesis, and adipocyte metabolism.

In this research, the main goal was to extend these findings and to address the following fundamental issues regarding agouti and skin carcinogenesis:

a) Identify the changes in gene expression patterns in the skin of K14-Agouti transgenic mice using cDNA microarray and real-time quantitative

reverse transcriptase – polymerase chain reaction (qRT-PCR) analyses in order to identify molecular pathways associated with the promotion of skin carcinogenesis by the agouti protein.

b) Determine if the over-expression of agouti in the skin of K14-Agouti transgenic mice is affecting the regulation and progression of the hair-follicle cycle.

c) Investigate if changes in gene expression profiles in the skins of the transgenic mice are associated with specific stages in the hair-follicle cycle.

The agouti protein acts as a tumor promoter in the skin of K14-Agouti mice, as evidenced by the fact that skin tumors develop only following chemical initiation. In the untreated skin of K14-Agouti mice there is ongoing promotion due to the over-expression of agouti protein in the skin, but these mice do not develop skin tumors in the absence of initiation. Therefore, any gene expression changes detected in the untreated skin of K14-Agouti mice may be associated with the tumor promotion stage of skin carcinogenesis, and provide insight into the genetic pathways through which the agouti protein may be functioning in skin cancer. Differentially expressed genes in the skin of K14-Agouti transgenic mice are identified by microarray analysis, and the results of microarray analysis are validated and extended with qRT-PCR analysis.

Gene expression analysis using microarray technology can determine differential expression levels of thousands of genes simultaneously in a high-throughput fashion. Microarray analysis has been a valuable tool for identifying changes in the expression of genes under different experimental circumstances, such as following a genetic alteration, during different stages of disease, or in

response to various treatments. The identification of split hand/foot malformation (ectrodactyly) type 1 (*Shfm1)* as a TPA responsive gene expressed in keratinocytes by microarray analysis is only one of many examples of the application of the microarray technique for novel gene discovery [151]. Additionally, microarray analysis can be utilized for establishing molecular signatures associated with different disease states for the purpose of diagnosis and for predicting disease outcome [152, 153]. In cancer research, microarray-based techniques have been used in a wide range of applications, such as to identify biomarkers associated with different cancer stages, responses to therapy, and disease prognosis. In a recent study, microarray analysis was used to identify differentially expressed genes in mouse squamous skin cancer induced by DMBA and TPA treatment. Differential expression of four genes — annexin A1 (*Anxa1*), lipocalin 2 (*Lcn2*), S100 calcium binding protein A8 (calgranulin A) (*S100a8*), and serine/threonine kinase 38 like (*Stk38l*) — identified in mice were also shown to be differentially expressed in human skin tumor samples, demonstrating the strength of microarray analysis for the identification of novel cancer related genes [154].

In the following chapter, the design and results of this study are presented. The non-initiated skins of K14-Agouti and control mice were examined by cDNA microarray analysis, resulting in the identification of 181 genes that had significantly different expression levels in K14-Agouti mice compared to control mice. These gene expression changes are associated with the promotion stage of skin carcinogenesis in K14-Agouti mice. Constitutive signaling by agouti protein in the skin of K14-Agouti mice, independent of any effects of K14-Agouti on skin morphology or on timing of hair-follicle stages, caused these gene expression differences. Additionally, qRT-PCR analysis demonstrated that the levels and temporal patterns of expression of 10 genes

previously associated with skin and/or other epithelial cancers were significantly altered in the skin of K14-Agouti transgenic mice. Agouti-induced over-expression of these proto-oncogenes in the skin of K14-Agouti mice is proposed to be associated with their increased susceptibility to skin carcinogenesis.

In the last chapter, the results obtained from the microarray and qRT-PCR experiments are integrated into a hypothetical model to explain the mechanism of action of the agouti protein as a tumor promoter in skin carcinogenesis. Finally, strategies for future follow-on experiments to further investigate the role of agouti in tumor promotion and to test aspects of the proposed model are presented.

CHAPTER II

DIFFERENTIAL EXPRESSION OF SKIN CANCER AND HAIR-FOLLICLE CYCLE REGULATED GENES IN TUMOR SUSCEPTIBLE K14-AGOUTI MICE

This chapter is a slightly revised version of a paper by the same title, which is ready for submission by Yesim Aydin-Son and Edward J. Michaud.

My use of "we" in this chapter refers to my co-author and myself. My primary contributions to this paper include: (1) identification, optimization and application of the experimental approaches, (2) some of the mouse husbandry, (3) tissue collections from mice, (4) most of the RNA isolation and cDNA preparation, (4) microarray experiments and analysis, and data mining, (5) most of the real-time qRT-PCR experiments, (6) interpretation of the qRT-PCR data, (7) hair-cycle synchronization experiments, (8) histological staging of the hair-follicle cycle, (9) most of the gathering and interpretation of literature, and (10) writing the manuscript.

INTRODUCTION

The mouse nonagouti (*a*) gene, hereafter referred to as agouti, encodes a paracrine-acting signaling molecule in the skin that regulates coat-color pigmentation [1, 2]. The agouti gene is expressed in the dermal papilla of hair follicles in a transient manner only during the early anagen (growth) stage of the hair-follicle cycle, when hair follicles proliferate and differentiate [3, 4]. Agouti signaling causes hair-follicle melanocytes to switch from black to yellow pigment production by signaling through the melanocortin 1 receptor (MC1R) [5]. As a result of this switch mechanism, individual hairs of wild-type mice have an agouti pigmentation pattern, which is black with a sub-apical yellow band [155]. Importantly, the skin of mice is also more susceptible to carcinogenesis during the anagen stage of the hair-follicle cycle [139, 141, 143], a time when many signaling pathways are normally activated in hair follicles (reviewed in [156-158]).

Dominant regulatory mutations in the mouse agouti gene, such as lethal yellow (A^{y}) and viable yellow (A^{vy}), cause ectopic over-expression of the wild-type gene product in every tissue of the body [1, 2, 59]. This alteration in agouti expression results in yellow-haired mice that are obese, diabetic and have increased susceptibility to hyperplasia, and spontaneous and chemically induced neoplasia [8, 9] (reviewed in [11, 16, 17]) in a wide variety of tissues (e.g., skin [20, 21], liver [22-28], lung [23, 27], mammary gland [22, 23, 29-31], and urinary bladder [32]).

Liver-specific expression of the agouti gene in transgenic mice was shown to promote hepatocellular carcinogenesis, even in the absence of obesity and diabetes [98]. We recently extended these findings to show that expression of the

agouti gene in the skin of transgenic mice also promotes skin cancer in the absence of obesity and diabetes [99]. In K14-Agouti transgenic mice, the wild-type agouti gene is over-expressed in the skin under the regulatory control of the keratin complex 1, acidic, gene 14 (*Krt1-14*, abbreviated as K14) promoter. These transgenic mice have yellow hair as a result of continuous agouti antagonism of MC1R on melanocytes, but do not exhibit the obesity or diabetes phenotypes observed in mice carrying the A^{y} or A^{vy} mutations [150]. The obesity and diabetes phenotype exhibited by mice harboring the A^{y} and A^{vy} mutations is likely caused by over-expression of agouti in the brain and antagonism of MC4R [83]. In two-stage skin carcinogenesis experiments with K14-Agouti transgenic mice, the agouti protein was shown to act as a tumor promoter since it promoted the development of skin tumors only after the skin was initiated with a single sub-carcinogenic dose of 7,12-dimethylbenz[a]anthracene (DMBA) [99]. Although agouti has been shown to promote the development of hyperplasia and neoplasia in numerous tissues, the mechanism of action of agouti protein in tumor promotion remains largely uncharacterized [12].

Our hypothesis for the role of the agouti protein in tumor promotion is that agouti may be inducing the up-regulation of proto-oncogenes as a consequence of high levels of constitutive paracrine signaling of cells and pathways that may or may not normally encounter agouti protein. As a first step to test this possibility, we used cDNA microarray and real-time qRT-PCR analyses in order to examine gene expression levels in the skin of non-initiated K14-Agouti transgenic mice compared to non-transgenic littermate control mice. Our goal was to identify changes in K14-Agouti skin at the molecular level due to over-expression of the agouti gene in the skin, which in turn could be associated with tumor promotion following spontaneous or chemical initiation. We identified a set of genes that are significantly up-regulated in the skin of K14-

Agouti mice and that have previously been shown to play a role in skin, and epithelial cancer biology.

MATERIAL and METHODS

Mice

Mice of the transgenic line TG2579K14iA [150] were backcrossed to C57BL/6J mice for more than 10 generations in order to obtain transgenic mice that are congenic on the C57BL/6J genetic background, which are hereafter referred to as K14-Agouti transgenic mice. K14-Agouti transgenic mice are hemizygous for the transgene and are non-agouti (*a/a*) at the endogenous agouti locus, as determined by Southern blot analysis [1]. C57BL/6J (*a/a*) littermates were used as controls for K14-Agouti transgenic mice. All mice were maintained in the Mammalian Genetics Research Facility, Oak Ridge National Laboratory, Oak Ridge, TN, and all experiments involving mice were conducted under approved Institutional Animal Care and Use Committee protocols.

RNA Isolation and cDNA Preparation

Dorsal skin sections from adult male K14-Agouti transgenic mice and *a/a* control mice were dissected and snap-frozen in liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen), followed by on-column DNAse treatment and a purification step with the Qiagen RNeasy Mini Kit. SuperScript II Reverse Transcriptase (Invitrogen Life Technologies) was used to generate cDNA according to the manufacturer's instructions.

Microarray Analysis

Ontario Cancer Institute cDNA microarrays (OCI, Toronto, Canada) containing the NIA/NIH 15K mouse EST clone set [159] were used for microarray experiments. Labeling and hybridization of cDNAs were performed by following the TIGR Protocol for indirect amino-allyl labeling of cDNAs [160]. An optimized version of this protocol is available at http://web.utk.edu/~saydin/protocols. Three biological replicates were used to minimize experimental and individual variations. Total RNA from three different eight-week-old male K14-Agouti transgenic mice was hybridized individually against a pooled total RNA sample from three sex-matched, age-matched non-agouti control mice. All three hybridizations were repeated with dye swapping to minimize dye bias in the microarray analysis. Since each one of the NIA 15K mouse ESTs is spotted in duplicate on the microarray slides, 12 data points were generated for the expression level of each gene, which were combined for statistical analysis. Fluorescence intensities were measured using ScanArray (GSI Lumonics), and Imagene 5.2 (BioDiscovery) was used for image acquisition and quantification. Composite images were generated by Imagene software.

Statistical Analysis

BRB-ArrayTools developed by Dr. Richard Simon and Amy Peng Lam, and SAM (significance analysis of microarrays) [161] software packages were used for the statistical analysis of gene expression data. After local background correction with Imagene 5.2 output, average log (base 2) intensities were used for normalization. Each dye-swap experiment was individually normalized with

LOWESS analysis using BRB-ArrayTools software. Statistical significance of alterations in gene expression levels between transgenic and control mice were determined by SAM. Threshold values (delta) were selected by considering the total number of genes on the array, the number of genes found significantly different at that threshold value, and the False Discovery Rate (FDR) for the threshold.

Real-Time qRT-PCR

The qRT-PCR method depends on the real-time measurement of PCR products amplified in every cycle, which is proportional to the amount of starting template [162-164]. We used the Cepheid SmartCycler real-time PCR machine for the qRT-PCR experiments. The TaqMan primers and probes were purchased from Applied Biosystems (TaqMan Assays-on-Demand Products Assay numbers: Mm00438337_m1, Mm00476174_m1, Mm00516876_m1, Mm00515219_s1, Mm00448100_m1, Mm00435540_m1, Mm00440911_m1, Mm00439518_m1, Mm00456961_m1, Mm00436931_m1). The 18S RNA (*Rn18s*) gene was used as an internal control to normalize experimental data. The threshold cycle (C_T) for all experimental genes was first normalized to the corresponding *Rn18s* C_T. Relative fold differences were then determined using the $2^{-\Delta\Delta CT}$ method [165] by comparing each experimental sample to the control sample.

Synchronization of Hair-Follicle Cycle

To repeat the qRT-PCR experiments on skin samples in which the exact stage of the hair-follicle cycle was determined by diagnostic histology, we

synchronized the hair-follicle cycle on the dorsal skin of eight-week-old female K14-Agouti transgenic and control mice by wax epilation, as described previously [166]. Mice were euthanized at eight time points (0, 3, 5, 8, 12, 17, 20 and 25 days) after wax epilation. Two transgenic and two control mice were analyzed at each time point, and the expression level of each gene was measured in duplicate for each mouse.

Histology

The samples of dorsal skin were fixed with Fekete's acid-alcohol-formalin, and embedded in paraffin blocks. Sections were stained with hematoxylin and eosin, and Giemsa. Slides were examined under routine light microscopy (Zeiss Axioskop2), and hair-follicle cycle stages were determined by following the guideline for the classification of murine hair follicles in distinct hair-cycle stages [123].

RESULTS

Microarray Analysis of Gene Expression in the Skin of Adult K14-Agouti and Control Mice

Changes in gene expression levels in the skin of non-initiated K14-Agouti mice were identified using 15K Toronto cDNA microarrays. BRB-ArrayTools and SAM software programs were used for the analysis of microarray data. We used self-to-self hybridization of control samples (data not shown) for quality assurance of the microarray technique and the statistical analysis of data. Data

generated from six different hybridization experiments of skin RNA from eight-week-old transgenic and control male mice were used collectively to identify genes with significantly different expression levels in K14-Agouti mice (Fig. 6). Microarray analysis resulted in the identification of 63 ESTs that were up-regulated (Table A-1 in Appendix) and 118 ESTs that were down-regulated (Table A-2 in Appendix) in the skin of K14-Agouti mice compared to the control mice at the delta threshold value of 0.4 with a FDR of 22% (see Materials and Methods). The complete data set was deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE4641 [167].

The identities of all 63 significantly up-regulated and an equal number of significantly down-regulated EST clones were determined using the NCBI Entrez Gene database [168]. Among the top 63 EST clones in both groups, only 40 up-regulated clones (Table 1) and 29 down-regulated clones (Table 2) were associated with known genes with previously determined biological functions.

qRT-PCR Analysis of the Temporal Expression Profiles of 10 Genes in the Skin of K14-Agouti and Control Mice

Since the microarray data were from the skin of mice at a single age, we next selected a subset of these genes, and others, for analysis of their temporal expression patterns using qRT-PCR. We selected four up-regulated genes from the microarray results (casein kinase II, alpha 2, polypeptide, *Csnk2a2;* Harvey rat sarcoma virus oncogene 1, *Hras1;* platelet derived growth factor, alpha, *Pdgfa;* and plasminogen activator, urokinase receptor, *Plaur*) that have known functions associated with skin cancer biology, cellular differentiation, cellular proliferation,

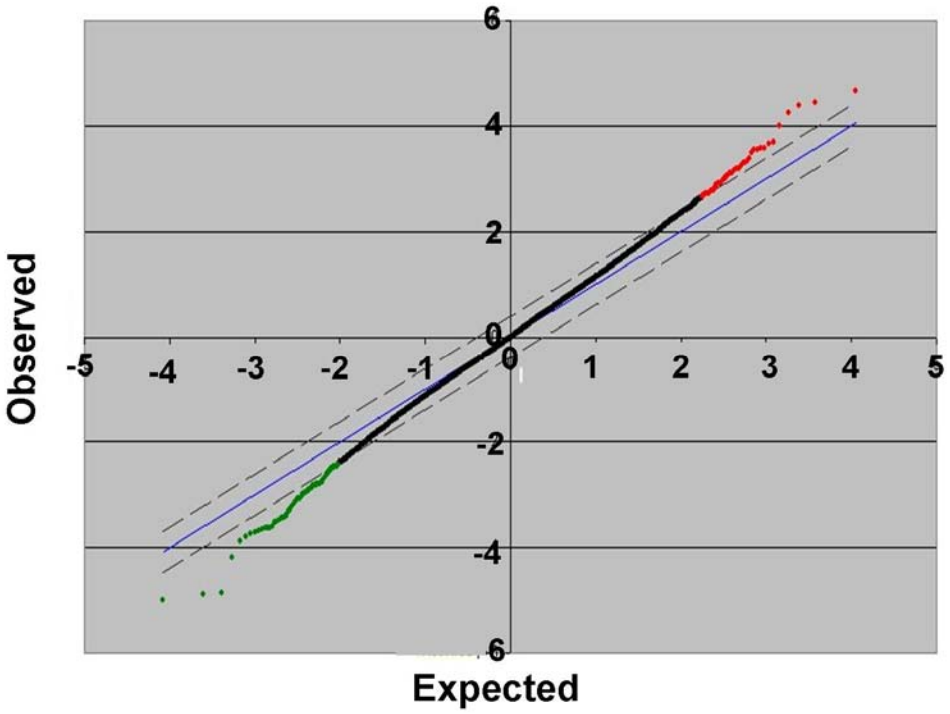


Figure 6. Identification of significantly altered gene expression levels in the skin of K14-Agouti transgenic mice. Data generated from six independent microarray hybridization experiments with skin RNA from transgenic and control mice on cDNA microarrays were analyzed by SAM software as described in Materials and Methods. A scatter plot of the observed relative difference $d(i)$ versus the expected relative difference $d_E(i)$ is shown. Solid line indicates no differential expression. Dashed lines mark the cutoff value for significant differential expression when the threshold value (delta) was 0.4 and the FDR was 22%. This analysis identified 181 significantly altered genes in K14-Agouti mice compared to non-transgenic control mice, which are plotted outside of the dashed lines. Out of 15,264 mouse ESTs, 63 genes were up-regulated (in red, upper-right of plot) while 118 genes were down-regulated (in green, lower-left of plot).

