

## REVIEW

## Biological, cellular, and molecular characteristics of an inducible transgenic skin tumor model: a review

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The genetically initiated Tg.AC transgenic mouse carries a transgene consisting of an oncogenic *v-Ha-ras* coding region flanked 5' by a mouse  $\zeta$ -globin promoter and 3' by an SV-40 polyadenylation sequence. Located on chromosome 11, the transgene is transcriptionally silent until activated by chemical carcinogens, UV light, or full-thickness wounding. Expression of the transgene is an early event that drives cellular proliferation resulting in clonal expansion and tumor formation, the unique characteristics now associated with the Tg.AC mouse. This *ras*-dependent phenotype has resulted in the widespread interest and use of the Tg.AC mouse in experimental skin carcinogenesis and as an alternative carcinogenesis assay. This review examines the general biology of the tumorigenic responses observed in Tg.AC mice, the genetic interactions of the *ras* transgene, and explores the cellular and molecular regulation of  $\zeta$ -globin promoted transgene expression. As a prototype alternative model to the current long-term rodent bioassays, the Tg.AC has generated a healthy discussion on the future of transgenic bioassays, and opened the doors for subsequent models for toxicity testing. The further exploration and elucidation of the molecular controls of transgene expression will enhance the usefulness of this mouse and enable a better understanding of the Tg.AC's discriminate response to chemical carcinogens.

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### What is the Tg.AC transgenic mouse?

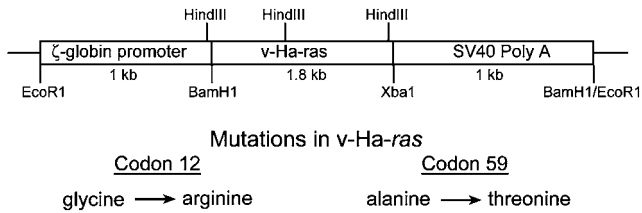
The Tg.AC transgenic mouse was developed in the Leder laboratory (Leder *et al.*, 1990) to examine gene regulation in embryonic development. The line was one of several created on an FVB/N strain mouse background by pronuclear injection of a transgene construct containing a *v-Ha-ras* gene flanked 5' by a mouse

$\zeta$ -globin promoter and 3' by an SV-40 polyadenylation sequence (Figure 1). The Tg.AC mouse was created with two goals in mind: to define the putative promoter region of the mouse  $\zeta$ -globin gene, and to create an embryonic erythroid cell line from the  $\zeta$ -globin/*v-Ha-ras*-induced tumors (Leder *et al.*, 1990). Four transgenic lines were created carrying 3–10 copies of the transgene (Leder *et al.*, 1990). Three of the lines showed no transgene expression or apparent phenotype. A fourth, Tg.AC, expressed the transgene in day 12 embryonic blood, the hematopoietic fetal liver, and the placenta. In addition, low levels of transgene expression were detectable in the adult bone marrow (Leder *et al.*, 1990). It was noted that the Tg.AC mice developed dorsal squamous cell papillomas at the site of bite wounds inflicted by cage mates. Transgene expression in normal Tg.AC skin was not detected (Leder *et al.*, 1990).

Wound repair and chronic irritation are known tumor promoters (Pullinger, 1945; Hennings and Boutwell, 1970; Argyris, 1985). The observed promotional response to wound stimuli in Tg.AC was further explored through the use of the known chemical tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). When treated topically with TPA, Tg.AC mice developed multiple papillomas, some of which progressed to malignancies (Leder *et al.*, 1990; Spalding *et al.*, 1993). Tg.AC mice also develop papillomas in response to full-thickness wounding (Leder *et al.*, 1990; Hansen and Tennant, 1994a; Cannon *et al.*, 1997). UVA/B radiation (Trempus *et al.*, 1998a; Battalora *et al.*, 2001) and to a lesser extent, UVA alone (Chignell *et al.*, 2003b) can generate a tumorigenic response in Tg.AC, an effect that can be diminished by pretreatment with an inhibitor of the epidermal growth factor receptor (EGFR) (El-Abersari *et al.*, 2005). Other wavelengths of radiation have been found to be tumor suppressive (Ohara *et al.*, 2003). As the parent FVB/N strain does not develop tumors in response to these promotional stimuli in the absence of initiation, the tumorigenic response in Tg.AC mice must be due to the presence of the *v-Ha-ras* transgene.

Experimental skin carcinogenesis is typically a multi-stage process consisting of initiation, promotion, and progression. Initiation is thought to induce mutations in

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**Figure 1** Transgene construct in v-Ha-ras Tg.AC transgenic mice. The Tg.AC transgene consists of a 1 kb region corresponding to the mouse  $\zeta$ -globin promoter, a 1.8 kb v-Ha-ras sequence, and a 1 kb SV-40 polyadenylation signal sequence. Restriction sites found at the junctions between each component and at the ends of the construct are indicated. The *ras* gene contains two activating mutations found at amino acids 12 and 59

critical genes which confer a selective growth advantage to specific cells, which, during the proliferative process of tumor promotion, can acquire additional genetic or epigenetic changes resulting in tumor formation and a potential progression to malignancy (Deelman, 1927; Barry *et al.*, 1935; Berenblum, 1941; Boutwell, 1964). Chemically induced mutations in the *c-Ha-ras* gene have been detected following initiation with the mutagen 7,12-dimethylbenz[a]anthracene (DMBA) in mouse skin carcinogenesis studies (Balmain and Pragnell, 1983). Because the tumorigenic response observed in Tg.AC mice occurred independent of an initiation step, and is due to the presence of the v-Ha-ras transgene, these mice have been characterized as a 'genetically initiated' model for mouse skin tumorigenesis. Tg.AC mice can develop papillomas within 20 weeks of topical applications of either mutagenic or nonmutagenic carcinogens (Leder *et al.*, 1990; Spalding *et al.*, 1993). Hemizygous and homozygous Tg.AC mice have been found to have similar tumorigenic responses (Hansen *et al.*, 1998; Spalding *et al.*, 1999).

In addition to the wound or chemically induced skin tumors observed on Tg.AC mice, a number of other tumor types have been reported. Spontaneous tumors develop at the sites of chronic abrasion, such as the mouth, nares, eyelids, and genitalia (Leder *et al.*, 1990). Approximately 36% of homozygous Tg.AC males and females will develop odontogenic tumors within their first year of life, a phenomenon uncommon in mice (Wright *et al.*, 1995), and likely due to local injuries caused by continuous gnawing. One type of odontogenic tumor appears to arise from cells in the periodontal ligament, which is located in the mid-root region of the incisor (Wright *et al.*, 1995; Mahler *et al.*, 1998). In a more recent investigation, it was found that genes displaying differential expression in the ameloblastoma-like tumors of Tg.AC mice were also the same genes thought to be important in the odontogenesis and odontogenic tumor formation in human ameloblastomas (Dodds *et al.*, 2003). Other reported spontaneous tumors include squamous cell carcinomas (SCC) of the salivary gland, ovarian teratomas, a rare ovarian yolk sac carcinoma, squamous cell papillomas of the forestomach and lung alveolar/bronchiolar adenomas

(Hansen *et al.*, 1996; Mahler *et al.*, 1998). The incidence of erythroleukemia in control hemizygous Tg.AC mice is 1–4% (Mahler *et al.*, 1998). The erythroleukemia have an associated hepatosplenomegaly due to leukemic infiltration. No clear evidence of erythroleukemia has been associated with any particular chemical treatment (Trempeus *et al.*, 1998b), and erythroleukemias have not been observed in the parental FVB/N line. Tumors also form in the forestomach (Cannon *et al.*, 2000).

