Multi-stage chemical carcinogenesis in mouse skin: Fundamentals and applications

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Abstract

For more than 60 years, the chemical induction of tumors in mouse skin has been used to study mechanisms of epithelial carcinogenesis and evaluate modifying factors. In the traditional two-stage skin carcinogenesis model, initiation is accomplished by the application of a subcarcinogenic dose of a carcinogen. Subsequently, tumor development is elicited by repeated treatment with a tumor promoting agent. The initiation protocol can be completed within 1–3 hours depending on the number of mice used, while the promotion phase requires twice weekly treatments (1–2 hours) and once weekly tumor palpation (1–2 hours) for the duration of the study. A highly reproducible papilloma burden is expected within 10–20 weeks with progression of a portion of the tumors to squamous cell carcinomas within 20–50 weeks. In contrast to complete skin carcinogenesis, the two-stage model allows for greater yield of premalignant lesions as well as separation of the initiation and promotion phases.

INTRODUCTION

Historical Perspective

The mouse skin model of multi-stage chemical carcinogenesis represents one of the best established *in vivo* models for the study of the sequential and stepwise development of tumors. In addition, this model can be used to evaluate both novel skin cancer prevention strategies and the impact of genetic background and genetic manipulation on tumor initiation, promotion, and progression. The multistage nature of the carcinogenic process was first clearly demonstrated in the mouse skin model[reviewed in 1]. In the 1920s it was noted that wounding of mouse skin that had previously been treated with carcinogenic tar could lead to the appearance of tumors. These findings suggested a role for cell proliferation and hyperplasia in a multi-step evolution of cancer, and ultimately, led to the development of the two-stage protocol for mouse skin tumorigenesis (Fig. 1). From these seminal studies to the present, mouse skin carcinogenesis has become one of the most extensively analyzed rodent models of chemically-induced cancer[reviewed in 2,3–10]. Studies in this model have yielded, and continue to yield, insight into the fundamental biology of cancer, and much of our understanding of the multi-stage nature of epithelial cancers is rooted in the analysis of chemically-induced skin tumors in mice.

"Complete" or "two-stage" carcinogenesis protocols have been developed for the study of skin tumors in mice (i.e., tumor incidence, latency, multiplicity, and progression). In complete carcinogenesis protocols, tumor development occurs after either the administration of a single high dose (or repeated applications of a lower dose) of a carcinogen or by continuous exposure to ultraviolet (UV) light. Additional treatments with promoting agents are not required for tumor development, indicating that both the initiating and promoting components are present during complete carcinogenesis. However, the interpretation of complete carcinogenesis studies is complicated by the inability to distinguish events or effects related to the tumor initiation versus tumor promotion stages. In two-stage skin carcinogenesis experiments, initiation occurs following a single subcarcinogenic dose of a carcinogen such as 7,12-dimethylbenz[a]-anthracene (DMBA) (Fig. 1). This event is irreversible; however, no visible tumors will appear until 'promoted' by the repeated application of a tumor promoting agent such as the phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Thus, in two-stage skin carcinogenesis experiments, the initiation and promotion stages can be distinctly separated both operationally and mechanistically.

An additional advantage of the two-stage skin carcinogenesis model is that tumor development can be conveniently monitored visually throughout the life span of the mouse; tissue harvest and pathological analysis are only necessary at the termination of the study or when an animal requires sacrifice. Since the tumor response is highly reproducible, the efficacy of chemopreventive agents or the impact of dietary manipulation can be assessed[11–13]. Additionally, the role of various genes and cell-signaling pathways can be explored in this model through the use of genetically engineered mouse models (GEMMs) as well as small molecule inhibitors[14–23]. A number of established short-term markers of skin carcinogenesis aid in determining the stage at which modifying factors affect tumorigenesis and give clues as to the molecular mechanisms involved[reviewed in 1,24]. In light of the extensive characterization of this model as well as its versatility, two-stage skin carcinogenesis in mice continues to serve as a useful model of human cancers of epithelial origin[reviewed in 2,25].