Table 1. List of 40 genes with known biological functions that are significantly up-regulated in the skin of K14-Agouti mice. Shown for each gene are the GeneID number, gene symbol, gene name, score (d), and fold difference of gene expression in K14-Agouti skin compared to controls. Genes are sorted based on score (d), or d (*i*), which is assigned by the SAM program according to the change in gene expression relative to the standard deviation of repeated measurements. Four genes selected for further analysis are marked by double borders.

List of Significantly Up-regulated Genes				
Gene ID	Gene Symbol	Gene Name	Score (d)	Fold Difference
13000	*Csnk2a2*	casein kinase II, alpha 2, polypeptide	4.65	5.12
18201	*Nsmaf*	neutral sphingomyelinase	4.23	2.71
22113	*Phlda2*	pleckstrin homology-like domain, family A, member 2	4.00	2.40
50875	*Tmod3*	tropomodulin 3	3.69	2.78
19116	*Prlr*	prolactin receptor	3.65	1.81
214597	*Sidt2*	SID1 transmembrane family, member 2	3.56	3.37
16882	*Lig3*	ligase III, DNA, ATP-dependent	3.54	2.7
18793	*Plaur*	plasminogen activator, urokinase receptor	3.54	6.45
286931	*Unc13c*	unc-13 homolog C (C. elegans)	3.48	2.16
54651	*Usp27x*	ubiquitin specific protease 27, X chromosome	3.31	1.98
12393	*Runx2*	runt related transcription factor 2	3.2	2.23
15461	*Hras1*	Harvey rat sarcoma virus oncogene 1	3.19	1.70
76380	*Ccdc46*	coiled-coil domain containing 46	3.15	2.15
81896	*Ift122*	intraflagellar transport 122 homolog (Chlamydomonas)	3.15	1.81
53421	*Sec61a*	Sec61 alpha 1 subunit (S. cerevisiae)	3.10	2.69
407785	*Ndufs6*	NADH dehydrogenase (ubiquinone) Fe-S protein 6	3.09	1.70
244668	*Sipa1l2*	signal-induced proliferation-associated 1 like 2	3	2.34
12167	*Bmpr1b*	bone morphogenetic protein receptor, type 1B	2.99	1.50
71807	*Tarsl1*	threonyl-tRNA synthetase-like 1	2.98	2.30
18139	*Zfml*	zinc finger, matrin-like	2.96	1.82
22083	*Sh2bp1*	SH2 domain binding protein 1 (tetratricopeptide repeat c	2.95	2.36
72748	*Hdhd3*	haloacid dehalogenase-like hydrolase domain containing	2.92	1.93
18590	*Pdgfa*	platelet derived growth factor, alpha	2.89	1.76
11764	*Ap1b1*	adaptor protein complex AP-1, beta 1 subunit	2.89	1.78
94066	*Mrpl36*	mitochondrial ribosomal protein L36	2.82	2.28
66797	*Cntnap2*	contactin associated protein-like 2	2.78	1.50
216285	*Cart1*	cartilage homeo protein 1	2.76	2.53
19355	*Rad1*	RAD1 homolog (S. pombe)	2.75	1.87
11603	*Agrn*	agrin	2.75	1.69
13872	*Ercc3*	excision repair cross-complementing rodent repair deficie	2.74	2.49
52123	*Agpat5*	1-acylglycerol-3-phosphate O-acyltransferase 5 (lysophos	2.74	1.67
269181	*Mgat4a*	mannoside acetylglucosaminyltransferase 4, isoenzyme A	2.73	1.43
57746	*Piwil2*	piwi-like homolog 2 (Drosophila)	2.72	1.45
227394	*Slco4c1*	solute carrier organic anion transporter family, member 4	2.71	2.38
56309	*Mycbp*	c-myc binding protein	2.70	1.83
23964	*Odz2*	Oz/ten-m homolog 2 (Drosophila)	2.69	2.02
18673	*Phb*	prohibitin	2.67	1.46
27984	*Efhd2*	EF hand domain containing 2	2.67	2.55
56306	*Tera*	teratocarcinoma expressed, serine rich	2.66	1.88
57783	*Tnip1*	TNFAIP3 interacting protein 1	2.64	2.10

Table 2. List of 29 genes with known biological functions that are significantly down-regulated in the skin of K14-Agouti mice. Shown for each gene is the GeneID number, gene symbol, gene name, score (d), and fold difference of gene expression in K14-Agouti skin compared to controls. Genes are sorted based on score (d), or d (*i*), which is assigned by the SAM program according to the change in gene expression relative to the standard deviation of repeated measurements.

List of Significantly Down-regulated Genes				
Gene ID	Gene Symbol	Gene Name	Score (d)	Fold Difference
217835	*Rin3*	Ras and Rab interactor 3	-4.94	-4.55
17449	*Mor2*	malate dehydrogenase 1, NAD (soluble)	-3.73	-2.56
27028	*Ermap*	erythroblast membrane-associated protein	-3.58	-5.88
59079	*Erbb2ip*	Erbb2 interacting protein	-3.23	-3.85
110147	*Ehmt2*	euchromatic histone lysine N-methyltransferase 2	-3.20	-1.96
68810	*Nexn*	nexilin	-3.18	-2.08
14756	*Gpld1*	glycosylphosphatidylinositol specific phospholipase D1	-3.15	-1.41
66229	*Rpl7l1*	ribosomal protein L7-like 1	-3.13	-2.00
235439	*Herc1*	hect (homologous to the E6-AP (UBE3A) carboxyl terminus	-3.09	-2.00
211347	*Pank3*	pantothenate kinase 3	-3.05	-10.00
66959	*Dusp26*	dual specificity phosphatase 26 (putative)	-2.96	-1.61
20382	*Sfrs2*	splicing factor, arginine/serine-rich 2 (SC-35)	-2.95	-2.27
217030	*Ap1gbp1*	AP1 gamma subunit binding protein 1	-2.95	-2.33
68943	*Pink1*	PTEN induced putative kinase 1	-2.94	-3.23
12307	*Calb1*	calbindin-28K	-2.94	-2.13
14704	*Gng3*	guanine nucleotide binding protein (G protein), gamma 3	-2.91	-3.03
17425	*Foxk1*	forkhead box K1	-2.90	-3.57
233724	*Tmem41b*	transmembrane protein 41B	-2.86	-3.70
12319	*Car8*	carbonic anhydrase 8	-2.86	-2.38
93739	*Gabarapl2*	gamma-aminobutyric acid (GABA-A) receptor-associated p	-2.83	-2.04
76843	*Dtl*	denticleless homolog (Drosophila)	-2.82	-1.64
22381	*Wbp5*	WW domain binding protein 5	-2.82	-2.00
12974	*Cs*	citrate synthase	-2.81	-3.13
321022	*Cdv3*	carnitine deficiency-associated gene expressed in ventricle	-2.80	-2.56
53376	*Usp2*	ubiquitin specific protease 2	-2.79	-4.17
78455	*Helz*	helicase with zinc finger domain	-2.79	-2.38
54525	*Syt7*	synaptotagmin VII	-2.79	-7.69
110521	*Hivep1*	human immunodeficiency virus type I enhancer binding p	-2.78	-2.13
67877	*Nat5*	N-acetyltransferase 5 (ARD1 homolog, S. cerevisiae)	-2.75	-3.03

and/or hair-follicle cycle events. Additionally, we included two markers of cellular proliferation (proliferating cell nuclear antigen, *Pcna;* and telomerase reverse transcriptase, *Tert*), two markers of keratinocyte differentiation (*Krt1-14* and involucrin, *Ivl*), and two transcription factors (signal transducer and activator of transcription 1, *Stat1;* and signal transducer and activator of transcription 3, *Stat3*) previously shown to be up-regulated by ectopic agouti expression in adipose tissue [97]. Each of these 10 genes has been associated with skin cancer and/or other epithelial cancers (Table 3). The expression profiles of these genes were examined in transgenic and control mice ranging in age from 4.5 to 26.5 weeks (Table 4).

The data in Table 4 demonstrated that all 10 genes were up-regulated in the skin of K14-Agouti mice compared to control mice, but not at all ages. To better understand the impact of K14-Agouti transgene expression on the expression patterns of these 10 genes, we next examined their wild-type temporal expression profiles in control mice (Fig. 7A and B). For this analysis, the expression level of each gene in the skin of 4.5-week-old control mice was assigned the value of 1.0 and the expression levels of each gene at all later ages were reported relative to this level. This analysis revealed that the wild-type expression profiles of these genes exhibited two general patterns. In Group I genes (*Hras1, Krt1-14, Ivl, Pdgfa, Plaur, Stat1,* and *Stat3*), expression remained at a baseline level from about 4.5-11.4 weeks of age, followed by up-regulation at later ages (Fig. 7A). The hair follicles of mice develop postnatally in a synchronous manner through two cycles of regression (catagen), resting (telogen), and growth (anagen), up until they are about 12 weeks old. After this time, hair follicles continue to cycle but in an asynchronous manner relative to each other [123]. The first catagen and telogen stages occur prior to 4.5 weeks of age, which is the youngest age of mice in which we examined gene expression

Table 3. Summary of known functions of 10 genes with altered temporal expression patterns in the skin of K14-Agouti mice

Gene Symbol	Role in Epithelial and/or Skin Cancer Biology	Expression and Role During the Hair-Follicle Cycle
Csnk2a2	Transformation of mammary epithelium due to altered expression [169].	Acts in casein kinase signal transduction pathway in keratinocytes [170].
Hras1	Activating point mutations are present in 30% of all human tumors; 10-20% of SCC and BCC carry an *Hras1* mutation [171].	Expression peaks at early anagen [172].
Krt1-14	Altered expression in squamous cell skin cancer reflecting the differentiation stage [173].	Expressed at anagen, early differentiation marker [127].
Ivl	In epidermal papillomas, expression level correlates with degree of histological differentiation [174].	Expressed during anagen, late differentiation marker [127].
Pcna	Increased expression in squamous type skin cancers [175, 176].	Expressed in proliferating hair follicle cells [177, 178].
Pdgfa	Stimulates tumor cell growth, transformation [179, 180]; expressed in many epithelial tumors [181, 182]; altered expression in squamous cell cancer [183].	Expressed by both FK and DPC [182]; role in formation of dermal papilla [184].
Plaur	Over-expression is correlated with tumor progression and metastasis; expressed in squamous cell skin cancers [185].	Expressed in all cells of hair follicle [186].
Stat1	Constitutively active in many human tumors with Stat3 [187].	Increases promoter activity of *Ivl*, which is a keratinocyte differentiation marker [188].
Stat3	Constitutively active in squamous cell cancers [183, 189, 190]; required for skin cancer development [190, 191].	Expressed during transition from telogen to anagen stage [192].
Tert	Reactivated in 90% of human cancers [131]; telomerase-deficient mice are resistant to skin carcinogenesis [193].	Rapid transition from telogen to anagen [131].

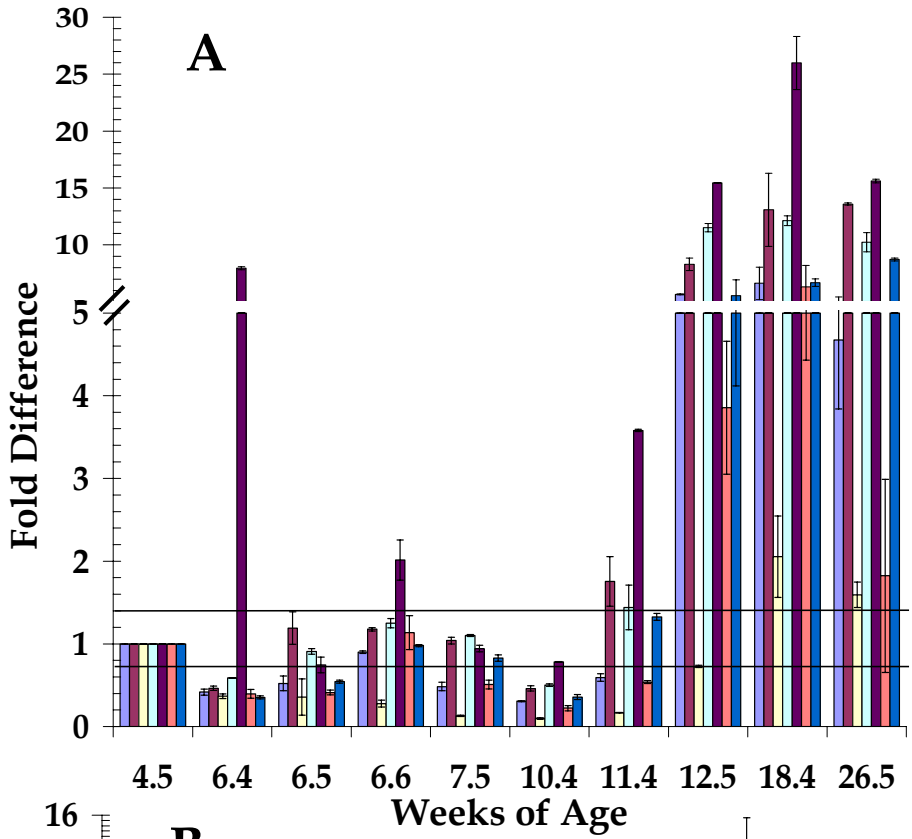
Table 4. Differential expression levels of 10 selected genes in the skin of K14-Agouti mice compared to control mice at various ages. Fold differences for each gene between transgenic animals and their littermate controls are shown. Expression levels that are significantly up-regulated in transgenic mice based on qRT-PCR analysis are labeled in red. Expression levels that are significantly down-regulated in transgenic mice are labeled in green, and expression levels that do not differ significantly between transgenic and control mice are labeled in blue. Shown in the bottom panel are the hair-follicle cycle stages corresponding to the ages of C57BL/6 control mice based on the published literature [123]. The genes were placed into two groups based on their age-related expression patterns in control mice (see Fig. 7).

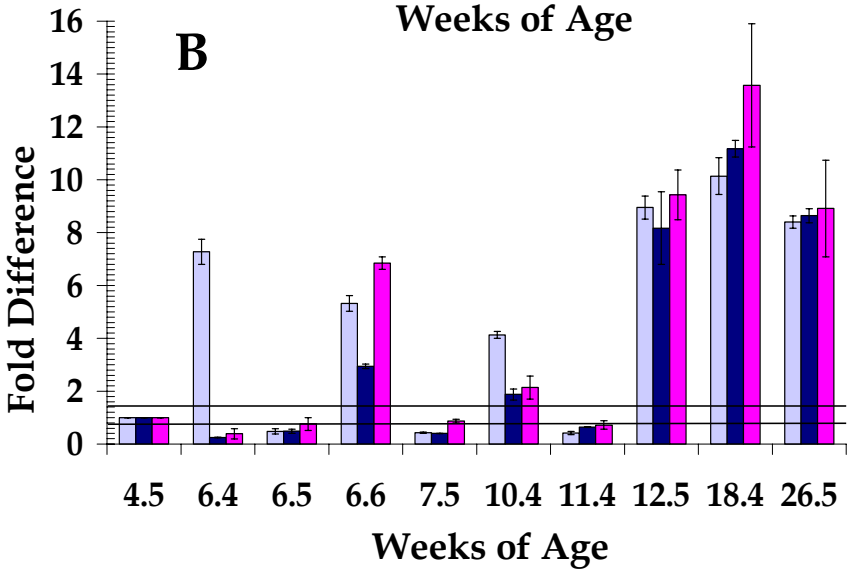
Age	GROUP I							GROUP II		
(weeks)	*Hras1*	*Krt1-14*	*Ivl*	*Pdgfa*	*Plaur*	*Stat1*	*Stat3*	*Csnk2a2*	*Pcna*	*Tert*
4.5	0.87	1.40	1.91	0.89	0.91	0.40	0.80	0.72	0.86	1.40
4.5	1.27	2.40	1.11	1.26	1.52	0.60	1.10	0.68	1.25	1.46
6.4	1.74	1.13	2.93	1.20	1.33	0.90	0.90	1.05	2.78	3.23
6.4	1.48	1.38	2.17	0.77	1.18	0.85	0.80	0.56	1.27	1.44
6.5	0.42	0.39	1.06	0.53	0.21	0.63	1.09	0.53	0.35	0.46
6.5	1.40	1.18	4.27	4.29	2.24	3.67	4.00	2.17	1.71	2.79
6.5	1.95	2.23	5.05	2.17	5.58	2.39	2.71	3.17	1.76	2.84
6.5	1.64	1.40	5.08	2.64	4.06	3.34	2.64	4.76	2.28	4.54
6.6	0.81	0.80	2.58	1.88	2.56	1.20	1.00	1.50	3.15	1.31
6.6	0.72	0.70	5.22	0.97	1.30	0.80	0.80	1.40	3.20	1.36
7.5	2.09	1.59	5.41	2.87	4.11	2.08	2.49	3.42	2.50	1.68
7.5	2.79	3.13	7.52	4.32	6.87	5.37	3.53	4.55	4.39	2.40
10.4	16.62	4.97	11.47	9.82	10.97	7.70	5.70	0.90	2.29	2.08
10.4	2.47	3.31	8.34	4.26	3.99	5.30	2.70	2.72	3.84	2.51
10.4	7.70	7.43	8.14	10.74	7.21	9.70	6.00	1.95	2.15	2.05
11.4	1.31	0.75	4.32	0.93	1.34	1.21	0.94	2.23	2.11	1.20
11.4	0.66	0.74	2.06	1.34	0.48	0.52	0.58	1.31	0.97	1.17
12.5	3.02	5.64	12.17	11.79	3.76	4.04	4.27	3.77	4.63	1.83
12.5	1.22	2.15	8.37	4.10	2.04	1.13	1.92	1.82	1.79	1.46
18.4	1.41	2.55	5.72	4.42	0.52	0.86	2.31	1.02	0.79	1.80
18.4	0.53	0.85	0.81	0.99	0.91	1.29	1.26	0.70	0.66	0.45
26.5	1.64	0.92	3.63	0.99	0.88	3.48	0.66	1.08	1.69	1.48