Tg.AC transgene expression is detected in day 12 embryonic blood, the hematopoietic fetal liver, placenta, kidney, and at low levels in adult bone marrow (Leder *et al.*, 1990; Hansen *et al.*, 1996; Delker *et al.*, 1999). Transgene expression was not detected in normal Tg.AC skin, but was detected in skin papillomas, carcinomas, malignancies, and all spontaneous tumors examined (Leder *et al.*, 1990; Hansen and Tennant, 1994a). RT-PCR has failed to detect transgene expression in the liver (Hansen *et al.*, 1996; Delker *et al.*, 1999), although some adult Tg.AC spleens will test positive for transgene expression by RT-PCR or by an RNase protection assay (Cardiff *et al.*, 1993; Hansen *et al.*, 1996). It should be noted that not all Tg.AC mouse tissues respond with a tumorigenic outcome following wounding. Despite constitutive transgene expression in the kidneys, chloroform-induced kidney damage was not sufficient to generate a renal tumorigenic response (Delker *et al.*, 1999). FVB/N mice are not susceptible to spontaneous kidney tumors (Mahler *et al.*, 1996) nor were spontaneous kidney tumors seen in 33–34-week-old control hemizygous Tg.AC mice (Mahler *et al.*, 1998).

Skin tumorigenesis in Tg.AC mice is strongly age dependent (Battalora *et al.*, 2001). Limited (two to four) doses of TPA on mice 5 or 10 weeks of age develop a much lower tumor incidence and multiplicity when compared to mice 21 and 32 weeks of age treated in a similar manner. The age-dependent increase in response occurs following TPA exposure, wounding, or UV irradiation (Battalora *et al.*, 2001). Young mice receiving a second treatment later in life will respond in a manner to similarly dosed older mice. It is interesting to note that FVB/N mice do not demonstrate an age-dependent response when treated with DMBA/TPA (Battalora *et al.*, 2001).

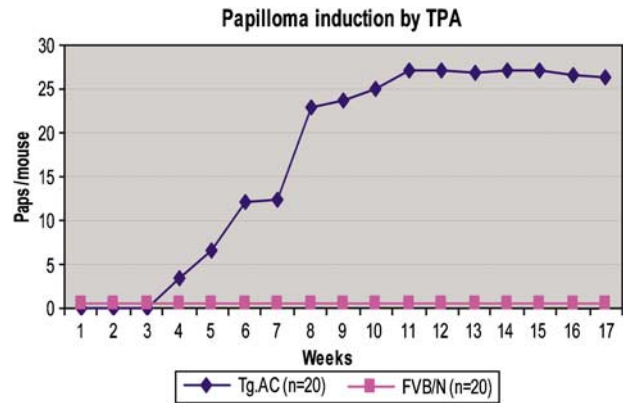
Tg.AC mice can develop cutaneous malignancies, the sites of which have always been associated with spontaneous or induced papillomas (Hansen *et al.*, 1996; Asano *et al.*, 1998). Malignancies are primarily either squamous cell carcinomas or spindle cell carcinomas (Hansen *et al.*, 1996), although malignant fibrous histiocytoma-like (MFH-like) spindle cell tumors have been reported (Cardiff *et al.*, 1993; Asano *et al.*, 1998). Based on subsequent immunohistochemical staining for keratins, as well as the presence of desmosomes, the spindle cell carcinomas have been shown to be of epithelial origin (Asano *et al.*, 1998). Transgene expression, while usually found at the basal region of papillomas, may be found throughout spindle or squamous cell carcinomas (Hansen *et al.*, 1996).

## Tg.AC and chemical carcinogenesis

The Tg.AC mouse, along with additional transgenic mice, has been proposed as an alternative or adjunct to the rodent 2-year bioassay (Tennant *et al.*, 1995; Tennant, 1997), a standardized scientific procedure developed by the National Toxicology Program (NTP) for identifying toxic and carcinogenic compounds that are hazardous to human health. The International Life Sciences Institute (ILSI) sponsored a collaborative evaluation program to examine alternative models for carcinogenicity testing (Cohen *et al.*, 2001; Robinson and MacDonald, 2001; Tennant *et al.*, 2001). With the ILSI initiative and individual lab testing, a wide variety of chemicals have been tested on Tg.AC mice in an effort to validate the model (Tennant *et al.*, 1996; Spalding *et al.*, 1999, 2000). An extensive analysis of those chemical tests has recently been performed (reviewed in Pritchard *et al.*, 2003; Sistare *et al.*, 2002) and a compilation of tests and references can be found on the Tg.AC website at [http://dir.niehs.nih.gov/dir-lecm/TgAC/TgAC\\_Home.htm](http://dir.niehs.nih.gov/dir-lecm/TgAC/TgAC_Home.htm). The data from these studies show that a variety of chemical carcinogens can generate a tumorigenic response in Tg.AC mice, including both genotoxic and nongenotoxic carcinogens. The Tg.AC data, and the ILSI initiative have demonstrated transgenic assays to be valuable adjuncts to the 2-year bioassay. There are concerns when the model is being utilized for human risk assessment of dermal formulations applied to human skin. Positive responses are likely to occur, if these formulations contain components, or their expected metabolites, that have been previously identified as promoters in the two-stage mouse skin carcinogenesis model. As noted, '... treatments that are tumor promoting in a two-stage skin carcinogenesis model would be expected to induce papillomas in this system, if the mutated *ras* gene products can be expressed and cooperates with events that follow the promotional stimulus (Sistare *et al.*, 2002)'.

The induction of papillomas on topically treated Tg.AC skin serves as a reporter phenotype that defines the carcinogenic activity of a chemical compound (Figure 2). TPA, the most well-defined promoter in two-stage carcinogenesis protocols, is the most common promoter utilized to generate an experimental tumorigenic response, and its use constitutes most of the positive control group dosing in bioassays and experimental protocols. However, the stratified epithelium of the forestomach is also a potential target. In accordance with the NTP bioassay, dimethyl vinyl chloride (DMVC) was able to significantly increase the incidence of forestomach papillomas in Tg.AC mice in both a time and dose-related manner (Cannon *et al.*, 2000). All forestomach tumors examined expressed the transgene, while transgene expression in normal, nontumorigenic forestomach was not detected (Cannon *et al.*, 2000).