Description of the Model

During the first stage of chemically-induced skin carcinogenesis, referred to as 'initiation', key genes in epidermal keratinocytes acquire mutations as a result of exposure to a chemical mutagen. Currently, the most frequently utilized initiating agent is the polycyclic aromatic hydrocarbon, DMBA, but additional agents can serve as chemical initiators (Table 1). DMBA is most often applied topically, although systemic exposure is also effective[26]. The *Hras1* gene appears to be a primary target gene for the initiation stage, although mutations in *Kras* have been demonstrated in lesions initiated with DMBA and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and *Nras* mutations have been observed in lesions initiated with UV light, (Table 1)[27–29]. Activating mutations *in Hras1* can be detected in the epidermis as early as 3–4 weeks following treatment with DMBA[30] and are observed in the majority of papillomas that develop initially following tumor promoter treatment[31]. While DMBA predominantly induces an A → T (182) transversion in codon 61 of the *Hras1* gene, various other initiating agents each produce a unique spectrum of activating *Hras1* mutations[32]. The observation that skin tumors are induced in TG.AC mice (transgenic mice that express a *v*-Ha-ras under the control of the zeta-globin promoter) following promotion by a variety of agents without prior treatment with an initiating agent, supports mutation of *Hras1* as a critical event in skin carcinogenesis[33]. Keratinocyte stem cells, which are primarily found at the base of epidermal proliferative units in the interfollicular epidermis and in the bulge region of the hair follicles, are believed to be the primary cellular targets of the initiation stage[34].

Following the initiation stage, the population of mutated cells is promoted to clonally expand during the second stage, referred to as "promotion". Tumor promotion is elicited by the repeated topical application of chemical agents or wounding that leads to sustained epidermal hyperplasia evidenced by an increase in the number of nucleated cell layers and an overall increase in thickness of the epidermis[reviewed in 1,2–10,35]. During epidermal hyperplasia, initiated cells are believed to have a growth advantage over neighboring cells allowing for their selective expansion[36–38]. The stimulation of growth of initiated cells may be a direct effect of the tumor promoter on these cells and/or occur through indirect effects due to loss of cell populations[1,8,39]. The end result of the promotion stage is the development of clonal outgrowths of the skin called papillomas[reviewed in 1,2–10,35]. Papillomas consist of a stromal core surrounded by hyperplastic epidermis (Fig. 1). Promoting agents, which are structurally diverse as well as mechanistically diverse in action (Table 1), stimulate cell signaling, increase production of growth factors, and generate oxidative stress and tissue inflammation[reviewed in 1]. Therefore, short-term markers of tumor promotion include increased epidermal thickness, proliferation of basal keratinocytes, increased DNA synthesis, and inflammatory cell infiltration[reviewed in 1,2–10,35].

Papillomas generated during two-stage skin carcinogenesis protocols may progress to invasive squamous cell carcinomas (SCC) as early as 20 weeks after treatment with the promoting agent begins (Fig. 1). The frequency of malignant conversion is dependent on genetic background; for instance, as few as 1–10% of papillomas progress to SCC in SENCAR or BALB/c mice while up to 50% of papillomas that develop in skin of FVB mice may convert[40–42]. This is also highly dependent on doses of initiator and promoter, which influence papilloma burden[43,44]. Further progression can lead to formation of spindle cell carcinomas, although this is a relatively rare event[35]. During the conversion process, progressive chromosomal abnormalities occur independent of continued treatment with tumor promoting agents. The tumors become aneuploid ~30–40 weeks after the initiation protocol begins[45–47]. In this regard, the conversion of papillomas to SCCs is associated with trisomies of chromosomes 6 and 7 as well as mutations in *Trp53*[48,49]. SCCs are downward invading lesions that are highly vascularized. Numerous gene expression changes are present, including those associated with epithelial-mesenchymal transition (EMT)[1,50–52]. Approximately 15–35% of mice with one or more carcinomas will also have metastases to organs such as lung or lymph nodes, and the frequency of metastasis appears to be under genetic control[53].