Age of Mice (weeks)	4.5	6.4	6.5	6.6	7.6	10.4	11.4	12.5	18.4	26.5
Hair cycle stages	Anagen	Catagen			Telogen	Telogen to anagen	Anagen		Unsynchronized	

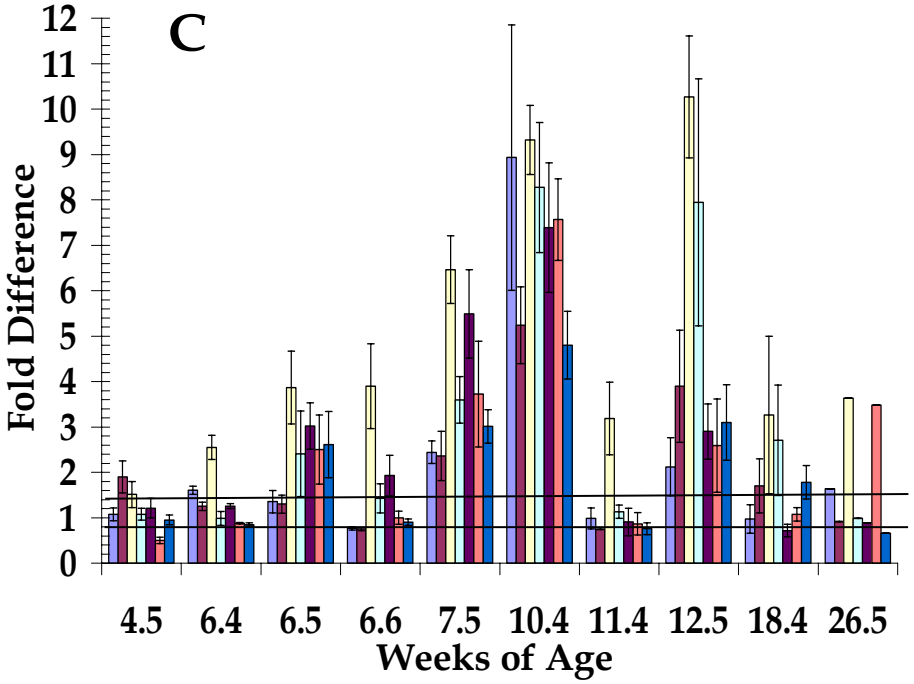
sig. up	>= 1.40
sig. down	<= 0.72
no sig difference	

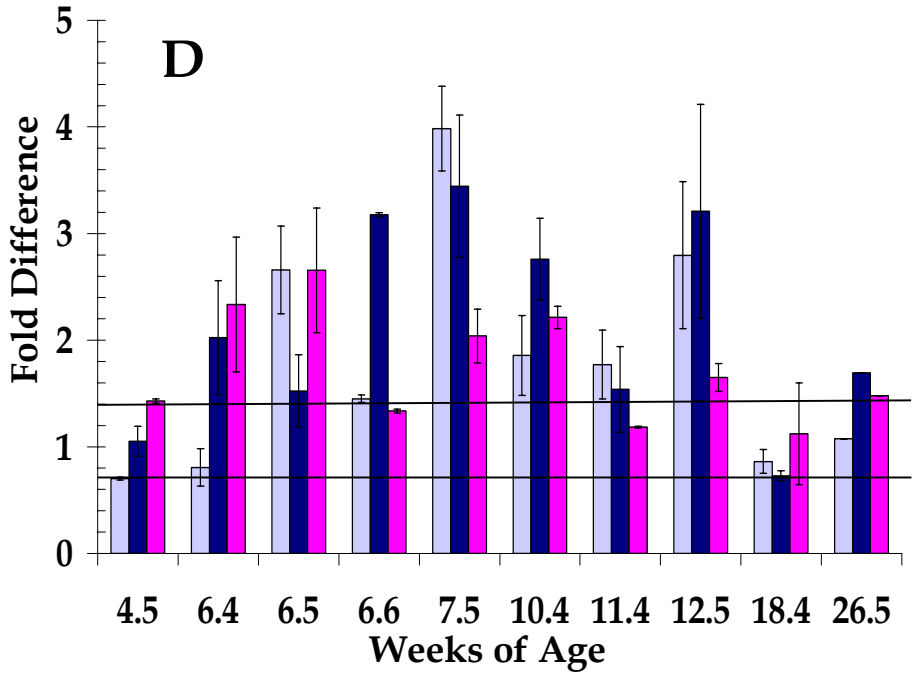
Figure 7. Age-related differences in the expression patterns of 10 genes in the skin of K14-Agouti mice. Gene expression levels in control mice (A and B) and in K14-Agouti mice relative to control mice (C and D) were determined by qRT-PCR analysis. For control mice in graphs A and B, the expression level of each gene at 4.5 weeks-of-age was assigned the value of 1.0, and the expression levels at later ages are reported relative to this value. In graphs C and D, the relative fold differences between K14-Agouti and control mice are presented for each age. In all graphs, the y-axis represents average fold differences in gene expression levels (mean ± SEM), and the x-axis represents the age of mice in weeks. In A and B, N= 2 at 4.5, 6.4, 6.6, 7.5, 10.4, 11.4, 12.5, and 26.5 weeks of age and N=4 at 6.5, and 18.4 weeks of age. In C and D, N=4 at 4.5, 6.4, 6.6, 7.5, 11.4, and 12.5 weeks of age, N=8 at 6.5 weeks of age, N=6 at 10.4 weeks of age, and N=2 at 26.5 weeks of age. Gene expression levels greater than 1.4 fold or less than 0.72 fold (each indicated by a black horizontal line) indicate significant changes in expression. Shown in the bottom panels are the hair-follicle cycle stages corresponding to the ages of C57BL/6 control mice based on the published literature [123].





Age of Mice (weeks)	4.5	6.4	6.5	6.6	7.6	10.4	11.4	12.5	18.4	26.5
Hair cycle stages	Anagen	Catagen			Telogen	Telogen to anagen	Anagen		Unsynchronized	





Age of Mice (weeks)	4.5	6.4	6.5	6.6	7.6	10.4	11.4	12.5	18.4	26.5
Hair cycle stages	Anagen	Catagen			Telogen	Telogen to anagen	Anagen		Unsynchronized	

(Fig. 7, bottom panel). Thus, the wild-type expression levels of most Group I genes were significantly up-regulated at a time when the hair follicles are in the second postnatal anagen stage (Fig. 7A). Group II genes (*Csnk2a2, Pcna,* and *Tert*) exhibited a cyclical wild-type pattern, with up-regulated expression possibly coinciding with the transitions between different hair-follicle cycle stages, or with other age-related differences in the skin (Fig. 7B).

Having determined the temporal expression patterns of these 10 genes in control mice, we next determined the relative differences in the expression levels of these genes in K14-Agouti mice compared to the control mice (Fig. 7C and D). In K14-Agouti mice, Group I genes were significantly up-regulated at three different time points (7.5, 10.4, and 12.5 weeks) (Fig. 7C). Thus, the expression levels of Group I genes were normally up-regulated in control mice during anagen, but in the K14-Agouti mice these genes were significantly over-expressed during telogen, prior to the onset of anagen, and also significantly over-expressed at two time points during anagen. Group II genes in K14-Agouti mice had more of a linear profile, showing uniform over-expression during most of the hair-follicle cycle stages, including significant over-expression at telogen, prior to the onset of anagen (Fig. 7D). Taken together, these data demonstrate that 10 genes associated with cancer biology and/or cellular differentiation and proliferation events exhibited dynamic patterns of gene expression in the skin of control mice that change with age, and that their expression levels and patterns are altered in the skin of tumor susceptible K14-Agouti transgenic mice.

Examining Gene Expression Profiles in K14-Agouti Skin at Precisely Determined Stages of the Hair-Follicle Cycle

Since the agouti protein is a secreted signaling molecule normally expressed during anagen, we hypothesized that the observed differences in gene expression in K14-Agouti mice could be caused in one of two ways. First, constitutive over-expression of agouti could be impacting other signal transduction pathways, resulting in the differential expression of these genes. Second, over-expression of agouti could be altering the timing of the hair-follicle cycle stages, which in turn would alter the expression of genes associated with specific stages of follicle growth. To choose between these possibilities, we synchronized the hair-follicle cycle for all follicles in the dorsal skin of eight-week-old K14-Agouti and control mice by hot wax epilation. At eight weeks of age all dorsal skin hair follicles are in telogen, and epilation induces synchronous anagen development of follicles over the entire epilated region, followed by the spontaneous entry into catagen and telogen at defined times over a 25 day period [123]. Mice were euthanized at eight time points (0, 3, 5, 8, 12, 17, 20 and 25 days) after wax epilation, the precise stage of hair follicle development was determined at each time point by histological examination of the skin and hair follicles, and qRT-PCR analysis was performed for the 10 genes at each time point.

Histological analysis of the hair follicles in K14-Agouti mice following synchronization showed no differences in hair-follicle cycle progress compared to their littermate controls. Additionally, the timing of each hair-follicle cycle stage for transgenic and control mice was consistent with the published literature for female C57BL/6 mice (Table 5).

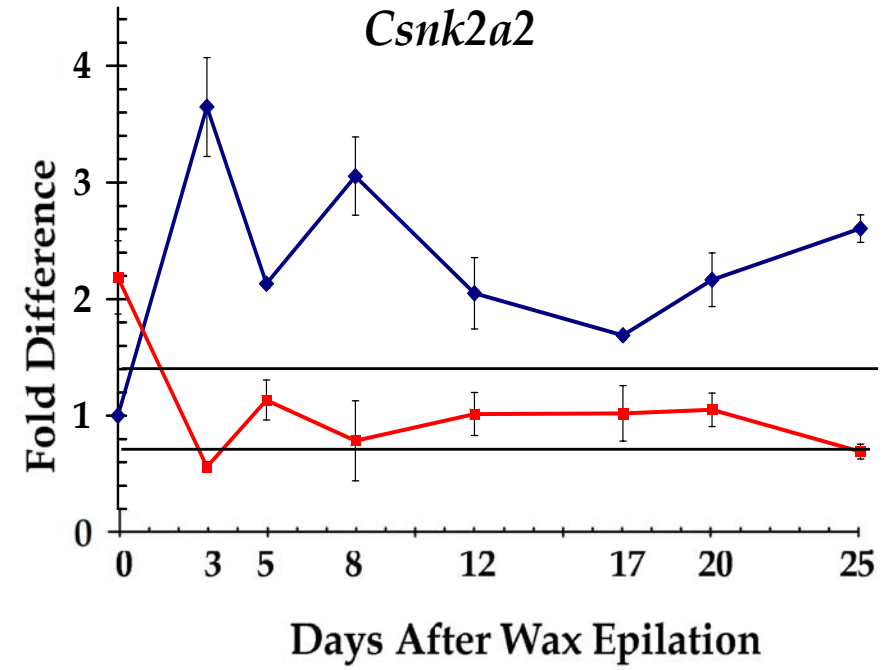
Table 5. Progress of the hair-follicle cycle in K14-Agouti and C57BL/6J (B6) control mice. The hair-follicle cycle stages in the dorsal skin of K14-Agouti mice and C57BL/6J (B6) control mice are shown at eight time points (days) following wax epilation. Two transgenic and two control mice were euthanized at each of eight time-points post-epilation, and the stage of the hair-follicle cycle was determined by histological analysis of the skin. The hair-follicle stages did not differ between transgenic and control mice at any time, and they were also essentially the same as previously published for C57BL/6 mice (Reference B6) [123].

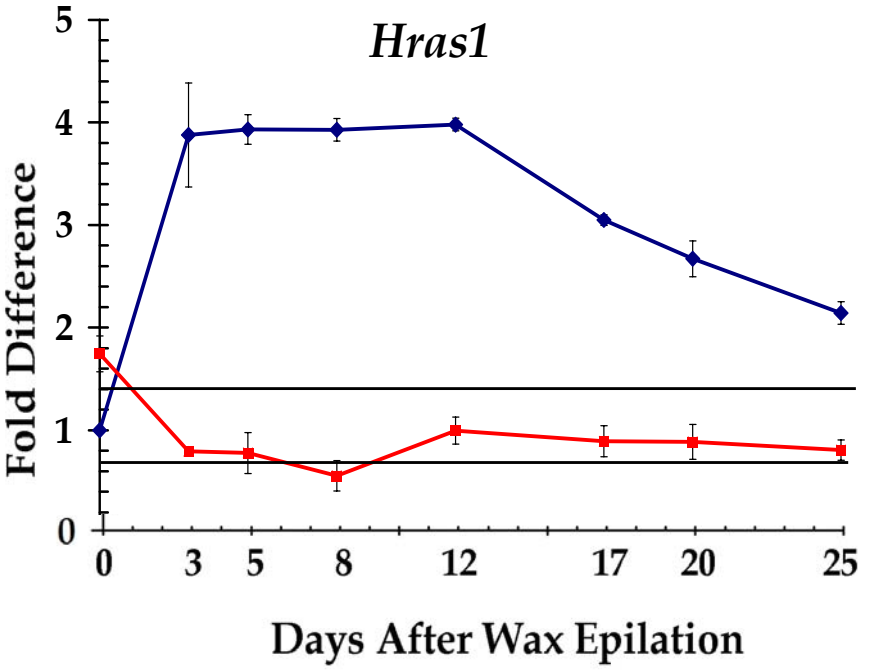
DAYS AFTER WAX-EPILATION		0	3	5	8	12	17	20	25
Stages of Hair Follicle Cycle	K14-Agouti	Telogen	Anagen 2-3a	Anagen 3a-3c	Anagen 6	Anagen 6	Catagen 1-3	Catagen 6-8	Telogen
	Control B6 (*a/a*)	Telogen	Anagen 2-3a	Anagen 3a-3c	Anagen 6	Anagen 6	Catagen 1-3	Catagen 6-8	Telogen
	Reference B6 (*a/a*)	Telogen	Anagen 2	Anagen 4	Anagen 6	Anagen 6	Catagen	Catagen	Telogen

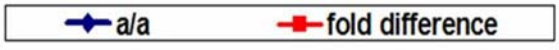
Gene expression analysis of K14-Agouti and control skin following epilation was performed by qRT-PCR. For each of the 10 genes, two temporal expression profiles are shown for the eight time-points post-epilation; the wild-type pattern of expression in the control mice, and the relative fold differences in gene expression in the transgenic mice compared to the control mice (Fig. 8). For all genes except *Stat3*, wax epilation induced a significant up-regulation of wild-type gene expression in control mice at day 3 (early anagen), which generally remained up-regulated at most subsequent time points. Notable among these nine genes were the expression patterns of *Tert* and *Pcna*. Wild-type *Tert* expression was up-regulated 3,000-fold on days 3 and 5 post-epilation (early anagen), followed by expression levels that ranged between 3- and 8-fold up-regulated on subsequent days. Wild-type *Pcna* expression was up-regulated 120-fold on day 3 post-epilation and ranged between 57- and 138-fold up-regulated on subsequent days.