The truncation of the tumorigenic timeline and the discriminate response to carcinogens highlights the use of Tg.AC mice as a short-term bioassay for identifying potential chemical carcinogens. The relevance to hu-



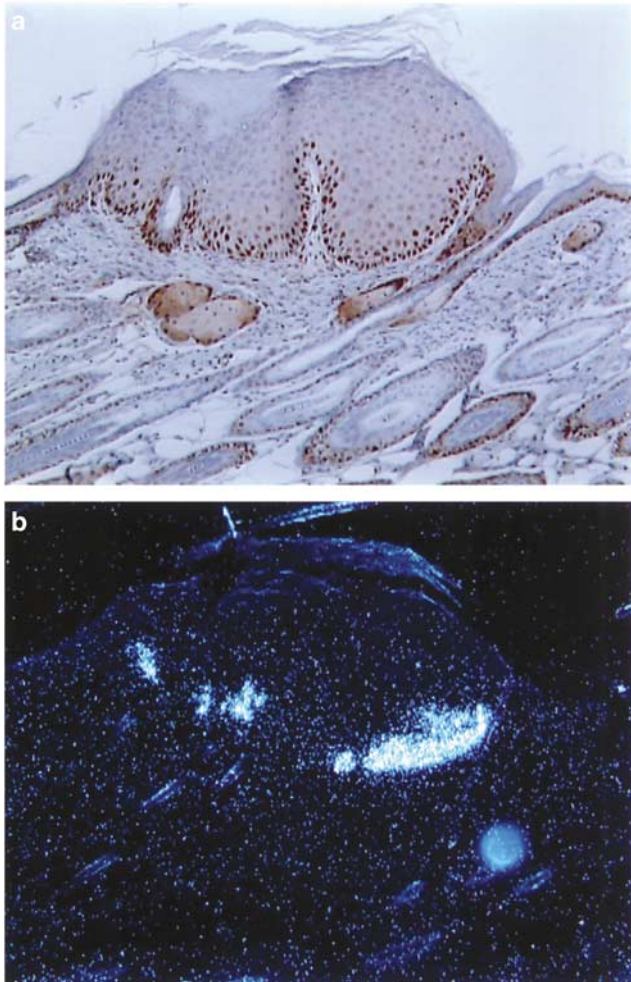
**Figure 2** Papilloma induction by TPA. TPA (2.50  $\mu$ g) was applied to the shaved dorsal surface, three times a week for up to 20 weeks or until a maximum tumor response of 30 papillomas is reached. Animals having 30 papillomas before the end of treatment were euthanized

mans can be correlated to the finding that 30% of human tumors contain activating point mutations in the *Ha-ras* gene (Bos, 1987). While skin carcinogenesis in the mouse is not orthologous to that in humans, this correlation of *ras*-dependent pathways in the tumorigenic process strengthens the utility of the Tg.AC bioassay. Understanding the cellular targets and the molecular mechanisms by which the  $\zeta$ -globin promoter/*v-Ha-ras* transgene confers the tumorigenic phenotype in Tg.AC mice is paramount in its continued use as a model for carcinogenesis and as an adjunct to the 2-year bioassay.

## Cellular aspects

A major focus of current research has been to identify the specific cells at risk for transgene-dependent proliferation. Untreated Tg.AC skin is grossly and histologically indistinguishable from that of FVB/N skin (Hansen and Tennant, 1994b). Also indistinguishable between the two strains is the hyperplasia induced by TPA treatment, as well as the early activation and subsequent downregulation of protein kinase C (PKC) (Hansen and Tennant, 1994a). However, TPA-promoted Tg.AC mice develop focal hyperplasias that eventually develop into squamous papillomas, while uninitiated FVB/N fail to do so. All tumors tested to date, whether spontaneous or induced, express the *ras* transgene, as determined by RT-PCR, Western blots, Northern analysis, and *in situ* hybridization, with the highest level of transgene expression associated with the proliferating cells of the basal epidermis (Figure 3) (Hansen and Tennant, 1994a; Wright *et al.*, 1995; Hansen *et al.*, 1996; Cannon *et al.*, 1997, 2000).

Transgene expression is not detectable in normal whole skin, but can be detected in focal hyperplasias and papilloma precursors that develop following a promotional stimulus. This observation can be accounted for in one of two ways. First, the *ras* transgene is not



**Figure 3** Expression of *ras* transgene message and PCNA. Tissue from treated Tg.AC skin was removed, fixed in 10% neutral-buffered formalin and assayed for proliferating cell nuclear antigen expression by immunohistochemistry (panel a) and *ras* transgene message by *in situ* hybridization (with a transgene-specific riboprobe) (panel b). Note that transgene expression is localized to the basal compartment of the lesion, coincident with proliferating cells

expressed in normal, untreated skin, but is induced in a small population of susceptible cells, which then clonally expand into *ras* transgene-expressing papillomas. Second, it is possible that transgene expression is constitutively present in a small population in the epidermis, but due to the small numbers, transgene expression is below the lower limit of detection using current methodologies. In the absence of additional signaling, neoplastic proliferation does not occur. In most cases, using whole skin, the earliest transgene expression detected is 18 days following the first of four TPA treatments (Hansen and Tennant, 1994a) or 16 days after a full-thickness wound (Cannon *et al.*, 1997), concomitant with global hypomethylation (Cannon *et al.*, 1998). However, using keratinocytes harvested from TPA-treated skin, it has been shown that the transgene can be detected by RT-PCR 14 days after treatment with TPA in older, more responsive mice, and as early as 9 days after TPA

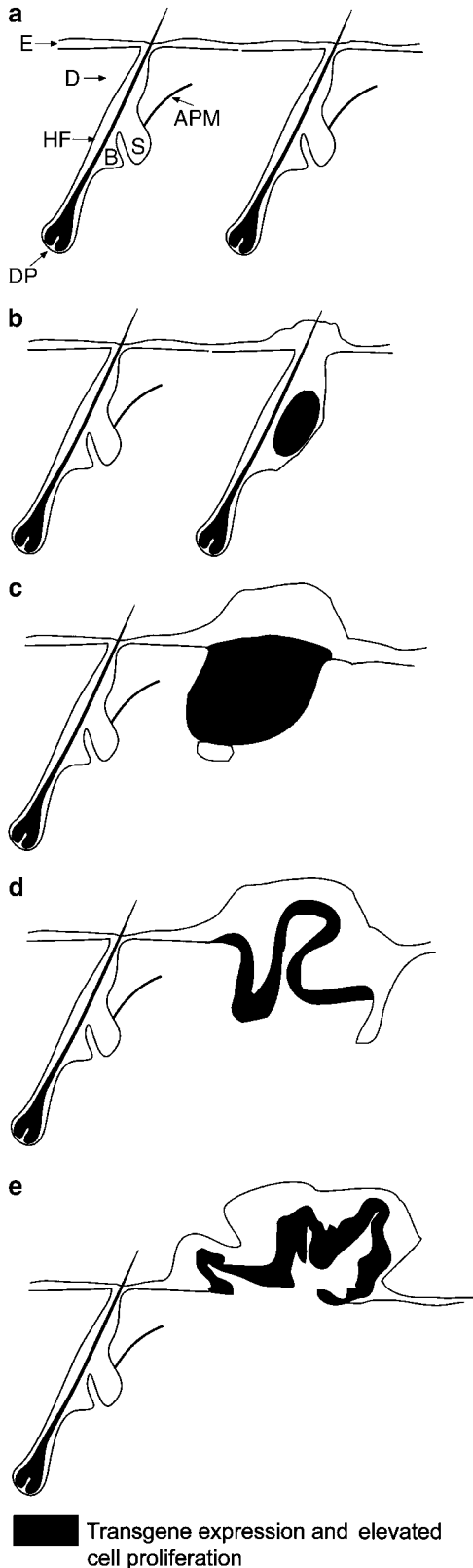
treatment using fractionated keratinocytes (Battalora *et al.*, 2001). These results provide clues as to the temporal kinetics of transgene expression, but – given detection limits – it is likely that induction of the *ras* transgene occurs earlier in the neoplastic process than we have measured, and probably in specific cells ‘at risk’ in the epidermis and/or hair follicle.