The expression levels of 7 of the 10 genes (*Csnk2a2*, *Hras1*, *Krt1-14*, *Ivl*, *Pcna*, *Pdgfa*, and *Tert*) in K14-Agouti skin were significantly up-regulated compared to control levels immediately after wax epilation (day 0) when dorsal hair follicles were in telogen (Figs. 8 and 9). The expression levels of 9 of the 10 genes were significantly down-regulated in K14-Agouti skin compared to control skin during one time point in early or mid anagen (day 3: *Csnk2a2, Pcna, Pdgfa*, *Plaur*; day 8: *Hras1*, *Ivl, Stat1*, *Stat3*, *Tert*) (Fig. 9). With a few exceptions, the expression levels of the 10 genes in K14-Agouti skin did not differ significantly from the levels in control skin at the remaining time points. Overall, the hair-follicle cycle synchronization experiments revealed that the initiation and duration of the different hair-follicle cycle stages did not differ between K14-Agouti transgenic mice and control mice. However, the expression levels of

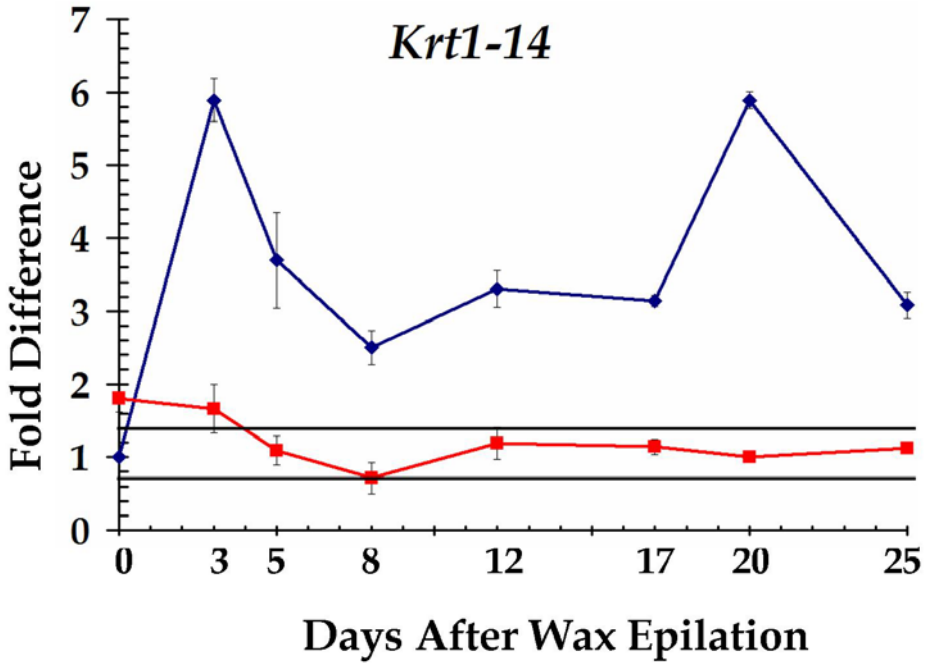
Figure 8. Temporal expression patterns of 10 genes in the skin of K14-Agouti mice and control mice during specific stages of the hair-follicle cycle following wax epilation. Gene expression levels in control mice (shown in blue) and in K14-Agouti mice relative to control mice (shown in red) were determined by qRT-PCR analysis. Each graph corresponds to a single gene, and the gene symbol is shown in the top of the graph. For each of the 10 genes, the expression level in control mice at day 0 after epilation was assigned the value of 1.0, and the expression levels at later days are reported relative to this value. In addition to the wild-type pattern of expression for each gene, the relative fold differences between K14-Agouti and control mice are shown at each time point. In all graphs, the y-axis represents average fold differences in gene expression levels (mean ± SEM), and the x-axis represents the number of days following wax epilation. Gene expression levels greater than 1.4 fold or less than 0.72 fold (each indicated by a black horizontal line) indicate significant changes in expression. Shown in the bottom panel are the hair-follicle cycle stages that correspond to each time point after wax epilation, as determined by histological analysis of the skin. N = 4; each gene analyzed in duplicated in two animals at each time point.

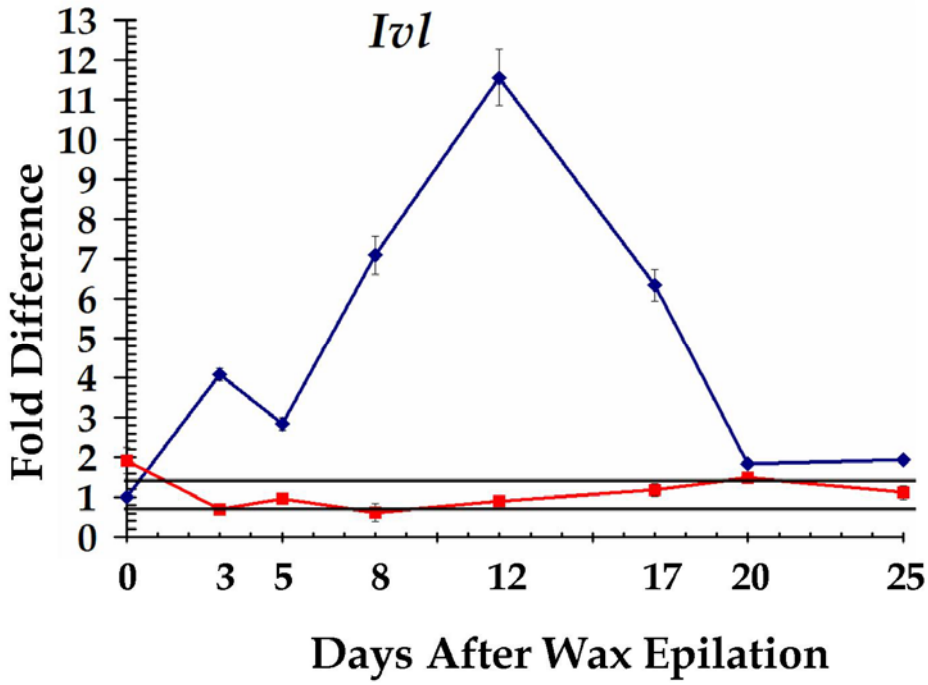


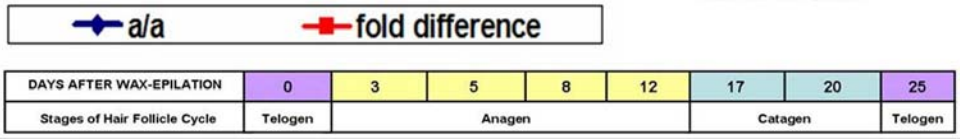




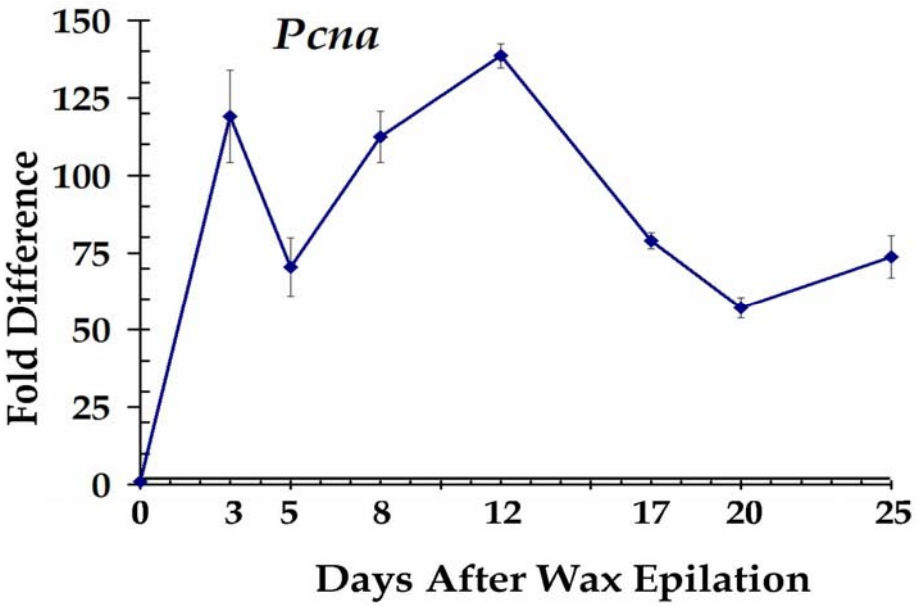
DAYS AFTER WAX-EPILATION	0	3	5	8	12	17	20	25
Stages of Hair Follicle Cycle	Telogen	Anagen				Catagen		Telogen

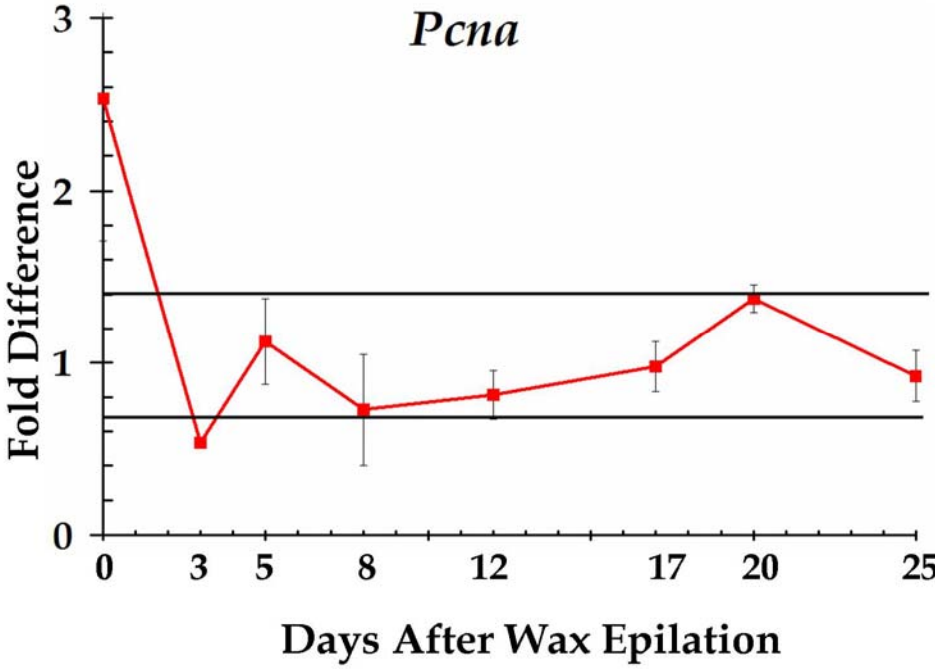






DAYS AFTER WAX-EPILATION	0	3	5	8	12	17	20	25
Stages of Hair Follicle Cycle	Telogen	Anagen				Catagen		Telogen





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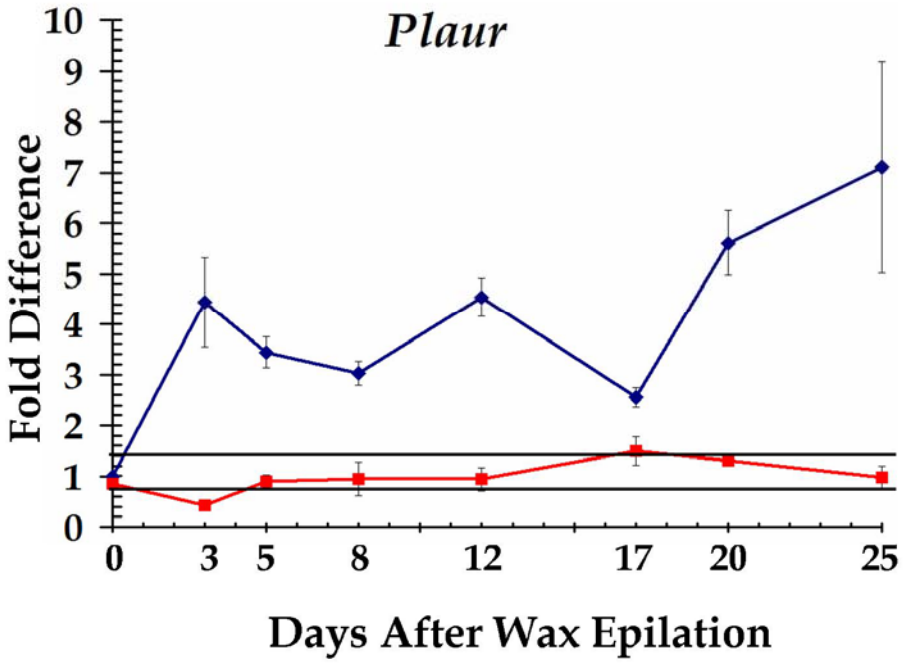
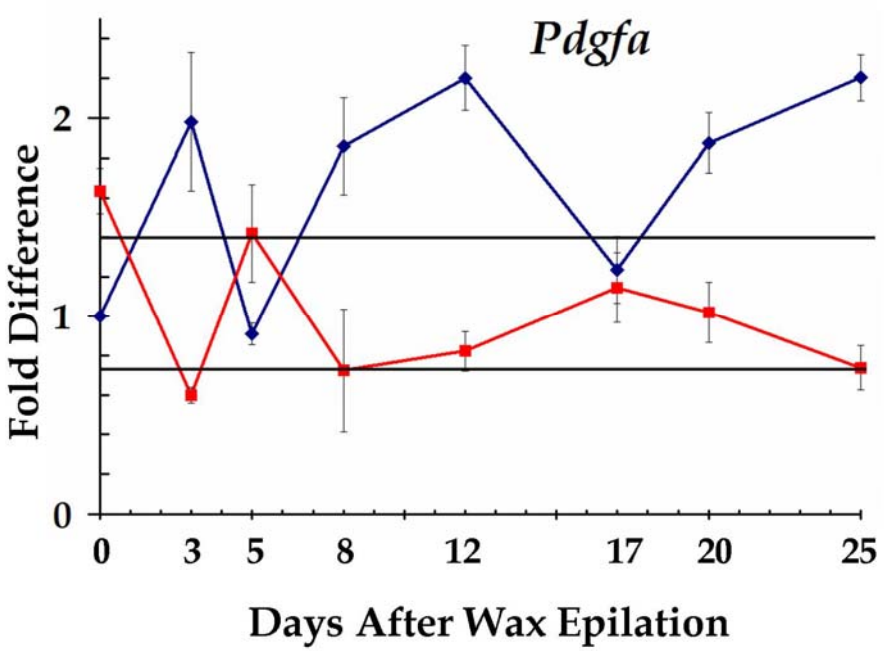
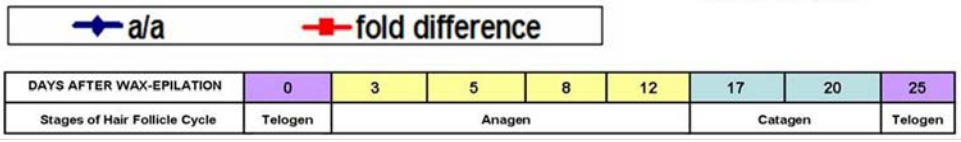
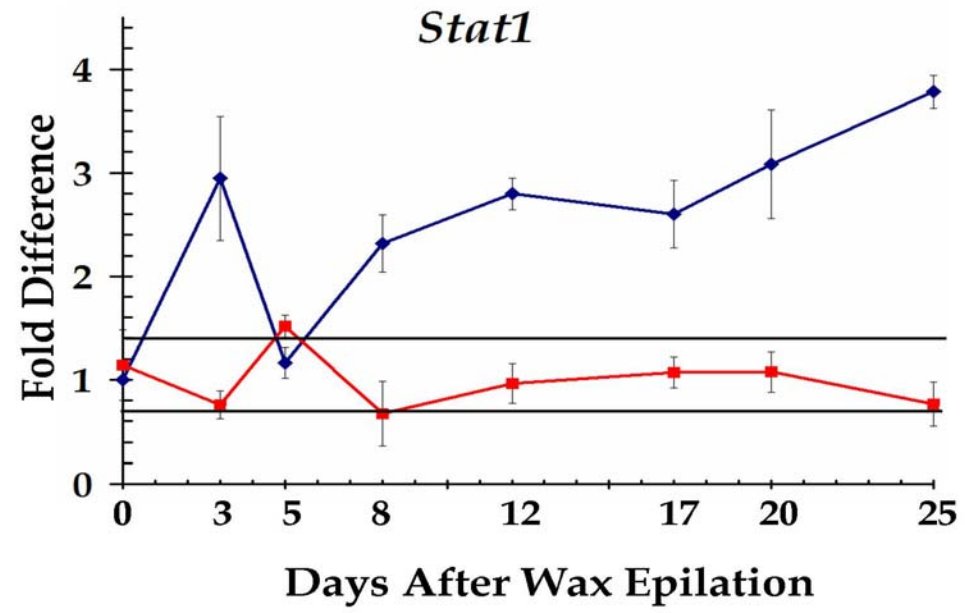
DAYS AFTER WAX-EPILATION	0	3	5	8	12	17	20	25
Stages of Hair Follicle Cycle	Telogen	Anagen				Catagen		Telogen