The two-stage murine epidermal carcinogenesis model provides insights into the cellular origin of tumors. Initiated cells persist in the skin presumably for the lifetime of the animal despite renewal of the epidermis every 6 days (Potten, 1983). In order for latent neoplastic cells to persist in the skin, they must be slowly cycling to avoid removal to the suprabasal layers and loss by terminal differentiation, and they must reside within a protected niche or microenvironment within the skin (Lavker and Sun, 2000). Therefore, carcinogen target cells possess some qualities of a stem cell population (Morris, 2000). The most likely repository of keratinocyte stem cells (KSC) in the skin is the bulge region, which is located in the permanent portion of the hair follicle below the sebaceous gland at the point of attachment of the arrector pili muscle. Bulge cells are biochemically distinct in that they express keratin 15 (Lyle *et al.*, 1998), and they are slowly cycling (label-retaining cells) (Cotsarelis *et al.*, 1990). The bulge region itself is protected from cyclical changes in the hair follicle as well as from environmental damage (Cotsarelis *et al.*, 1990).

The first evidence for a follicular origin of papillomas in Tg.AC mice was the observation of transgene expression in early focal hyperplasias, in a region consistent with the bulge (Hansen and Tennant, 1994b; Hansen *et al.*, 1995). Transgene expression was also localized to the 20% highest expressing  $\beta$ -1 integrin population in keratinocytes harvested from TPA-treated, nontumor-bearing skin (Battalora *et al.*, 2001). It has been demonstrated that the 20% ‘ $\beta$ -1 bright’ keratinocytes possess a subset of cells that are slowly cycling and clonogenic in culture, thereby providing a selectable marker for KSCs (Jones and Watt, 1993). Data localizing expression to this population was evidence that the earliest transgene-expressing cells are in the pool of cells containing stem and progenitor cell characteristics (Figure 4).

Induction of the *ras* transgene in the skin via the hematopoietic  $\zeta$ -globin promoter was unexpected. In order to provide a biological rationale for the transcriptional activation of a blood-borne promoter in the skin, the hematopoietic system was examined for insights into the follicular microenvironment. As described below, members of the GATA family of transcription factors that are typically associated with the hematopoietic system have been found to coexpress with the transgene (Trempey *et al.*, 1998b; Delker *et al.*, 1999; Humble *et al.*, submitted). It has also been reported that a reduction in GM-CSF reduces tumor multiplicity in Tg.AC mice (Germolec *et al.*, 1997). The expression of CD34, a 105–120 kDa glycoprotein expressed in hematopoietic stem and progenitor cells, was examined in the skin and intense membrane staining was found in cells in

the follicular bulge region that colocalized with both slowly cycling (label retaining) and keratin-15-expressing cells (Trempeus *et al.*, 2003). CD34 expression has



been used in combination with  $\alpha$ -6 integrin, which is expressed on basal keratinocytes, and fluorescent-activated cell sorting (FACS) to isolate living bulge keratinocytes that are both slowly cycling and form large colonies in culture (Trempeus *et al.*, 2003) and unpublished observations). Preliminary evidence suggests that CD34<sup>+</sup> keratinocytes express the *ras* transgene following TPA treatment, providing additional support for the hypothesis that KSCs are the carcinogen target cell population (Carol Trempeus, unpublished observations).

The identification of TPA-inducible genes that contribute to tumor development is important with regard to understanding the biology of the neoplastic process. The identification of CD34 as a selectable determinant of follicular bulge cells allowed us to identify several important candidate genes that may contribute to tumor development. CD34<sup>+</sup> keratinocytes were isolated from either TPA-treated or untreated Tg.AC skin, and a gene expression profile was developed using cDNA nylon arrays (Wei *et al.*, 2003). A total of 11 genes were identified as either upregulated (nine genes, including galectin 7, nucleoside diphosphate kinase B, keratin 14, Dss1, and a double-strand break repair RAD21 homolog) or downregulated (two genes, including keratin 15 and apolipoprotein E precursor) in CD34<sup>+</sup> cells from TPA-treated mice. Further characterization of Dss1 has revealed that (1) Dss1 is overexpressed in TPA-induced hyperplastic skin, papillomas, and cutaneous malignancies; (2) constitutive expression of Dss1 promoted cell proliferation and soft agar growth in preneoplastic epidermal cell lines, demonstrating *in vitro* transformation potential and defining a possible role for Dss1 in early TPA-induced neoplastic development (Wei *et al.*, 2003).

#### Molecular aspects

In addition to identifying the cell(s) responsible for transgene-dependent tumorigenesis, it is also important to gain an understanding of the molecular controls regulating transgene expression and tumor formation. Only one of the four founder lines in the creation of the  $\zeta$ -globin/*v-Ha-ras* transgenic mouse exhibited the unique skin tumorigenic response observed in the Tg.AC (Leder *et al.*, 1990), leading to a hypothesis that the transgene-driven tumorigenic response is the result of a position effect. To explore the possibility that transgene

**Figure 4** Follicular origin of papillomas in Tg.AC mice (adapted from (Hansen *et al.*, 1995)). (a) Hair follicles of normal skin (E: epidermis; D: dermis; HF: hair follicle; DP: dermal papilla; S: sebaceous gland; APM: arrector pili muscle; B: bulge region). (b) Following a promotional stimulus, a small population of transgene-expressing cells in the bulge region of a follicle begin to proliferate. (c) A focal hyperplasia within the hair follicle is established. (d) The focal hyperplasia begins to push above the surface of the skin. (e) Formation of a pedunculated papilloma of follicular origin

expression is initiated not by the transgene's  $\zeta$ -globin promoter, but rather by the promoter of a nearby gene or possibly the promoter of a gene in which the transgene construct is integrated, RNA was isolated from Tg.AC erythroleukemic liver and spleen (leukemic infiltrates) as well as from Tg.AC carcinoma cell lines and examined for the start of transgene transcription by an S1 protection assay (Trempeus *et al.*, 1998b). Results indicated that the start of transcription initiated from the natural start site within the transgene mouse  $\zeta$ -globin promoter, and not from an adjacent gene in the flanking chromosome.

#### *The role of the $\zeta$ -globin promoter*

The regulatory portion of the Tg.AC  $\zeta$ -globin proximal promoter has three regions with adjacent GATA and Sp1-binding sites, as well as an AP1-binding site, all of which are occupied by transcription factors according to DNase I footprinting assays (Humble *et al.*, submitted). Functional assays utilizing deleted  $\zeta$ -globin promoter/secreted alkaline phosphatase (SEAP) reporter vectors demonstrated the importance of the most proximal and distal GATA–Sp1 sites for transgene promoter function, while the central GATA–Sp1 contributed little to promoter function (Humble *et al.*, submitted).

The specific transcription factors interacting with the Sp1, GATA, and AP1 sequences within the regulatory region of the transgene  $\zeta$ -globin promoter may account for the tissue-specific gene expression observed in the Tg.AC mouse. Electrophoretic mobility shift assays have identified Sp1 binding to the three Sp1 sequences in the regulatory promoter, as well as a cooperativity between Sp1 and a GATA-binding factor (Humble *et al.*, submitted). Expression of the GATA family of transcription factors is typically associated with hematopoietic tissues and cells, but has been observed in additional nonhematopoietic tissues such as kidney, developing jaw, brain (Orkin, 1992), and hair follicle (Kaufman *et al.*, 2003). In Tg.AC mice, GATA-1 has been reported to coexpress with the transgene in erythroleukemias (Trempeus *et al.*, 1998b). GATA-3 expression has been found in the kidney of Tg.AC mice, a site where transgene expression has been shown to be constitutively expressed without tumor development (Delker *et al.*, 1999). GATA-3 expression has also been identified in Tg.AC papillomas and carcinomas by RT-PCR (Humble *et al.*, submitted). The tissue-specific expression of GATA-3 and the observed cooperative binding between Sp1 and a GATA-binding factor at sites within the transgene  $\zeta$ -globin promoter may help to account for the spatial and temporal expression of the Tg.AC transgene.

#### *The palindrome*

An important feature within the transgene array is a palindromic repeat formed from two transgene copies joined in a head-to-head inverted orientation ( $\leftarrow \rightarrow$ ) (Thompson *et al.*, 1998). A variety of 100 bp and larger symmetric or asymmetric deletions across the *EcoRI*

center of symmetry within the palindromic  $\zeta$ -globin promoters eliminates the tumorigenic response in hemizygous Tg.AC mice resulting in a nonresponding (NR) phenotype (Thompson *et al.*, 1998). In homozygous Tg.AC mice, the presence of an intact transgene array on one allele and a deleted palindrome on the other allele creates a heterozygous nonresponder, with the deleted palindrome being recessive to the dominant intact palindrome. The effect of the NR allele in these mice is masked, as the remaining intact transgene palindrome enables transgene expression and a tumorigenic phenotype.

The NR phenotype was originally reported following poor TPA responses in positive control Tg.AC hemizygous mice (Weaver *et al.*, 1998), and may have been an unknown confounding factor in other studies (Blanchard *et al.*, 1998). Subsequent examination of the entire Tg.AC line demonstrated a germline transmission of the deleted palindrome. Careful monitoring of the breeding lines by DNA blotting for the presence of the NR allele (Kantz *et al.*, 1999) has reduced the appearance of NR hemizygous Tg.AC mice to less than 2% per generation.

Palindromic sequences are known to be unstable and prone to deletion (Leach, 1994; Collick *et al.*, 1996; Lewis *et al.*, 1999). It has been reported that a Tg.AC carcinoma cell line continued to express the transgene despite loss of the promoter palindromes (Humble *et al.*, 2000). To examine palindrome stability over time and its role in sustained transgene expression, DNA was isolated from normal and TPA-treated hemizygous Tg.AC skin, papillomas, carcinomas, and malignancies, and examined for the presence of intact or deleted palindromes. The results demonstrated a quantifiable but incomplete loss of the palindrome in carcinomas and malignancies (Moon and Cannon, 2001; Thompson *et al.*, 2001).

An intact  $\zeta$ -globin promoter palindrome is required for the Tg.AC discriminate response to promotional stimuli, yet the function of the promoter palindrome is not known. It has been suggested that a stem-loop or hairpin structure may form as a result of the palindromic sequences, and that this structure in some manner enables transgene expression (Thompson *et al.*, 1998). It is also possible that the palindromic transgene promoters act much the same as a traditional locus control region (LCR) by initiating an open chromatin domain for gene expression. LCRs may be many kilobases away from a gene, and may function by altering the topography of the chromatin domain, or by directly interacting with genes by a looping model (reviewed in Fraser and Grosveld, 1998; Grosveld, 1999). Deletion across the central palindrome may remove important transcription factor-binding sites as well as remove the palindrome's ability to initiate chromatin remodeling, rendering the transgene quiescent and noninducible and creating the nonresponder phenotype. Sequence analysis of the 5' end of the  $\zeta$ -globin promoter indicates transcription factor-binding sites, particularly GATA and AP1 sites, within the first 100 bp (Humble *et al.*, submitted).

Flanking chromosomal sequences, such as LCRs, regulate the expression of a number of genes, including the  $\alpha$ - and  $\beta$ -globin genes (Tuan and London, 1984; Forrester *et al.*, 1987; Tuan *et al.*, 1989; Higgs *et al.*, 1990; Jarman *et al.*, 1991). The 5' regulatory sequences, such as the  $\alpha$ -globin LCR, were not included with the  $\zeta$ -globin promoter in the Tg.AC transgene, which may account for transgene expression in nonhematopoietic tissues (Leder *et al.*, 1990; Delker *et al.*, 1999). To explore the possibility that *cis*-flanking regions near the integration site on chromosome 11 were involved in transgene expression, radial transformation-associated recombination (TAR) cloning (Kouprina *et al.*, 1998) in yeast was utilized to create orientation-specific Yeast Artificial Chromosomes (YACs)/Bacterial Artificial Chromosomes (BACs) containing variable lengths of 5' or 3' flanking chromosome 11 DNA and the Tg.AC transgene (Humble *et al.*, 2000). BACs were assayed for their ability to promote transcription of the transgene following stable transfection into an FVB/N carcinoma cell line. A transgene-specific RT-PCR assay demonstrated that all Tg.AC BACs expressed the transgene in this *in vitro* system regardless of their flanking DNA content (Humble *et al.*, 2000). This suggests that transcriptional activity may not require *cis* elements flanking the transgene's integration site. It was noted, however, that the transgene promoter palindromes were deleted in the YAC/BACs at some point in the cloning or propagation process, likely due to the instability of palindromic sequences in *Escherichia coli* (Humble *et al.*, 2000). Although necessary for *in vivo* induction of the transgene, the absence of intact promoter palindromes did not hinder transgene expression *in vitro*. It was postulated that conditions within the FVB/N carcinoma cell line may circumvent the need for the intact palindromes as these cells would have already achieved a carcinogenic potential and thus may not mimic the cellular environment or conditions of the nontransformed/promoted cell *in vivo*.