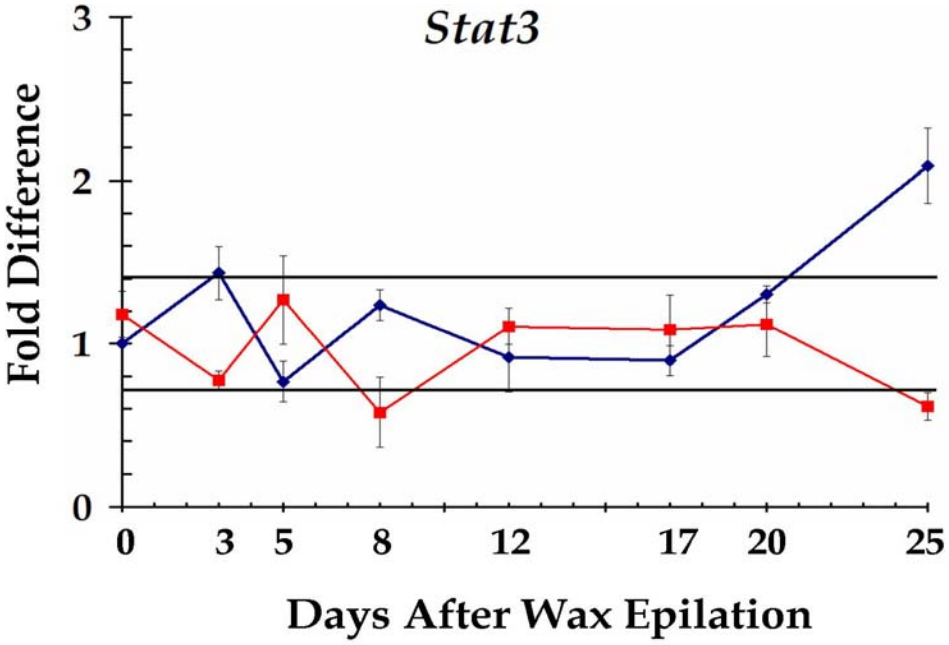






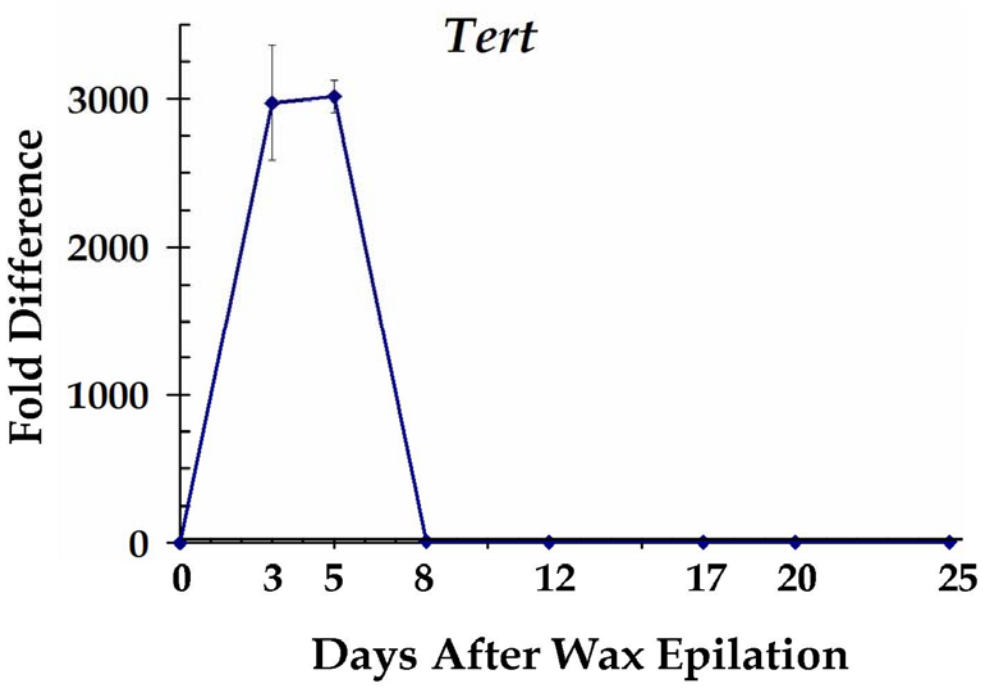
DAYS AFTER WAX-EPILATION	0	3	5	8	12	17	20	25
Stages of Hair Follicle Cycle	Telogen	Anagen				Catagen		Telogen

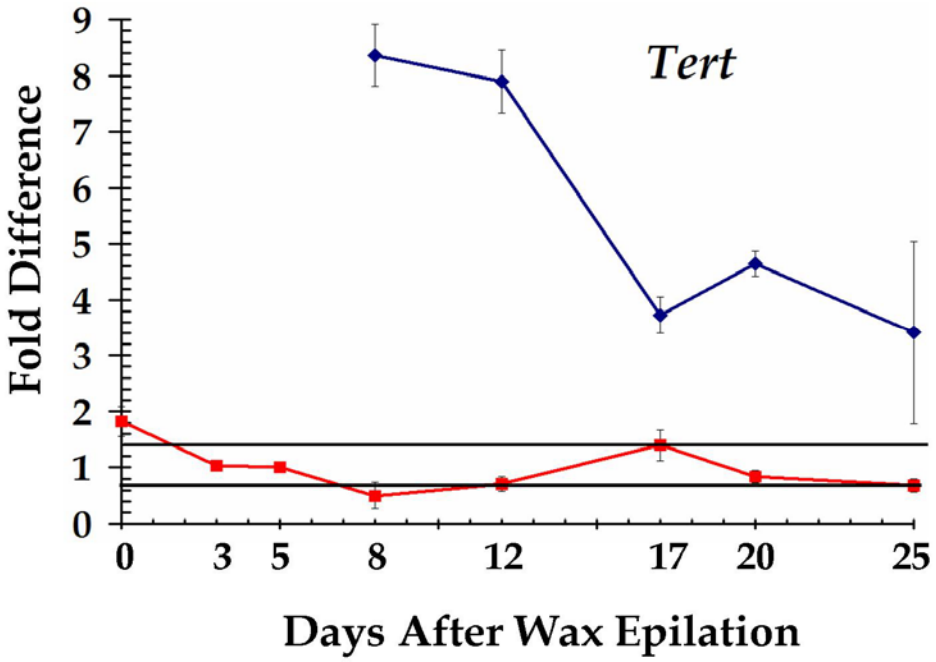




a/a — fold difference

DAYS AFTER WAX-EPILATION	0	3	5	8	12	17	20	25
Stages of Hair Follicle Cycle	Telogen	Anagen				Catagen		Telogen





a/a — fold difference

DAYS AFTER WAX-EPILATION	0	3	5	8	12	17	20	25
Stages of Hair Follicle Cycle	Telogen	Anagen				Catagen		Telogen

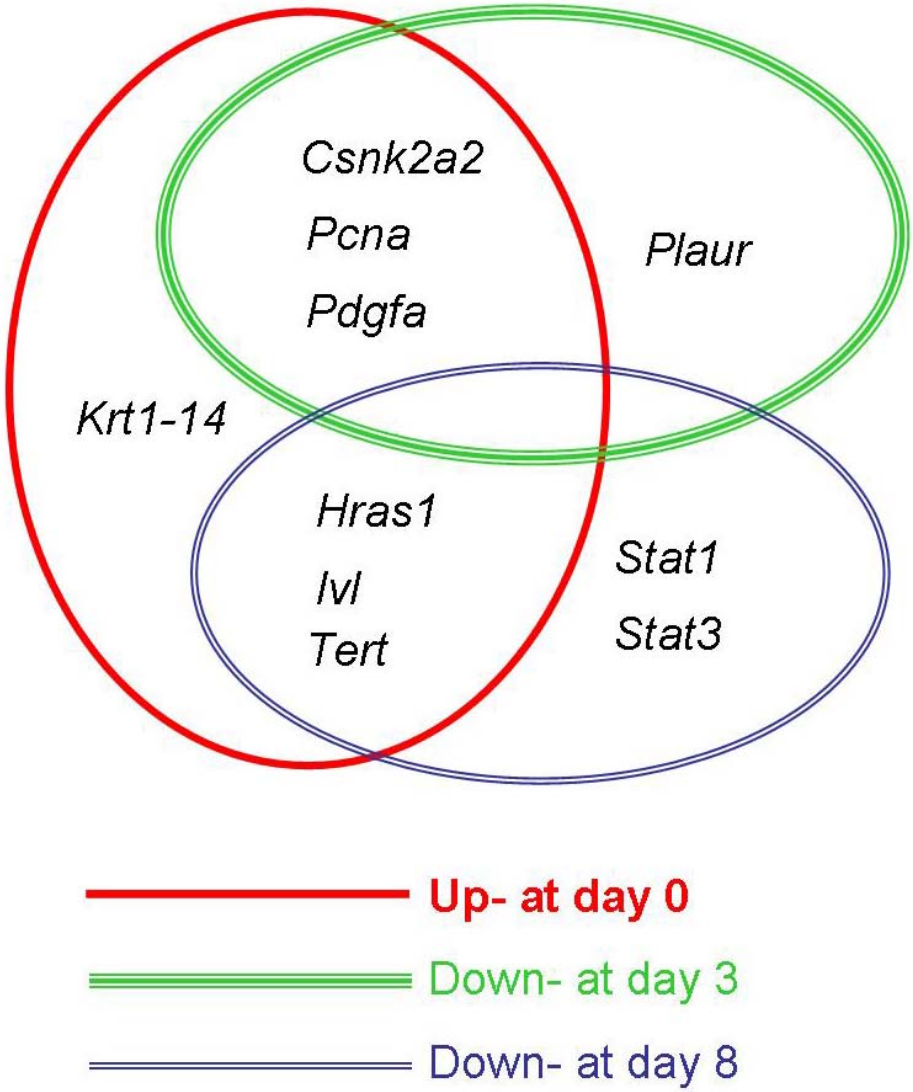


Figure 9. Changes in the temporal profiles of 10 genes in the skin of K14-Agouti mice compared to control mice following wax epilation. Genes are grouped according to their differential expression levels at the start of the hair-follicle cycle synchronization experiment and during the early and mid anagen stages of the hair-follicle cycle. In K14-Agouti skin, seven genes were significantly up-regulated at day 0, four genes were significantly down-regulated at day 3, and five genes were significantly down-regulated at day 8.

many genes that are normally up-regulated in early anagen in wild-type mice were up-regulated at an earlier time point (telogen) in the transgenic mice.

DISCUSSION

The agouti protein was previously shown to act as a tumor promoter in the skin of K14-Agouti transgenic mice since it promoted the development of papillomas in two-stage skin carcinogenesis experiments, but only after the skin was chemically initiated [99]. We speculated that high levels of constitutive paracrine signaling by agouti protein in the skin of K14-Agouti mice might act as a tumor promoter by in-turn up-regulating the expression of other proto-oncogenes. Here, our goal was to determine molecular level changes in the non-initiated skin of K14-Agouti transgenic mice using cDNA microarray and qRT-PCR analyses. Since over-expression of agouti in the skin of K14-Agouti mice acts as a tumor promoter and skin tumors do not develop in the absence of initiation, we reasoned that any gene expression differences detected in the skin will possibly be associated with the promotion stage of two-stage carcinogenesis.

Through microarray analysis we identified a total of 181 differentially expressed genes that may be associated with the promotion stage of skin carcinogenesis in K14-Agouti transgenic mice. Forty of the significantly up-regulated genes and 29 of the significantly down-regulated genes have been functionally annotated previously. According to their gene ontology terms, these differentially expressed genes are primarily involved in the following cellular processes: cell proliferation (*Hras1*, *Pdgfa*), regulation of progression through the cell cycle (*Csnk2a2*, *Pdgfa*), signal transduction (*Csnk2a2*, *Hras1*, *Plaur*, Erbb2 interacting protein, *Erbb2ip*; guanine nucleotide binding protein (G protein),

gamma 3 subunit, *Gng3*), protein phosporylation (*Csnk2a2*; PTEN induced putative kinase 1, *Pink1*), regulation of peptidyl-tyrosine phosphorylation (*Pdgfa*), DNA repair (RAD1 homolog (S. pombe), *Rad1*; excision repair cross-complementing rodent repair deficiency, complementation group 3, *Ercc3*), protein targeting/transport (Sec61 alpha 1 subunit (S. cerevisiae), *Sec61a1*, adaptor protein complex AP-1, beta 1 subunit, *Ap1b1*; *Erbb2ip*; AP1 gamma subunit binding protein 1, *Ap1gbp1*), ubiquitin-dependent protein catabolism (ubiquitin specific peptidase 27, X chromosome, *Usp27x*; ubiquitin specific peptidase 2, *Usp2*), regulation of transcription, DNA-dependent (zinc finger, matrin-like, *Zfml*; cartilage homeo protein 1, *Cart1*; c-myc binding protein, *Mycbp*), and the tricarboxylic acid cycle (malate dehydrogenase 1, NAD (soluble), *Mdh1*; citrate synthase, *Cs*).

Alterations in the first three of these cellular processes — cell proliferation, regulation of progression through the cell cycle, and signal transduction — are known to be associated with the promotion stage of carcinogenesis. *Csnk2a2*, *Hras1*, *Pdgfa*, and *Plaur* are each involved in at least one of these cellular processes, they play important roles in many normal as well as pathological processes, including carcinogenesis, and they were significantly up-regulated in the skin of K14-Agouti transgenic mice. For these reasons, we selected *Csnk2a2*, *Hras1*, *Pdgfa*, and *Plaur* for more detailed analyses, which included examining the expression levels of these genes in the skin of K14-Agouti and control mice ranging in age from about one month old to over six months old. We also examined the expression levels of these genes in the skin of two-month-old K14-Agouti and control mice during precisely determined stages of the hair-follicle cycle over a 25 day period following synchronization of follicle development in the dorsal skin by wax epilation. In these analyses, we also included four markers of cellular proliferation and differentiation (*Pcna, Tert, Krt1-14* and *Ivl*),

and two transcription factors (*Stat1* and *Stat3*) previously shown to be up-regulated by ectopic agouti expression in adipose tissue [97]. All 10 genes under investigation have previously been associated with epithelial carcinogenesis and/or cellular differentiation, proliferation and hair-follicle cycle events, as summarized in Table 3.

qRT-PCR analysis of these 10 genes in the skin of transgenic and control mice over a wide range of ages revealed that, in all cases, both the expression levels and age-dependent dynamic expression patterns of these genes were altered in the skin of K14-Agouti mice. The wild-type expression levels of most of these genes were normally up-regulated in the skin of control mice at 12.5 weeks of age, a time when dorsal hair follicles are in anagen, or the growth stage of the hair-follicle cycle. However, in K14-Agouti mice, all of these genes were significantly up-regulated compared to control levels at 7.5 weeks of age, a time that corresponds to telogen, or the resting stage of the hair-follicle cycle. For the majority of these genes, expression levels remained significantly elevated in the skin of transgenic mice compared to control mice at 10.4 weeks (early anagen) and 12.5 weeks (late anagen), but not at 11.4 weeks.

The major findings from the qRT-PCR analysis of these 10 genes in the skin of transgenic and control mice during defined stages of the hair-follicle cycle following epilation are that most of these genes were significantly up-regulated in K14-Agouti skin compared to control skin at the telogen stage, prior to the onset of anagen. In control mice, wax epilation induced telogen hair follicles to enter anagen at three days post-epilation, which also caused a significant up-regulation in the wild-type expression levels of 9 of the 10 genes (all except *Stat3*) during early anagen (Fig. 8; 3 days post-epilation). These results were in agreement with the age-dependent experiments (Fig. 7), where the wild-type expression levels of these genes were also up-regulated in association with the

transition from telogen to anagen. In K14-Agouti transgenic mice, wax epilation also induced telogen hair follicles to enter anagen at three days post-epilation, and there were no differences in hair-follicle cycling between transgenic and control mice. However, seven of the 10 genes (*Csnk2a2*, *Hras1*, *Krt1-14*, *Ivl*, *Pcna*, *Pdgfa*, and *Tert*) were significantly up-regulated in K14-Agouti skin compared to control skin during telogen (Fig. 8; 0 days post-epilation). This result also correlates with the age-dependent expression profiles of these genes (Fig. 7); namely, that the expression of K14-Agouti in the skin of mice is inducing significant up-regulation in the expression of genes associated with epithelial carcinogenesis at an earlier time (telogen) and at abnormally elevated levels compared to control mice.

For nine of the 10 genes (all except *Krt1-14*), there was a brief period during early or mid anagen when their expression levels were significantly lower in K14-Agouti mice compared to control mice. This result may be similar to what we observed in mid anagen for the age-dependent experiments, where the expression levels of most genes did not differ significantly between transgenic and control mice in mid anagen (Fig. 7; 11.4 weeks). Unlike the age-dependent experiments, where the expression levels of all genes were significantly up-regulated in the skin of K14-Agouti mice in late anagen (Fig. 7; 12.5 weeks), the expression levels of all genes did not differ significantly in transgenic and control mice in late anagen following epilation (Fig. 8; 12 days). The reason for this difference is unclear, but it suggests that, in the age-dependent experiments, over-expression of these 10 genes in the skin of transgenic mice in late anagen is associated with some age-dependent factor in the skin other than the stage of the hair follicles.

Over-expression of K14-Agouti in the skin of transgenic mice had no effect on the hair-follicle cycle based on histological analysis of the skin, but it did

cause over-expression of numerous genes associated with epithelial cancers. Not only were these genes over-expressed in the skin of transgenic mice at ages associated with anagen, a time when these genes are normally activated in control mice, they were also over-expressed in the skin of transgenic mice during telogen, resulting in the exposure of transgenic skin and hair follicles to these proto-oncogenes over a prolonged period of the hair-follicle cycle. These results suggest that K14-Agouti may have a more direct role as a paracrine signaling molecule in altering the expression of the genes under investigation.

Notable among the genes over-expressed in the skin of K14-Agouti mice are *Csnk2a2*, *Hras1*, *Pdgfa*, *Plaur*, and *Stat1*. *Csnk2a2* is one of the catalytic subunits of protein casein kinase 2 (*Ck2*) [194]. CK2 is a pleiotropic, ubiquitously expressed, protein Ser/Thr kinase with 307 known substrates [195], and involved in many cellular functions, such as cell proliferation, division, and development (reviewed in [196, 197]); cell survival [198]; inhibition of apoptosis [199, 200]; and neoplasia (reviewed in [201]). The catalytic *Csnk2a2* subunit also has oncogenic activity [201]. Altered expression of *Csnk2a2* results in the transformation of mammary epithelium [169] and increases the transformation rate in fibroblasts [202].

Activation of RAS proteins initiate different signal transduction pathways that regulate cellular proliferation, transformation, differentiation, and apoptosis (reviewed in [203, 204]). Over-expression of wild-type *Ras* or mutations that constitutively activate *Ras* have been observed in a wide spectrum of cancers [205-207] including skin cancer [208, 209]. Mutations in the *Hras1* gene have been observed in non-melonoma skin cancers with up to 20% frequency (reviewed in [210]). Additionally, the skin of v-Ha-ras transgenic TG.AC mice has been shown to have enhanced sensitivity to chemically induced tumorigenesis when there is

ubiquitous over-expression of agouti protein due to the presence of the A^{vy} allele [21].

PDGF and its protein tyrosine kinase receptors have been linked to a variety of disorders including carcinogenesis [203]. PDGF is a mitogenic protein and its alpha chain is known to cause transformation in both autocrine [180, 211] and paracrine fashions on its target cells (reviewed in [212]). Activation of PDGFA signaling has been shown to be critical for the development of a wide range of tumors such as glioblastoma, leukemia, gastro-intestinal carcinoma, prostate and breast cancer, malignant melanoma, and squamous cell carcinomas ([181], reviewed in [179, 180, 183]).

PLAUR is also involved in a wide range of physiological functions, such as regulation of cell surface plasminogen activity, cellular adhesion, cell motility, angiogenesis, wound healing, tissue regeneration, tumor invasion, and metastasis (reviewed in [213-215]). PLAUR and its ligand have been associated with malignant transformation of the ovarian epithelium [216]. Elevated levels of PLAUR have been reported in several malignancies ([217, 218], reviewed in [215, 219]), including squamous cell cancer of the skin [185, 220].

Although loss of STAT1 signaling has been shown in many human tumors [221, 222], it has been found to be constitutively active in a variety human tumors with STAT3 [188]. In squamous epithelial cells, aberrant activation of epidermal growth factor receptor (EGFR) has been shown to constitutively activate both *Stat1* and *Stat3* [189, 223]. Over-expression of these proto-oncogenes in the skin of K14-Agouti mice are highly likely to play a role in K14-Agouti-induced skin tumor promotion.

In conclusion, we used cDNA microarray analysis to identify 181 differentially expressed genes in the skin of K14-Agouti mice. We used qRT-PCR analysis to identify and quantify changes in the expression levels and temporal profiles of 10 genes (*Csnk2a2, Hras1, Krt1-14, Ivl, Pcna, Pdgfa, Plaur, Stat1, Stat3,* and *Tert*) in the skin of K14-Agouti mice, which have previously been associated with epithelial neoplasia in other models. These genes were significantly up-regulated in the skin of K14-Agouti mice during telogen, prior to the normal up-regulation of these genes during anagen in control mice. These genes were also over-expressed in the skin of K14-Agouti mice at ages associated with the anagen stage of the hair-follicle cycle, a time when agouti is normally transiently expressed in the skin and when mouse skin is known to be more susceptible to carcinogenesis. Thus, the increased susceptibility to skin carcinogenesis observed in K14-Agouti transgenic mice is proposed to be associated with the altered expression levels and patterns of these genes. Further studies with the tumor susceptible K14-Agouti mouse model will prove useful for extending these findings and for determining the molecular genetic network through which the unregulated activation of the agouti gene acts as a tumor promoter in skin carcinogenesis.

CHAPTER III

Conclusions and Future Perspectives

In mice carrying the spontaneous dominant mutations A^{y} or A^{vy}, ubiquitous over-expression of the agouti gene causes an increased susceptibility to tumorigenesis in numerous tissues, including the skin. These mice are also obese and diabetic because of ectopic agouti expression in the brain. In mice carrying the albumin-Agouti or K14-Agouti transgenes, over-expression of the agouti gene in the liver or skin, respectively, does not cause obesity or diabetes. However, albumin-Agouti and K14-Agouti mice are significantly more susceptible to liver or skin cancer, respectively, following chemical initiation of the liver or skin. These data suggest that the agouti protein may act as a tumor promoter in various tissues by directly impacting other signal transduction pathways in the tissue in which it is expressed. Although the association between the agouti protein and tumor susceptibility has been known for many decades and the agouti gene was cloned 14 years ago, the molecular basis of the agouti protein in tumorigenesis remains largely unknown. Therefore, the goal of my research was to elucidate molecular mechanisms associated with agouti-induced skin tumor promotion.

In the previous chapter, cDNA microarray analysis was used to identify significant changes in the expression levels of 181 genes in the skin of K14-Agouti transgenic mice compared to non-transgenic control mice. Real-time qRT-PCR experiments demonstrated that 10 genes previously associated with various epithelial cancers exhibited dynamic patterns of gene expression in the skin of control mice that changed with age, and their expression levels and patterns were altered in the skin of tumor susceptible K14-Agouti transgenic mice. These experiments provide significant insight into the potential role of the agouti protein as a tumor promoter in skin carcinogenesis. Taken together, these data suggest that over-expression of these proto-oncogenes in the skin of the transgenic mice are possibly associated with the increased susceptibility of K14-Agouti mice to skin carcinogenesis.

The genes with altered expression in K14-Agouti skin can be grouped by gene ontology terms, which indicate the biological attributes of the gene and its gene product. Among the cellular processes into which these genes are grouped, the four with the greatest impact on tumorigenesis when they are altered in an individual cell are cell proliferation, DNA repair, ubiquitin-dependent protein catabolism, and energy metabolism. The agouti protein may act as a tumor promoter by altering one or more of these cellular processes.

Based on the genes that have altered expression patterns in the skin of K14-Agouti mice, an impact of agouti protein on cellular proliferation appears to be the strongest candidate for the underlying molecular mechanism of agouti-induced tumor promotion. For example, expression of the *Pdgfa, Plaur, Stat1, Stat3* and *Hras1* proto-oncogenes was altered in K14-Agouti skin. These genes are regulators of cell proliferation; they act through common signal transduction pathways, and also interact with intracellular pathways downstream of agouti

signaling, which are mainly described in adipocytes. Additionally, even though there were no gross histological differences in the hair-follicle cycle between K14-Agouti and control mice, there was an up-regulation of two cellular proliferation markers, *Pcna* and *Tert*, in the skin of K14-Agouti transgenic mice.

Here, I present a further dissection and analysis of the microarray results, and propose a model describing the main molecular mechanism for agouti-induced skin tumor promotion. It should be noted that changes in gene expression levels were measured in this study, but the model focuses on the expected outcomes of changes in certain corresponding protein levels, which were not measured in this study. The model depicts the main signaling pathways impacted by agouti protein, the manner in which these pathways interact, and how alterations in these pathways would lead to increased cellular proliferation, which I suggest is the main molecular mechanism for agouti signaling in skin tumor promotion. I also suggest future experimental approaches for validation of the gene expression results at the protein level, and for testing of this model.

MODEL SIGNAL TRANSDUCTION PATHWAYS FOR AGOUTI-INDUCED CELLULAR PROLIFERATION

Cells respond to different environmental signals (e.g., extracellular ligands, environmental changes, etc.) through different signal transduction pathways. The main components of a simple signal transduction pathway are an extracellular signal, a membrane receptor, an intracellular secondary messenger, and a transcription factor. Protein phosphorylation due to membrane receptor activation by extracellular signals initiates the signal transduction cascade. Protein tyrosine kinase (PTK), mitogen activated protein kinase (MAPK), and

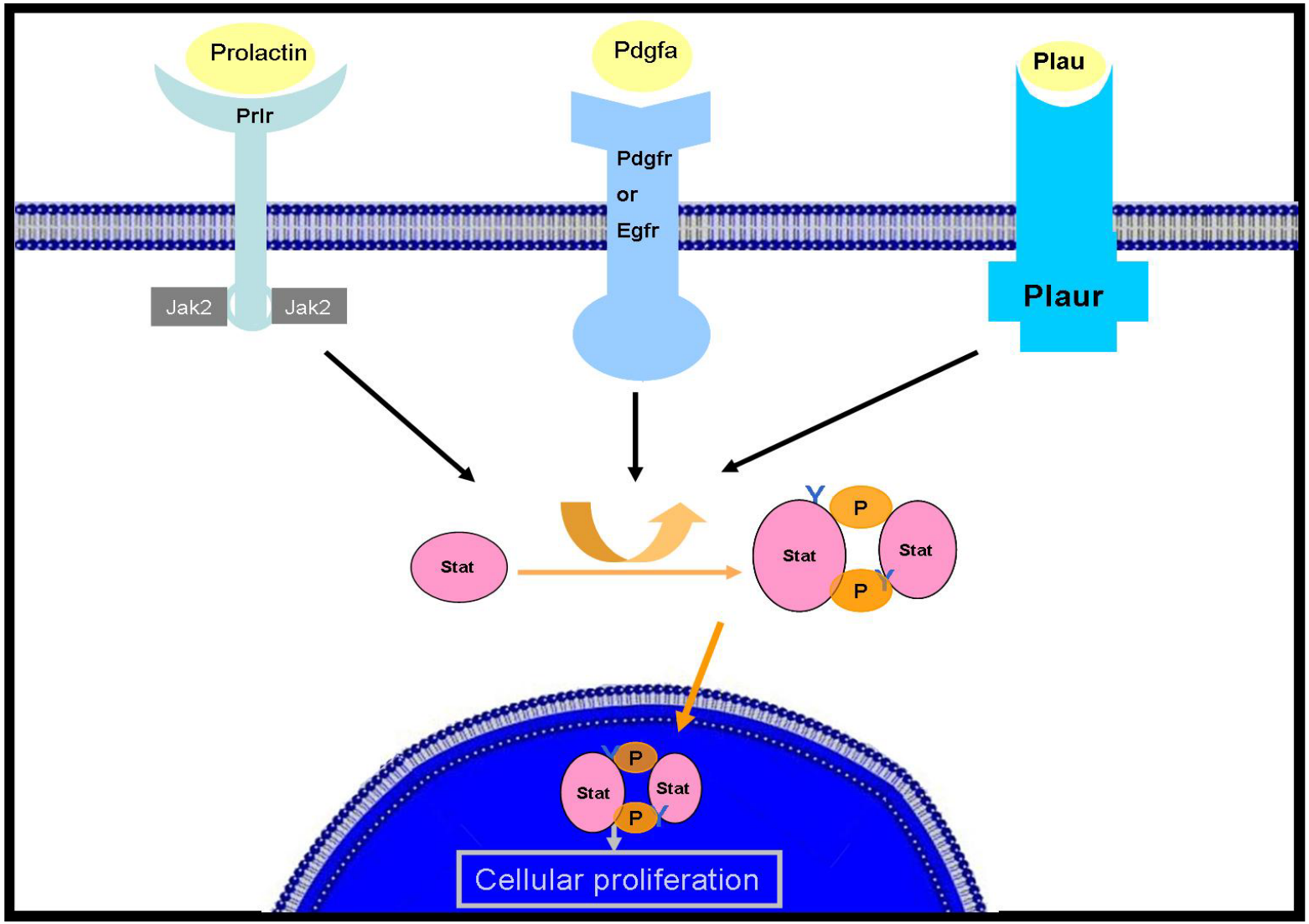
protein kinase C (PKC) are three major signal transduction pathways that regulate numerous important cellular functions, such as homeostasis, proliferation, differentiation and pathophysiological processes. Constitutive activation of these pathways often initiates intracellular signaling pathways that lead to uncontrolled cellular proliferation and transformation [224].

Signal Transducer and Activator of Transcription (STAT) Pathway

In the skin of K14-Agouti transgenic mice, a simple model for a signal transduction pathway that could lead to cellular proliferation is the activation of the protein tyrosine kinase (PTK) pathway, via platelet-derived growth factor alpha (PDGFA) signaling through a PTK receptor and transduction of the signal by STAT mediators (Fig.10). PDGFA is a potent mitogenic signal that initiates intracellular signaling pathways, leading to cellular proliferation through protein tyrosine kinase receptors [180, 211]. It activates PDGF receptor through ligand-mediated dimerization and auto-phosphorylation of the tyrosine residues on the cytoplasmic domain of the receptor. Some of the main downstream signal transduction molecules for PDGFA receptors are the signal transducer and activator of transcription (Stat) proteins [225].

Stat molecules moderate the cellular response to extracellular signals like cytokines and growth factors by transmitting the signal from the cell surface membrane to the nucleus. Upon activation of protein tyrosine kinase receptors, Stats are recruited by the PTK receptor and phosphorylated at the tyrosine residue. Phosphorylation of Stat molecules leads to homodimer or heterodimer formation, which directs translocation of Stats into the nucleus. In the nucleus,

Figure 10. Proposed mechanism for induction of STAT signaling in the skin of K14-Agouti transgenic mice. Up-regulation of the three protein tyrosine kinase receptors (PDGFR, PLAUR, and PRLR) in the skin of K14-Agouti mice is proposed to induce cellular proliferation through STAT signaling. The extracellular mitogenic signals are delivered to the cells by Prolactin, PDGFA, and PLAU ligands, and transferred to the intracellular compartments by activation of PTK receptors. Activated PTK receptors induce phosphorylation of STAT molecules. Phosphorylated STAT molecules can form dimers and translocate to the nucleus, where they induce cellular proliferation. All three PTK receptors are shown on the cellular membrane, which is drawn as a bi-layered dark blue line. Semi-circular blue area represents the nucleus, and is surrounded by the nuclear membrane. Phosphorylated forms of STAT molecules are labeled with the letter P, and tyrosine residues on STAT molecules are marked with the letter Y. Solid arrows represent activation of signaling molecules.


Prolactin
Prlr
Jak2
Jak2
Pdgfa
Pdgfr
or
Egfr
Plau
Plaur
Stat
Stat
P
Stat
P
Stat
P
Stat
P
Cellular proliferation

Stats directly bind to DNA to regulate transcription of target genes, depending on the signaling pathway and the target tissue (reviewed in [226]). It has been shown that PDGF signaling can induce phosphorylation of three STAT molecules: STAT1, STAT3 and STAT5 (reviewed in [227]). Up-regulation of *Pdgfa* expression due to ectopic over-expression of agouti might cause over-activation the PTK signal transduction pathway through altered STAT activation, which is important in skin tumor promotion.

STATs have been shown to be constitutively activated in skin papillomas and SCCs [189]. Recent studies have suggested that STAT3 has an essential role in two-stage skin carcinogenesis, and it is required during both initiation and promotion events [190]. STAT3 activation through the epidermal growth factor receptor (EGFR), which is a PTK receptor, during the promotion stage of skin carcinogenesis has been shown to stimulate epidermal hyperproliferation [190]. Activation of EGFR and up-regulation of its ligands have been observed as a common response to tumor promoting agents in mouse epidermis [228, 229]. Besides its own PTK receptors, PDGF can phosphorylate and activate EGFR [230, 231]. Transactivation of EGF signal by PDGF signaling has been shown to occur through heterodimer formation of EGFR and PDFGR [232-234]. Thus, agouti-induced over-expression of *Pdgfa* could result in altered signaling through both PDGF and EGF receptors, which could result in increased cellular proliferation, as shown in Figure 10.

Intracellular STAT signaling can also be activated by cytokine signals, such as prolactin, through tyrosine phosphorylation of JAK kinases. Binding of prolactin to the prolactin receptor (Prlr), which does not have any intrinsic tyrosine kinase cytoplasmic domain, induces receptor dimerization, tyrosine phosphorylation and activation of JAK kinase, and phosphorylation of Prlr [235].

Even though it was not validated by qRT-PCR, our microarray data showed that *Prlr* was up-regulated in K14-Agouti skin and had the fifth highest significance score. In different cell lines and tissues, PRLR has been shown to activate STAT1, STAT3 and STAT5. It has been suggested that prolactin activates STAT5 in a different manner than it does STAT1 and STAT3 [236, 237].

Plasminogen activator, urokinase receptor (*Plaur*), a glycosylphosphatidylinositol-anchored receptor, is another gene up-regulated by over-expression of agouti in the skin of K14-Agouti mice. PLAUR activation has been shown to activate different signal transduction pathways, including PTK [238], MAPK [239], and JAK/STAT pathways [240]. STAT1 activation through PLAUR has been demonstrated in a human kidney epithelial tumor line [240], and human vascular endothelial cells [241]. STAT1-STAT1 homodimers and STAT1 heterodimers with STAT2 and STAT4 have been observed upon PLAUR activation [242].

In the skin of K14-Agouti mice, up-regulation of the *Pdgfa* ligand, and *Plaur* and *Prlr* receptors, could each result in altered activation of STAT molecules that function in PTK pathways. Additionally, up-regulation of *Stat1* and *Stat3* were detected in the skin of K14-Agouti mice, which can directly contribute to the altered STAT signaling. Thus, unregulated STAT activation in the skin of K14-Agouti mice, caused by over-expression of agouti protein, is a candidate intracellular signaling mechanism that may lead to altered cellular proliferation (Fig. 10).

Mitogen Activated Protein Kinase (MAPK) Pathway

A variety of extracellular stimuli, such as environmental stress, mitogens, cytokines, and neurotrophins can be transmitted to the nucleus through intracellular mitogen activated protein kinase (MAPK) pathway signaling cascades (reviewed in [203, 243, 244]). Although the mechanisms that control the specificity of the cellular response to different stimuli through MAPK pathways are still not clear, regulation of cellular proliferation through Ras-mediated Raf/MEK/ERK signaling has been demonstrated in many studies (reviewed in [171, 245-248]). One of the major activators of Ras/ERK signaling is the phosphorylation of Ras by downstream mediators of PTK receptors [249]. Active Ras recruits Raf-1 to the plasma membrane, where it will be activated by phosphorylation [250]. In the next step of the cascade, Raf-1 directly activates MEK, which is followed by ERK phosphorylation [251]. As discussed above, PDGFA, PLAUR, and PRLR signals are activators of PTK signal transduction pathways. For all three molecules, activation of the Raf/ERK signaling cascade was previously proposed to explain effects of these signals on cellular proliferation [235, 239, 252, 253].