In addition to the presence of specific transcription factors, flanking control sequences, and chromatin packing, the control of gene expression is also affected by a gene's methylation status. Embryonic globin genes are hypomethylated while expressed, becoming methylated over time and silenced, while adult globin genes become hypomethylated as their expression begins ((Karlsson and Nienhuis, 1985; Yisraeli *et al.*, 1988) and references therein). A variety of stimuli have been shown to affect gene expression by altering CpG methylation (reviewed in Cannon *et al.*, 1998). The methylation status of the Tg.AC transgene was examined over the time course of wound-induced papillomagenesis (Cannon *et al.*, 1997). A site-specific hypomethylation of the transgene is detectable by DNA blots 23 days after wounding (Cannon *et al.*, 1998). This timing was in close correlation with additional time course parameters, primarily the RT-PCR detection of transgene mRNA 16 days post wounding, and blot detection of p21 v-Ha-*ras* protein 21 days post wounding (Cannon *et al.*, 1998). Thus, the Tg.AC transgene exists in a hypomethylated state in expressing cells.

### Initial induction of transgene expression

TPA is a multicyclic, phorbol ester containing a diacylglycerol-like moiety. Diacylglycerols (DAGs) are intracellular second messengers generated by the receptor-mediated hydrolysis of membrane phospholipids. *sn*-1,2-didecanoylglycerol (DIC<sub>10</sub>), a model DAG, readily formed tumors in 100% of Tg.AC mice, while neither glycerol nor *n*-decanoic acid, potential degradation products of DAGs, were able to promote tumors (Mills *et al.*, 1993; Owens *et al.*, 1995). A similar result was obtained in Tg.AC mice treated with an initiating dose of DMBA followed by DIC<sub>10</sub> promotion (Owens *et al.*, 1995). These results could implicate a role for phospholipid metabolism and the production of endogenous DAG signaling through protein kinase C (PKC) in Tg.AC tumorigenesis.

TPA and DAGs are known ligands for PKC. Upon activation by TPA, PKC sets in motion pathways leading to the upregulation and activation of transcription factors such as *c-fos* and *c-jun* (AP1), factors that may play an important role in initiating cell proliferation. AP1 may then bind sequences within the regulatory region of the  $\zeta$ -globin promoter as well as at the center of symmetry of the head-to-head  $\zeta$ -globin promoter palindromes. An AP1 sequence within the transgene  $\zeta$ -globin proximal promoter has been shown to be occupied in transgene-expressing tissues by DNase I footprinting (Humble *et al.*, submitted). AP1 upregulation and its subsequent binding to the transgene palindrome and promoter may embody the first step towards transgene induction and represent a common link between seemingly disparate promotional stimuli. An upregulation of a GATA factor in the skin, its binding to the transgene palindrome, and its cooperative binding with Sp1 to key elements within the  $\zeta$ -globin promoter (Humble *et al.*, submitted) may further account for the tissue specificity observed in the Tg.AC tumorigenic phenotype.

### Post-translational modification/activation of *ras*

Post-translational controls also play a role in v-Ha-*ras* transgene expression. Post-translational addition of a C<sub>15</sub> or C<sub>20</sub> isoprenoid moiety to the C terminus of *ras* proteins enables *ras* function and anchoring to the cell membrane. Addition of the C<sub>15</sub> moiety is accomplished by the enzyme farnesyl protein transferase, a target for anti-*ras* drug therapies. Homozygous and hemizygous Tg.AC mice were treated with a farnesyl transferase inhibitor (FTI), SCH 56582, prior to exposure to TPA (Trempeus *et al.*, 2000). SCH 56582 was able to reduce TPA-induced papillomagenesis, indicating post-translational farnesylation is involved in *ras*-transgene function.

A common phenomenon observed early in the Tg.AC tumorigenic response is the induction of cell proliferation within the epidermis. Topical exposure to tumor promoters (Leder *et al.*, 1990; Spalding *et al.*, 1993), chemical carcinogens (Tennant and Spalding, 1996), UV light (Trempeus *et al.*, 1998a; Chignell *et al.*, 2003a), full-

thickness surgical wounding (Cannon *et al.*, 1997), and depilation (Hansen and Tennant, 1994b) all result in epidermal hyperplasia. Within that hyperplasia, expression of the transgene in specific cells drives clonal expansion and tumor formation. Treatment of Tg.AC mice with FTI SCH 56582 did not affect TPA-induced epidermal hyperplasia (Tremplus *et al.*, 2000), implicating papillomagenesis to be a *ras*-dependent phenomenon, while TPA-induced hyperplasia is not. This is supported by the observation that a single low-dose treatment of TPA can induce hyperplasia, yet will not induce tumor formation or detectable transgene expression in Tg.AC mice. In addition, proliferating non-tumorigenic cells adjacent to the tumors or in other nontumor tissues can be observed that do not express the transgene (Hansen *et al.*, 1996).

A role for the endogenous *c-Ha-ras* gene in Tg.AC tumorigenesis has been explored. Sequence analysis of the endogenous *c-Ha-ras* gene in Tg.AC tumors revealed no activating mutations in codons 12, 59, or 61 (Hansen *et al.*, 1996). In addition, no codon 12, 13, 59, nor 61 mutations were observed in *c-Ha-ras* following DMBA/TPA treatment of Tg.AC mice (Owens *et al.*, 1995), although more tumors were generated in the DMBA-treated Tg.AC mice than in non-DMBA-treated mice. Additionally, no p53 mutations were detected.

Structural rearrangements within the Tg.AC or FVB/N genome were rarely observed (French *et al.*, 1994). A lack of trisomies on *c-Ha-ras* bearing chromosome 7 has been reported (French *et al.*, 1994), offering further support that endogenous *Ha-ras* is not likely playing a role in Tg.AC tumorigenesis. In addition, no report of trisomy 11 is seen, the chromosome on which the Tg.AC transgene is located. The absence of trisomy 7 in Tg.AC mice may be due to transgene *ras* expression since the multicopy transgene may compensate for a mutated *c-Ha-ras* (French *et al.*, 1994).

Trisomy 6 or 15 are frequently occurring alterations in Tg.AC and FVB/N malignancies, regardless of treatment or tumor type (spindle cell carcinomas or squamous cell carcinomas) (French *et al.*, 1994). Perhaps genes or a gene on chromosome 6 or 15

contributes to the development of malignant tumors. The fact that two chromosomes can be involved may indicate that multiple pathways exist in malignant conversions (French *et al.*, 1994).