The *Hras1* proto-oncogene is up-regulated in the skin of K14-Agouti mice. In addition to the activators of PTK signaling pathways, over-expression of wild-type *Ras* or activated *Ras* mutations is associated with tumorigenesis in different tissues [205-209]. Ras/Erk is a target pathway in tumor promotion, as altered regulation of mitogenic Ras/ERK signaling provides a growth advantage to the target cells.

STAT activation, induction of the Ras/ERK MAPK pathway through PTK receptors, and over-expression of *Hras1* may be independent but interacting

molecular mechanisms that could lead to altered cellular proliferation resulting from altered agouti signaling (Figure 11).

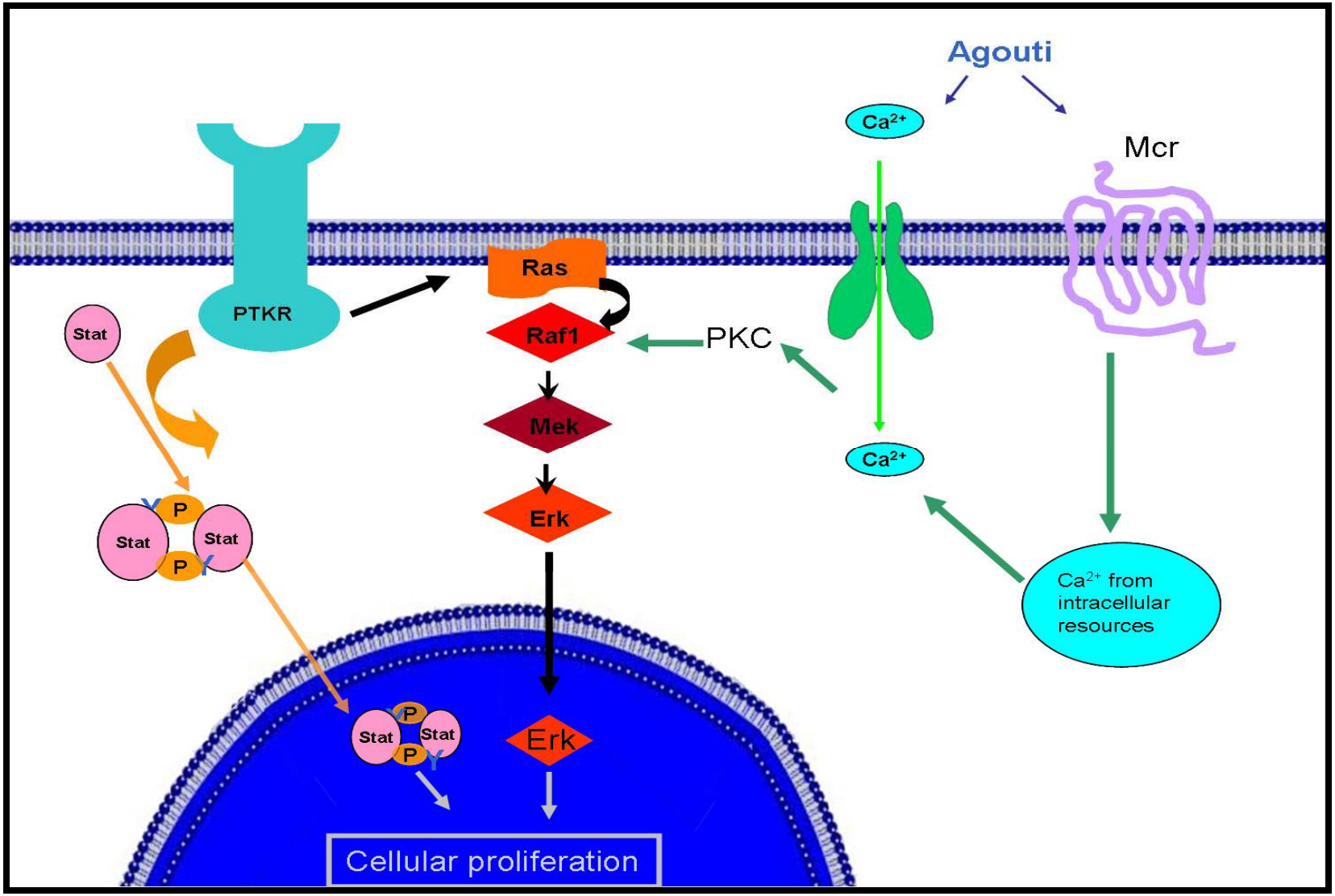
Protein Kinase C (PKC) Pathway

Protein kinase C (PKC) signaling has been shown to induce the Raf-1/MEK/ERK signaling pathway through activation of the Raf-1 molecule [254-256], which has been proposed as the key mediator connecting PTK signaling through growth factor receptors (such as PDGFR and EGFR) and PKC signals at the cell membrane [257, 258].

PKC signaling has been implicated in the control of different cellular functions, including cellular proliferation [249, 259, 260], and over-activation of PKC signaling can result in cellular transformation [261-263]. The conventional PKC isoforms, PKCα, PKCβ, and PKCγ, are diacylglycerol (DAG) sensitive, and calcium responsive. This group of PKCs are activated by increased intracellular Ca^{2+} levels due to Ca^{2+} influx through Ca^{2+} channels or due to release of Ca^{2+} from intracellular stores [264, 265]. PKC translocates to the membrane after binding to Ca^{2+} to interact with DAG, which transforms PKC into a fully active enzyme. PKCα has been reported to phosphorylate and activate Raf [251, 256, 266, 267], which then activates ERK and transmits a PKC-dependent proliferation signal ([263, 268], reviewed in [269]).

Activation of PKC in response to a sustained increase in intracellular calcium levels contributes to insulin resistance [249], which is also a possible mediator for the insulin resistance observed in A^{vy} mice [89, 270]. Similar to calcium-mediated PKC activation in insulin resistance, agouti might be

Figure 11. Intracellular signal transduction pathways proposed to be activated in K14-Agouti skin in response to agouti signaling. Activation of two intracellular signal transduction pathways, STAT and RAF/ERK, are proposed to induce cellular proliferation in K14-Agouti skin. STAT signaling can be activated through protein tyrosine kinase receptors (PDGFR, PLAUR, and PRLR) (Fig. 10), which is labeled as PTKR. In this model, over-expression of agouti is proposed to activate a calcium-dependent PKC pathway, which in turn can activate Ras-independent ERK signaling. ERK signaling can also be initiated by activation of either PTKR or RAS molecules in the skin of K14-Agouti mice. MCR, Ca^{2+} channels, and PTKRs are shown on the cellular membrane, which is drawn as a bi-layered dark blue line. Semi-circular blue area represents the nucleus, and is surrounded by the nuclear membrane. Phosphorylated forms of STAT molecules are labeled with the letter P and tyrosine residues on STAT molecules are marked with the letter Y. Solid arrows represent activation of signaling molecules.


Agouti
Ca2+
Mcr
PTKR
Ras
Raf1
PKC
Mek
Erk
Stat
P
Stat
Stat
P
Ca2+
Ca2+ from intracellular resources
Erk
Cellular proliferation

activating PKC signaling through Ca^{2+}-dependent pathways. Therefore, agouti-induced cellular proliferation through PKC/ERK signaling is another mechanism that may contribute to the tumor susceptibility phenotype in K14-Agouti, A^{y}, and A^{vy} mice (Figure 11).

Agouti-dependent elevation of intracellular Ca^{2+} has been observed in several different cell types, such as skeletal muscle myocytes [93], adipocytes [95], and pancreatic beta cells [271]. Agouti has been shown to act through both Ca^{2+} transportation channel-dependent [95], and melanocortin receptor-dependent pathways [89] to increase intracellular Ca^{2+} levels. Although agouti can act through both Ca^{2+} channels and melanocortin receptors for Ca^{2+}-dependent PKC activation, signaling through Ca^{2+} channels may be more likely to explain the fact that over-expression of agouti throughout the body of A^{y} and A^{vy} mice causes increased tumor susceptibility in numerous tissues. For example, agouti has been shown to cause a slow increase in intracellular Ca^{2+} levels only through MC1R and MC4R receptors, which are expressed in the skin and hypothalamus, respectively. On the other hand, Ca^{2+} channel receptors are ubiquitously expressed in many different tissues. Thus, PKC activation through Ca^{2+} channel receptors would not be limited to specific tissues, presenting a molecular mechanism that could explain tumor susceptibility in different tissues due to ectopic agouti signaling.

Agouti protein and the phorbol ester, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), are each tumor promoters in the skin of K14-Agouti mice. Additionally, the agouti and TPA tumor promoters act synergistically in the skin of K14-Agouti mice, decreasing the latent period of tumor formation and increasing the cumulative prevalence, multiplicity, and malignant conversion rate of skin papillomas [99]. It was suggested that agouti and TPA promote

tumor development through independent pathways, but that these pathways ultimately converge and lead to enhanced skin tumor promotion and progression [99]. In addition to the activation of conventional calcium-dependent PKCs by TPA, calcium-independent PKCδ can be induced in response to TPA treatment. TPA has been shown to regulate cellular hyperplasia through PKCδ induced Raf/ERK activation, as over-expression of c-Raf increases activation of Ras-independent MAPK signaling in response to TPA treatment [255]. TPA and agouti might be inducing independent intracellular signals through PKCδ and calcium-dependent PKCs, respectively, which in turn could activate Ras/Erk signaling in both cases. Therefore, the proposed mechanism for agouti-induced cellular proliferation through Ca^{2+}-dependent PKC activation might also be the molecular basis of the synergistic action of TPA and agouti in tumor promotion.

The induction of the STAT, MAPK and PKC signaling pathways by altered agouti signaling could each initiate cellular proliferation independently, in an additive manner, or these three pathways might be interacting with each other to amplify the cellular proliferation signal transmitted to the nucleus (Fig. 11).

Regulation of Gene Expression through Protein Kinase C

The *Plaur*, *Pdgfa*, *Stat1*, and *Stat3* genes, which are up-regulated in K14-Agouti skin due to over-expression of agouti, have also been shown to be regulated at the transcriptional level by phorbol esters, specifically, by TPA. Activation protein 1 (AP-1) is a group of transcriptional activator genes, including the c-fos and c-jun oncogenes, which are the early responding genes to TPA-induced PKC activation [272]. When activated, AP-1 proteins form dimers through their basic region-leucine zipper binding motif, which can recognize and

bind to TPA-response elements (TRE) found in the promoters of TPA-inducible secondary response genes ([273], reviewed in [274]).

The *PDGFA* gene was shown to be induced by phorbol esters in human glomerular mesangial cells [275] through TREs 1 and 2 in the promoter of *PDGFA* ([276, 277], reviewed in [278]). Additionally, phorbol ester and TPA-induced activation of *Plaur* transcription has been shown during the promotion stage of skin carcinogenesis, and in papillomas and carcinomas of mouse skin [224, 279, 280, 281]. *Plaur* expression is induced in response to phorbol esters, along with other urokinase genes, through PKC activation [279, 280]. Two different AP-1 binding sites in the promoter region of human *Plaur* were mapped, and induction of uPAR expression by phorbol esters was shown to require the AP-1 binding site in colon cancer cells [282]**.** As they are induced by the tumor promoting agent TPA, expression of urokinase genes by epithelial and stromal cells has been suggested to have an impact on the early steps of tumor promotion [220, 279].

In a recent study, activation of STAT1 and STAT3 transcription factors was observed in skin papillomas and SCCs after topical treatment with different tumor promoters, including TPA. In primary mouse keratinocyte cultures, an EGFR-dependent activation mechanism was demonstrated for TPA-induced *Stat3* transcription. However, the involvement of TPA-induced PKC activation in transcriptional regulation of *Stat1* and *Stat3* still needs to be determined [189].

Transcription of *Pdgfa* and *Plaur* were up-regulated in the skin of K14-Agouti mice in response to the over-expression of agouti protein. TPA has been shown to induce transcriptional activation of both *Pdgfa* and *Plaur* through activation of PKC. Agouti and TPA act synergistically during the promotion

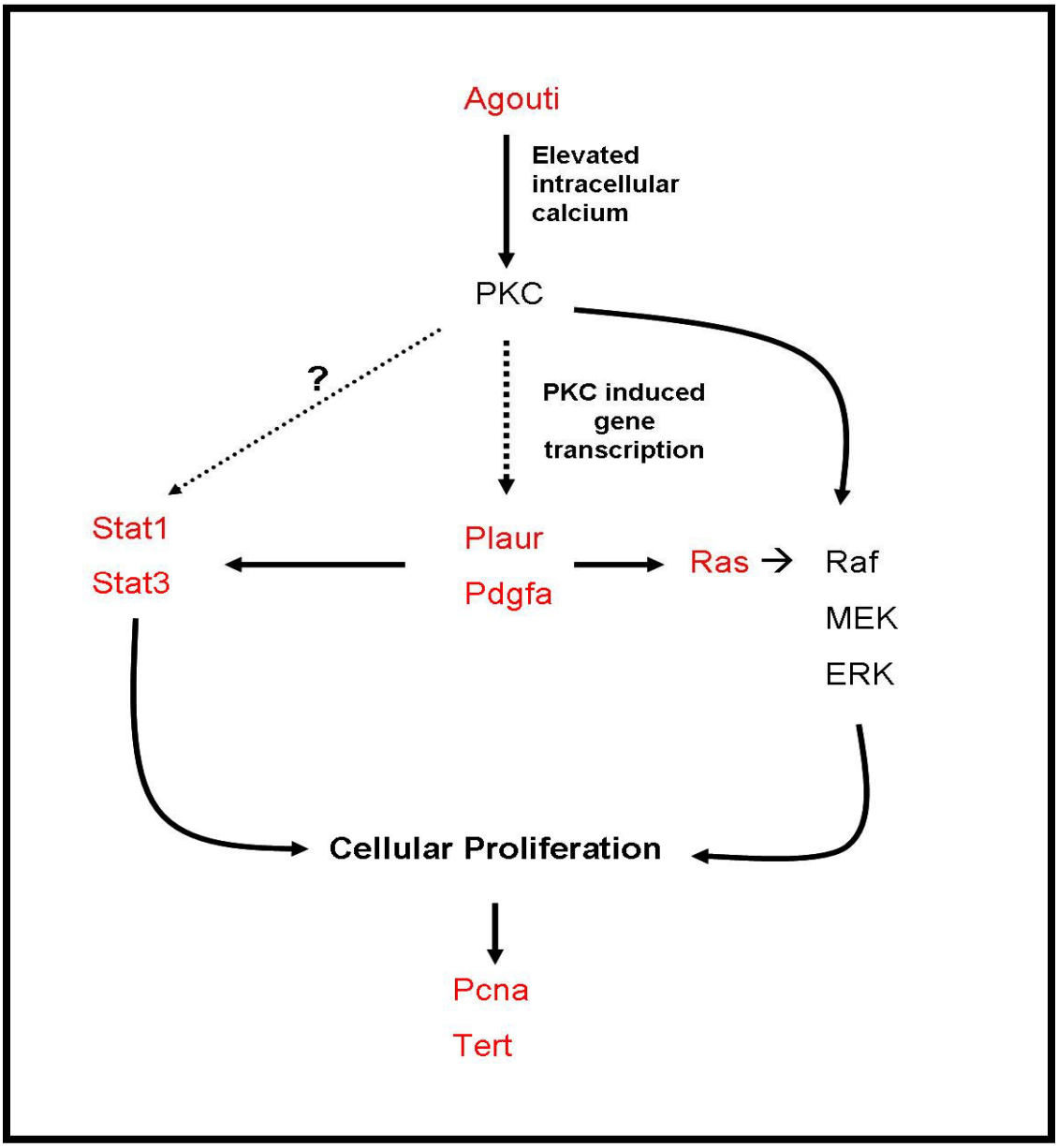
stage of skin carcinogenesis in K14-Agouti mice [99]. Therefore, it is possible that agouti-induced up-regulation of *Pdgfa* and *Plaur* transcription in the skin of K14-Agouti mice might be occurring through a PKC-activated signaling pathway. Up-regulation of the transcription of the *Pdgfa* and *Plaur* proto-oncogenes could be inducing altered signal transduction through the STAT and MAPK pathways, which could generate aberrant cellular proliferation signals.

In conclusion, I propose that agouti protein, like many other proto-oncogenes, may act as a tumor promoter by altering cellular signaling pathways that induce the proliferation of initiated cells. In Figure 12, I present a model for the role of agouti protein in cellular proliferation, which includes all of the proposed intracellular signaling pathways and interactions that were discussed above.

FUTURE PERSPECTIVES

In this study, I have shown differential expression of a set of genes in the skin of tumor susceptible K14-Agouti transgenic mice that have known roles in skin and/or other epithelial cancers. Based on the genes identified in these experiments, on their established roles in normal cellular processes and common signaling pathways, and on the known roles of agouti signaling in pigmentation and obesity, I have proposed a hypothetical model to explain the role of the agouti protein in tumor promotion. The model predicts that agouti may function as a tumor promoter by altering various intracellular signaling pathways that lead to unregulated cellular proliferation of initiated cells (Figure 12). Here, I briefly describe several experimental approaches to test the validity of this model and the proposed role of the agouti protein in tumor promotion.

Figure 12: Proposed model for intracellular signaling pathways involved in agouti-induced cell proliferation during tumor promotion. The main interactions between three signaling pathways (calcium dependent PKC, Raf/ERK and STAT) are shown in respect to each other. Genes that were found to be differentially expressed in K14-Agouti skin are shown in red. Pathway activation is represented with solid lines. Dashed lines indicate activation of gene transcription. Over-expression of agouti signaling activates calcium dependent PKC, which turns on transcription of *Pdgfa* and *Plaur*. Additionally, agouti signaling might be activating the transcription of *Stat1* and *Stat3* through PKC signaling. Altered levels of PDGFA and PLAUR proteins have the potential to induce cellular proliferation through both STAT and RAS-RAF/ERK signal transduction pathways. The RAF/EEK pathway can also be activated by PKC, and by over-expression of the Ras proto-oncogene.


Agouti
Elevated intracellular calcium
PKC
?
PKC induced gene transcription
Stat1
Stat3
Plaur
Pdgfa
Ras
Raf
MEK
ERK
Cellular Proliferation
Pcna
Tert

In the proposed model, increased transcription of *Pdgfa* and *Plaur* in the skin of K14-Agouti mice are considered to be the earliest events, which will then trigger activation of STAT and Raf/Erk signaling, resulting in increased cellular proliferation as determined by increases in the expression levels of cellular proliferation markers. The sequential expression of these genes can be examined by determining their order of activation after agouti stimulation of keratinocytes in a cell culture system, which is a well-established *in vitro* model system to study mouse skin biology and carcinogenesis [266, 283-286]. Changes in gene expression patterns at different time points following treatment of keratinocytes with recombinant agouti protein can be determined by qRT-PCR analysis. Examining a time-course of gene expression differences in keratinocytes following agouti stimulation would allow us to identify early and late responding genes, and to test the proposed model. The most important finding among the possible outcomes of this set of experiments would be the timing of expression of *Pdgfa* and *Plaur* compared to the other genes. I would expect to observe changes in the expression of *Pdgfa* and *Plaur* immediately following agouti protein stimulation, while up-regulation of the proliferation markers, *Pcna* and *Tert*, should occur at a later time for the proposed model to be valid. Determining the exact timing and order of the regulation of *Stat1*, *Stat3*, *Hras1*, *Pcna* and *Tert* will be helpful for fine-tuning the proposed interactions between the various signaling pathways.

Establishing differential regulation of these genes in keratinocyte cultures after treatment with recombinant agouti protein will provide us with a tractable system for following and testing the effects of agouti protein on calcium, PKC, and Raf/Erk signaling. First, it will be necessary to establish that treatment of keratinocytes with recombinant agouti protein will induce an increase in intracellular calcium levels (as it does in some other cell types), and activation of

PKC and Raf/Erk molecules. Then each of these three intracellular signals can be inhibited individually to determine their contribution to the overall picture, in which the end results can be followed by the measurements of *Pcna* and/or *Tert* expression, and cellular proliferation rates. During each inhibition experiment, analysis of the regulation of gene expression and the changes in the activation levels of the other two pathways will provide us with the necessary information to elucidate the dependence of these events on each other. For example, according to the model, when elevation of intracellular calcium is inhibited, PKC and Erk signaling, and the expression of *Pcna* and *Tert* should not be activated to the same levels as determined previously. Additionally, according to the proposed model, agouti induces transcription of *Pdgfa* and *Plaur* through activation of calcium-dependent PKC signaling. So, if this model is accurate, we would not expect to see any significant changes in the transcription of *Pdgfa* and *Plaur* in response to agouti signal when intracellular calcium and/or PKC signaling is inhibited. On the other hand, inhibition of Erk signaling should not affect the increase in intracellular calcium levels, the activation of PKC, or the transcription of *Pdgfa* or *Plaur* after treatment of keratinocytes with recombinant agouti protein.

Parallel to the experimental approaches described above, different keratinocyte cell lines deficient for *Hras1, Pcna, Pdgfa, Plaur, Stat1, Stat3,* and *Tert* can be generated *in vitro* by RNA mediated interference (RNAi), where long-term post-transcriptional inhibition of targeted mRNA can be achieved by transfection of cells with small interfering RNA duplexes (siRNA) expressed under the control of RNA Polymerase III [287-290]. Observation of changes in the activation of different signaling pathways, and regulation of other genes will provide valuable information about the contribution of each gene in the different

signaling pathways, and identify the key players of the proposed signaling pathway.

Changes in gene expression levels determined by qRT-PCR should be confirmed at the protein level. Additionally, analysis of the phosphorylation status of STAT, RAF and ERK proteins would be important to determine their activity levels. Western blot analysis is the classical approach for determination of single protein expression and phosphorylation levels, which should be the first choice in the analysis of a small set of genes, like in our experiments. However, identification of overall changes in the protein expression levels, and the post-translational modifications to these proteins with high-throughput techniques will also be valuable. While complementing the results of the microarray experiments, comparisons of changes in gene and protein levels will provide additional important information about the regulatory dynamics of the signaling pathways impacted by agouti signaling. Such comparisons may prove useful for mapping the signaling networks associated with agouti-induced tumor promotion [291].

Protein arrays, two-dimensional differential fluorescence gel electrophoresis (DIGE), and mass spectrometry are some of the high-throughput approaches that can be used for protein expression profiling. Protein arrays are produced by fixing antibodies on slides that are highly specific for their target proteins. When these arrays are treated with protein samples from tissues or cell cultures, proteins are bound by the antibodies to the array surface, which then can be detected with systems such as the streptoavidin-biotin complex. Comparison of protein binding levels between the experimental and control samples allows detection of the differentially expressed proteins [292, 293]. In the DIGE system, similar to cDNA microarray analysis, experimental and control

samples are labeled either with Cy3 or Cy5. Following 2D gel electrophoresis, where each protein is separated according to its charge and mass, each sample is individually imaged and measurements are compared in order to determine the expression levels of each protein in the experimental and control samples. An additional advantage of the DIGE system is that it has the capacity for detecting many different post-translational modifications of proteins, including phosphorylation status [294]. DIGE analysis can be followed by mass spectrometry technology for identification of proteins of interests. Additionally different mass spectrometry approaches such as stable isotope labeling and quantification using the absolute peptide ion intensity can be utilized for global protein analysis and identification of differential protein expression profiles [295, 296].

Findings of these experimental approaches should help us to accept, modify, or reject the proposed model. The new information gathered about agouti signaling should be used to adjust the shortcomings of the proposed model or to propose a new model.

After identification of the intracellular signaling pathways activated by agouti protein, the next challenge will be to demonstrate the role of the described signaling pathways in tumor promotion. Several of the reagents generated during elucidation of the signaling pathways impacted by agouti protein can be utilized in this effort. Comparison of *in vitro* cellular proliferation rates of agouti treated keratinocytes in which calcium, PKC, or Raf/Erk signaling is inhibited will be informative about the contribution of these signaling molecules on agouti-induced cell proliferation. Furthermore, determination of changes in the cellular proliferation rates in each RNAi keratinocyte cell line deficient for *Hras1,*

Pcna, Pdgfa, Stat, Stat1, Stat3, or *Tert* will establish the individual roles of these genes.

In vitro colony (focus) formation assays and *in vivo* transplantation of cells into nude mice are widely used experimental approaches for the analysis of tumorigenic capacity. These assays test the ability of cultured cells to form tumors after growth in a semisolid agarose bilayer in a petri dish or in athymic nude mice, respectively. In the colony formation assay, cells in culture started from a single cell are grown in soft agar and the rate of colony formation is measured. Similarly, cells can be injected subcutaneously into immunodeficient, hairless mice and their tumorigenic capacity can be measured by monitoring the formation of papillomas and squamous cell carcinomas. Since agouti is a tumor promoter, the tissues or cell cultures that are over-expressing the agouti signal will not give rise to increased numbers of tumors unless the cells are first initiated. Therefore, to be able to measure the differences in the tumorigenic capacities of the different cell lines in either assay, initiation of the cultured cells will be necessary. In order to overcome these problems and ensure the stability of introduced changes like gene silencing, and to eliminate the agouti treatment of the cell cultures, K14-Agouti mice can be crossed with mice null for several of the selected genes that showed potential to be the key players in agouti signaling in the previous experiments. These new transgenic lines, which are over-expressing agouti in the skin but deficient for one of the agouti-regulated genes, can be used for two-stage carcinogenesis. Any significant decrease in the rate of papilloma formation after initiation of the skin with a single DMBA application in the presence of over-expressed agouti signaling should demonstrate the importance of the knockout gene(s) during tumor promotion.

K14-Agouti transgenic mice represent a model system in which changes associated with the tumor promotion stage of skin carcinogenesis can be examined before the development of tumors. Such a model is useful for elucidating molecular mechanisms associated with the promotion stage of carcinogenesis. Since a large number of genetic changes will have occurred after the development of a skin tumor, it can be difficult to determine which molecular changes in tumors are associated with the promotion stage of tumor development. Elucidating the molecular mechanisms associated with tumor promotion is important, as tumor promotion is a reversible and rate-limiting event in carcinogenesis. The studies described here will provide insights into the mechanisms underlying agouti-induced tumor promotion. Ultimately, our goal is to achieving a better understanding of the promotion stage of carcinogenesis, which may provide new tools to better predict the risk factors associated with carcinogenesis, and to develop mechanistic-based strategies for prevention and treatment of cancer.

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APPENDIX

Table A-1: List of significantly up-regulated EST clones

	GenBank Accession	Score (d)	Fold Difference
1	BG069401	4.65	5.12
2	AW554157	4.23	2.71
3	BG064629	4.00	2.40
4	BG076699	3.69	2.78
5	BG069435	3.65	1.81
6	BG076669	3.60	3.24
7	BG069801	3.58	1.97
8	BG084801	3.56	3.37
9	BG068870	3.55	2.13
10	BG062978	3.54	2.70
11	BG078960	3.54	6.45
12	BG069400	3.48	2.16
13	BG068914	3.39	2.24
14	BG075404	3.31	1.98
15	BG066780	3.29	1.60
16	BG067185	3.20	2.23
17	BG070933	3.19	1.70
18	BG074710	3.19	2.51
19	BG067379	3.15	1.81
20	BG065574	3.15	2.15
21	AU045245	3.14	2.19
22	BG078140	3.10	2.69
23	BG075278	3.09	1.70
24	BG072416	3.06	1.90
25	BG081040	3.05	1.40
26	C78141	3.03	1.45
27	BG063752	3.00	2.34
28	BG072139	2.99	1.50
29	BG074480	2.98	2.30
30	BG065258	2.96	1.82
31	BG065416	2.95	2.36
32	BG075652	2.92	1.93
33	BG068523	2.91	2.00
34	AU040544	2.90	2.00
35	BG087899	2.89	1.76
36	BG073401	2.89	1.78

Table A-1 Continued

	GenBank Accession	Score (d)	Fold Difference
37	BG075147	2.89	2.26
38	AW546368	2.88	2.26
39	BG074375	2.87	2.12
40	AW558391	2.87	2.54
41	BG074155	2.82	2.28
42	BG065550	2.78	1.50
43	BG075009	2.78	1.53
44	BG078271	2.76	2.53
45	BG063602	2.76	1.77
46	BG085480	2.75	1.69
47	BG077083	2.75	1.87
48	BG078778	2.74	1.67
49	BG074261	2.74	2.49
50	BG069140	2.73	1.70
51	BG070161	2.73	1.43
52	BG088153	2.72	1.45
53	BG075704	2.72	1.66
54	BG069963	2.71	2.38
55	AW559010	2.71	7.59
56	BG065751	2.70	1.83
57	BG073392	2.69	2.02
58	BG077751	2.67	1.46
59	BG063024	2.67	2.55
60	AW556849	2.67	2.42
61	BG064824	2.66	1.88
62	BG075661	2.65	1.83
63	BG069325	2.64	2.10

Table A-2: List of significantly down-regulated EST clones

	GenBank Accession	Score (d)	Fold Difference
1	BG080756	-4.94	0.22
2	BG067459	-4.85	0.46
3	BG069326	-4.19	0.21
4	BG070908	-3.88	0.32
5	BG068321	-3.79	0.35
6	BG080961	-3.73	0.39
7	BG070437	-3.71	0.48
8	BG078393	-3.67	0.20
9	BG068281	-3.62	0.36
10	BG079049	-3.60	0.48
11	BG063061	-3.58	0.17
12	BG068227	-3.52	0.26
13	BG067575	-3.44	0.22
14	BG064789	-3.41	0.48
15	BG080372	-3.34	0.54
16	BG063376	-3.23	0.26
17	BG088216	-3.20	0.51
18	BG072510	-3.18	0.48
19	AA408197	-3.16	0.38
20	BG072581	-3.15	0.71
21	BG071958	-3.13	0.50
22	BG080565	-3.10	0.51
23	BG064250	-3.09	0.50
24	BG072525	-3.09	0.57
25	BG066404	-3.08	0.42
26	BG069987	-3.06	0.44
27	BG083259	-3.05	0.10
28	C85855	-3.02	0.36
29	BG080525	-2.98	0.50
30	BG070488	-2.96	0.62
31	BG068092	-2.96	0.45
32	BG065639	-2.95	0.44
33	BG082362	-2.95	0.43
34	BG072458	-2.94	0.31
35	BG072229	-2.94	0.47

Table A-2 Continued

	GenBank Accession	Score (d)	Fold Difference
36	BG076569	-2.92	0.28
37	BG080868	-2.91	0.33
38	BG082009	-2.91	0.38
39	BG067579	-2.90	0.28
40	BG066639	-2.89	0.22
41	AW558635	-2.89	0.40
42	BG067060	-2.87	0.61
43	BG069223	-2.86	0.27
44	BG072807	-2.86	0.42
45	BG067577	-2.86	0.63
46	BG065984	-2.83	0.49
47	BG067544	-2.82	0.61
48	BG086567	-2.82	0.50
49	BG063733	-2.81	0.32
50	BG084279	-2.81	0.48
51	BG066515	-2.80	0.39
52	BG088837	-2.80	0.48
53	BG070073	-2.80	0.42
54	BG070961	-2.79	0.24
55	BG066695	-2.79	0.42
56	BG073477	-2.79	0.13
57	BG066036:	-2.78	0.47
58	C87415	-2.78	0.56
59	AU018797	-2.76	0.21
60	AU043030	-2.76	0.41
61	BG068421	-2.75	0.33
62	C80656	-2.75	0.56
63	BG078783	-2.75	0.50
64	BG082144	-2.73	0.67
65	BG078394	-2.71	0.43
66	BG066960	-2.70	0.61
67	BG063430	-2.69	0.38
68	BG064752	-2.69	0.50
69	BG063715	-2.68	0.53
70	BG082354	-2.68	0.43
71	BG076744	-2.67	0.37

Table A-2 Continued

	GenBank Accession	Score (d)	Fold Difference
72	BG067204	-2.66	0.42
73	BG065962	-2.65	0.50
74	BG083351	-2.65	0.25
75	AW545830	-2.64	0.62
76	BG067124	-2.64	0.26
77	BG076025	-2.64	0.59
78	BG069716	-2.62	0.60
79	BI076595	-2.61	0.54
80	BG066161	-2.60	0.48
81	BG068687	-2.60	0.49
82	BG066468	-2.59	0.33
83	BG068412	-2.59	0.58
84	BG084956	-2.56	0.49
85	BG065203	-2.55	0.45
86	BG086529	-2.55	0.64
87	BG065689	-2.53	0.62
88	BG069589	-2.53	0.40
89	BG078672	-2.51	0.44
90	BG088006	-2.51	0.59
91	AW556334	-2.50	0.15
92	BG067320	-2.50	0.57
93	BG068547	-2.50	0.29
94	BG081646	-2.50	0.53
95	AW555555	-2.49	0.44
96	BG068764	-2.49	0.40
97	BG076373	-2.49	0.48
98	AU018221	-2.48	0.54
99	AU022767	-2.48	0.20
100	BG075725	-2.48	0.57
101	AU022997	-2.47	0.38
102	BG066568	-2.47	0.38
103	BG067890	-2.47	0.49
104	BG068433	-2.46	0.52
105	BG069583	-2.46	0.62
106	BG073971	-2.46	0.35
107	BG080589	-2.46	0.38

Table A-2 Continued

	GenBank Accession	Score (d)	Fold Difference
108	AA408305	-2.45	0.36
109	AW547303	-2.45	0.40
110	BG067557	-2.45	0.62
111	BG072014	-2.45	0.67
112	BG083842	-2.45	0.60
113	BG063053	-2.44	0.33
114	BG065759	-2.44	0.69
115	BG084201	-2.44	0.43
116	BG068506	-2.43	0.73
117	BG073713	-2.42	0.59
118	BG083368	-2.42	0.62

VITA

Yesim AYDIN SON, M.D., Ph.D.

Yesim AYDIN SON began her medical education at the University of Hacettepe Medical Faculty, Ankara TURKEY in September 1993 after graduating from T.E.D. Ankara College as a science major. She completed her medical studies in June 1998 and continued her medical education during her internship between June 1998 and 1999. She earned her medical degree (M.D.) in June 1999. Upon completion of her medical education, she moved to Knoxville, Tennessee, USA with her husband, where they both started their doctoral studies. She was accepted to the Ph.D. program in the Graduate School of Genome Science and Technology at University of Tennessee in August 2000 and worked with Dr. Edward J. Michaud on identification of changes in the expression of skin oncogenes during the promotion stage of tumorigenesis and the hair-follicle cycle in K14-Agouti transgenic mice. Throughout the course of this program, she served as a Graduate Teaching Assistant (2000-2002) and a Graduate Research Assistant (2002-2006). She completed the requirements for the Doctor of Philosophy (Ph.D.) degree in Life Sciences in May 2006. She is planning to continue her career as a medical scientist in Pasadena, California, where she is currently looking for a post-doctoral position.