#### Pathway interactions

In addition to the expression of the transgene, it is a certainty that the Tg.AC tumorigenic response is dependent on the expression and involvement of additional genes. To understand the cellular mechanisms regulating (and regulated by) Tg.AC transgene, the Tg.AC mouse has been crossed with additional transgenic mice in an effort to elucidate *ras*-dependent pathways and signaling (Table 1).

**Ornithine decarboxylase** Tumor promoters such as TPA induce the enzyme ornithine decarboxylase (ODC). ODC is the first and rate-limiting enzyme in the biosynthesis of polyamines. Overexpression of ODC in the epidermis of a transgenic mouse resulted in squamous papilloma formation following a single subthreshold dose of a carcinogen (O'Brien *et al.*, 1997).

The Tg.AC was crossed with a transgenic mouse that overexpressed ODC in the outer root sheath of the hair follicle (Smith *et al.*, 1998). The resulting K6-ODC/*ras* bitransgenics generated spontaneous papillomas and carcinomas in the skin, giving the appearance of being both genetically initiated (from the Tg.AC background) and genetically promoted (from the ODC background) (Smith *et al.*, 1998; Gilmour *et al.*, 1999). Tumor formation in the bitransgenics could be prevented by administration (via drinking water) of  $\alpha$ -difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ODC (Smith *et al.*, 1998). The mechanisms of ODC and *v-Ha-ras*, although independent, appear to act in concert driving progression through the cell cycle.

In addition to preventing tumors in ODC/*ras* bitransgenics, DFMO also was able to prevent tumor formation in wounded Tg.AC mice, indicating the potential involvement of polyamine synthesis and ODC in wound-induced promotion (Smith *et al.*, 1998).

**Table 1** Transgenic crosses with Tg.AC mice

Gene	Transgenic	Tg.AC F1 phenotype	Reference
Agouti	homo. lethal mutant alleles	Increase in tumor multiplicity	Hansen <i>et al.</i> (1998)
Bcl-2	bcl-2 deficient	Decreased tumor multiplicity	Tremplus <i>et al.</i> (1997)
Cyclin D1	Cyclin D1 null	Decreased tumor multiplicity	Robles <i>et al.</i> (1998)
Interleukin-1 $\alpha$	K14/IL-1 $\alpha$	Decreased tumor multiplicity	Murphy <i>et al.</i> (2003)
Kinase suppressor of <i>ras1</i>	KSR1 null	Decreased tumor multiplicity	Lozano <i>et al.</i> (2003)
Ornithine decarboxylase	K6/ODC	Spontaneous papillomas	Smith <i>et al.</i> (1998), Gilmour <i>et al.</i> (1999), and O'Brien <i>et al.</i> (1997)
Tyrosine kinase Ron	TK null	Smaller papilloma volume, reduction in malignant conversion	Chan <i>et al.</i> (2005)
Transforming growth factor- $\alpha$	TGF- $\alpha$ null	No significant tumorigenic effect	Humble <i>et al.</i> (1998)
Vascular endothelial growth factor	K6/VEGF	Earlier onset of papillomagenesis, increase in tumor size	Larcher <i>et al.</i> (1996, 1998), Tober <i>et al.</i> (1998)
Strain: C57Bl/6J	C57Bl/6J:Tg.AC	Decreased tumor multiplicity	Hansen <i>et al.</i> (1998)

Furthermore, it was found that administration of DFMO after the onset of tumorigenesis resulted in tumor regression, despite continued *v-Ha-ras* transgene expression (Smith *et al.*, 1998). Together, these data support a hypothesis that transgene expression, although a necessary precursor to papilloma formation, is in and of itself not sufficient for tumor formation.

**Cyclin D1** Mutated *ras* expression is known to affect the expression of a number of genes. Mutated *ras* increases the level of cyclin D and the associated kinase activity of cyclin D/Cdk4. In Tg.AC mice, the upregulation of D1 following TPA exposure has been shown in papillomas and follicular hyperplasias, but does not occur in uninvolved TPA-exposed skin (Robles *et al.*, 1998).

Tg.AC/cyclin D1-null bitransgenic mice were created to further explore the relationship between cyclin D1 and tumorigenesis (Robles *et al.*, 1998). Following TPA promotion, tumor multiplicity in cyclin D1-null bitransgenics was sixfold lower than in the matched cyclin D1-hemizygotes. In addition, the onset of tumors was delayed 1 week in D1 nulls. Similar results were obtained in the D1 nulls treated with DMBA followed by multiple applications with TPA, indicating the result was not simply a Tg.AC phenomenon.

Hyperphosphorylation of pRb (a blocker of G1 progression) by cyclin D1/Cdk4 allows cells to move through, but not out of G1. Elevated levels of ODC/polyamines indirectly activates or increases the kinase activity of cyclin E/Cdk2 in late G1, allowing the cells to move from late G1 to S phase DNA synthesis. Once a cell passes the G1-S checkpoint, progression through the cell cycle is independent of mitogenic stimulation. Elevated ODC/polyamines have the same effect at the S to G2 phase by increasing the kinase activity of cyclin A/Cdk2. p53 protein levels are increased in the skin of K6/ODC mice, indicating an increase in genomic instability or damage as the cells are pushed through the cell cycle (Gilmour *et al.*, 1999).

**Transforming growth factor  $\alpha$**  It has been shown that *v-Ha-ras*-mediated expression of cyclin D1 is also dependent on autocrine stimulation through the EGFR (Robles *et al.*, 1998). A member of the epidermal growth factor (EGF) family, transforming growth factor  $\alpha$  (TGF $\alpha$ ) binds to and activates the EGFR. A potent mitogen in many mesenchymal and epithelial cells, including keratinocytes, a body of evidence supports a role for TGF $\alpha$  in the promotional phase of multistage skin carcinogenesis (see references in Humble *et al.*, 1998). To determine if promotional stimuli (TPA and wounding) could induce papillomagenesis in Tg.AC mice deficient (null) in TGF $\alpha$ , Tg.AC mice were crossed with TGF $\alpha$ -null mice. F1 mice, hemizygous for *v-Ha-ras* and heterozygous or null for TGF $\alpha$ , were dosed with TPA or given full-thickness dorsal wounds. Papillomas were able to develop following both wounding and TPA treatment in TGF $\alpha$ -null mice, with some papillomas converting to squamous or spindle cell carcinomas.

Although no statistical difference was evident between dosing groups, trends were apparent, with TGF $\alpha$ -null mice developing fewer average papillomas with an increased latency time over sex-matched heterozygotes. Although not ruling out the importance of signaling through the EGFR in the tumorigenic process, these results may demonstrate that redundant members of the EGF family may be able to take the place of TGF $\alpha$  in its absence (Humble *et al.*, 1998), and that these EGF family members can stimulate cyclin D upregulation, permitting cells to move through G1 into S phase synthesis.

**Tyrosine kinase receptor signaling in Tg.AC** Kinase suppressor of *ras* (KSR) is a positive modulator of the map kinase pathway and appears to function by modulating the *ras/raf* activity of the mitogen-activated protein kinase signaling cascade (MAPK). To investigate its potential to modulate the *ras*-dependent tumorigenic response in Tg.AC mice, the *v-Ha-ras* transgene was crossed onto a KSR $-/-$  background (Lozano *et al.*, 2003). Mice that contained a transcriptionally active *v-Ha-ras* transgene but lacked KSR activity exhibited an abrogated TPA-induced tumorigenic response, indicating that KSR function appears to be required for TPA-induced papillomas in Tg.AC mice. Moreover, an interesting phenotypic observation was also noted; mice lacking KSR activity exhibited an unusual disorganized hair follicle phenotype similar to that of EGF knockout mice. Defects in EGFR signaling were also found in the mouse embryonic fibroblast (MEF). EGF-induced MAPK phosphorylation was blocked in KSR knockout mice demonstrating that KSR1 functions to integrate signaling through the Ras/MAPK complex via the EGFR receptor. Taken together, these data suggest that the *v-Ha-ras* transgene of Tg.AC mice signals through the MAPK pathway via the EGFR receptor.

It is also worth mentioning that another tyrosine kinase (TK) receptor, Ron, was found to modulate tumorigenesis in Tg.AC mice. Ron tyrosine kinase receptors are a family of multifunctional TK receptors that include the c-Met and c-Sea proto-oncogenes. These TK regulate very diverse biological responses, including proliferation, motility, invasiveness, and cellular dissociation. When a kinase domain-deficient Ron receptor was crossed onto Tg.AC, TPA-induced papillomas were found to be smaller in volume and less likely to convert to malignancy (Chan *et al.*, 2005).

**Bcl-2** The *bcl-2* gene product is a negative regulator of apoptosis, or programmed cell death. It has been shown that *bcl-2* is expressed in normal hair follicles in the mouse, the basal epidermis, and to a lesser extent in the postmitotic spinous epidermal layer (Stenn *et al.*, 1994). As an inhibitor of cell death, it was of interest to examine the role *bcl-2* plays in Tg.AC tumorigenesis. Tg.AC mice were crossed with mice deficient in *bcl-2*. The resulting F1 mice, hemizygous for the Tg.AC transgene and either homozygous or deficient in *bcl-2*,

were exposed to TPA promotion. At a low TPA dose (1.25  $\mu\text{g}$  twice weekly for 10 weeks), a statistically significant decrease in papilloma formation was seen for mice deficient in *bcl-2*. At a higher TPA dose (2.5  $\mu\text{g}$ ), no effect was seen. The decrease in papilloma development at the lower dose of TPA could be the result of an increase in apoptotic cell death due to the lowered *bcl-2* expression, or a possible enhancement of apoptosis through the upregulation of genes known to promote apoptosis and that are negatively controlled by *bcl-2*, such as *c-myc*, *c-fos*, and/or TGF $\beta$ . This possibility is supported by results obtained in a apoptosis-sensitive keratinocyte cell line (Marthinuss *et al.*, 1995).

**Vascular endothelial growth factor** The role of angiogenesis in the production of Tg.AC tumors has been explored through several means (Larcher *et al.*, 1996, 1998; Tober *et al.*, 1998). Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VGP), stimulates the formation of new capillaries and increases vascular permeability. A variety of splice variants exist for VEGF, designated by their amino-acid content as murine VEGF 120, 144, 164, 188, and 205. The smaller variants (120 and 144) are secreted and are mitogenic for endothelial cells, while the larger variants (188 and 205) are secreted yet remain adherent to the cell surface and likely regulate vascular permeability. VEGF derived from keratinocytes is the major angiogenic factor in the skin. VEGF expression has been shown to be mediated by oncogenic *ras* (Larcher *et al.*, 1996). In addition, TPA is able to upregulate VEGF/VGP expression in mouse skin (Larcher *et al.*, 1996). The four smaller splice variants of VEGF have been identified in Tg.AC and FVB/N skin, papillomas, and carcinomas (Tober *et al.*, 1998). Of interest is a fifth, larger splice variant present during the latter stages of tumor promotion and progression and perhaps related to the presence of v-Ha-*ras* (Tober *et al.*, 1998). Its presence in the latter stages may ensure that growth factors, oxygen, and nutrients are able to nourish the tumors by ensuring vascular permeability.

The overexpression of VEGF and its effect on Tg.AC tumorigenesis was examined through the use of a K6-VEGF transgenic mouse (Larcher *et al.*, 1998). The keratin-6 promoter utilized in this transgenic mouse confers a selective expression to the suprabasal layers of the epidermis in response to hyperproliferative stimuli. TPA-induced premalignant papillomas appear earlier in bitransgenic K6-VEGF/Tg.AC mice than in matched Tg.AC mice. Tumor size was greater in the majority of bitransgenic tumors, with total tumor mass also being significantly higher. Taken together, these studies demonstrate a role for VEGF in the tumorigenic process in Tg.AC mice.

### Future directions

Following proliferative stimuli and the change in regulation of additional genes such as ODC or VEGF,

specific transgene-expressing cells multiply to detectable levels, ultimately leading to clonal expansion and tumor formation. The fact remains, however, that only one in four founder lines carrying the  $\zeta$ -globin/v-Ha-*ras* transgene resulted in the unique phenotype now associated with the Tg.AC mouse. The influence of transgene position within the genome continues to be queried.

Transgene expression may be due to the fortuitous induction or expression of a neighboring gene on chromosome 11. This consequentially opens the chromatin surrounding or adjacent to the transgene integration site, resulting in  $\zeta$ -globin promoter regions becoming available to transcription factors. The multi-copy transgene array has been located proximal to the centromere on chromosome 11 (Humble *et al.*, 2000), and recently the specific site of integration is identified (Scott Barros, personal communication and Leder *et al.*, 2002). Examination of adjacent features within the flanking chromosome or the function of adjacent genes may offer insights into transgene induction. A continued examination of the nonresponder phenotype and the palindromic promoters may elucidate the role of this feature in transgene expression.

The advent and use of gene array technology has revealed TPA-inducible genes involved in the Tg.AC tumorigenic response (Wei *et al.*, 2003). Ongoing studies continue to explore the role Dss-1, NDPK-B, and other genes involved in the early stage of neoplastic development and skin tumorigenesis.

The correlation of Tg.AC tumorigenesis to *ras*-dependent pathways in the tumorigenic process strengthens the utility of the Tg.AC mouse as a model for experimental skin carcinogenesis. The utility of the Tg.AC as an adjunct to the 2-year bioassay will continue to be explored and debated. As a prototype alternative model, it has generated a healthy discussion on the future of transgenic bioassays, and opened the doors for subsequent models for toxicity testing. The continued exploration and elucidation of the cellular and molecular controls of transgene expression will enhance the usefulness of this mouse and enable a better understanding of the discriminate response to chemical carcinogens exhibited by Tg.AC mice.

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