Susceptibility to two-stage skin carcinogenesis in mice is known to be highly dependent on genetic background (Table 2)[reviewed in 1]. Early studies by Boutwell supported the hypothesis that specific genes modify susceptibility to two-stage skin carcinogenesis[54]. Different stocks and strains of mice do not significantly differ in the capacity of epidermis to metabolize initiating agents (e.g., polycyclic aromatic hydrocarbons such as DMBA). In addition, the formation and removal of covalent DNA-adducts during skin tumor initiation appears to be similar. Thus, the primary genetic determinants of susceptibility to two-stage skin carcinogenesis likely lie in response to tumor promotion[reviewed in 55]. Supporting this hypothesis, mice initiated with direct acting carcinogens, such as MNNG or UV-light, show the same distribution in susceptibility to two-stage epidermal carcinogenesis[reviewed in 55]. These results indicate that the strain-dependent response to two-stage skin carcinogenesis is not due to differences in metabolic activation of the initiating agent; rather, it is most likely due to variation in the effects elicited by treatment with promoting agent.

The majority of studies assessing tumor promotion susceptibility in mice have used phorbol esters such as TPA. The distribution pattern for sensitivity to tumor promotion by TPA (SENCAR > DBA/2 ≥ CD-1 > C3H/He >> C57BL/6) has been well documented[reviewed in 55]. A number of chromosomal loci underlying this complex trait have

been identified in various stocks and strains of mice[56–63]. A similar distribution pattern for the sensitivity to different classes of promoting agents suggests that common genetic factors modify the response to tumor promotion by different classes of promoting agents[64]. For example, the distribution pattern for sensitivity to skin tumor promotion by chrysarobin, benzoyl peroxide, and full thickness skin wounding is SENCAR > DBA/2 > C57BL/6[64–66]. Because of these genetic differences in response to skin tumor promoters, careful attention needs to be paid to the genetic background of the mouse strain being studied when selecting a dose of the promoting agent. For example, a higher dose of TPA and a different treatment regimen (i.e., three times weekly application) may be used when studying C57BL/6 mice compared with SENCAR mice[67]. As will be discussed, the choice of mouse strain and doses of initiating and promoting agents as well as the timing of interventions are critical design options that affect the outcome and interpretation of two-stage skin carcinogenesis studies in mice.

Potential Applications of the Model

Multiple lines of evidence suggest that epithelial cancers in humans are the result of a multistage process[68,69]. Cancer is widely believed to arise from the expansion of stem cell populations targeted during a DNA-mutating event such as carcinogen exposure[70]. In the case of colon cancer, the accumulation of numerous genetic lesions in an increasingly aberrant subset of tumor cells reflects the multiple steps required for epithelial carcinogenesis, and these genetic changes are reflected in progressive histopathological changes from hyperplasia to adenoma to true carcinomas[71–73]. The two-stage skin carcinogenesis model in mice recapitulates features of multi-stage carcinogenesis in humans. For example, similar to a number of solid tumors in humans, it appears that the occurrence of activating mutations within stem cell niches is the first step in a cascade of events leading to tumor formation in this model[74,75]. Additionally, there are a number of similarities to human cancers at the genetic or molecular level, including activating mutations in *ras* family members, activation of the epidermal growth factor receptor (EGFR), activation of Stat3 and Akt-mediated signaling pathways, elevated expression of transforming growth factor β1, and, at later stages, *Tp53* mutations[2]. Likewise, the two-stage skin carcinogenesis protocol is a good model for human cancers because humans are typically exposed to multiple low doses of both carcinogens and promoting agents[76]. The long latency associated with most human cancers also strongly supports a promotional component for tumor development[77,78]. Therefore, this extensively characterized model can be utilized to study the mechanistic basis of human epithelial cancers.

Recent advances in the generation of GEMMs have provided valuable tools in furthering our understanding of the carcinogenic process. Our laboratory and others have used GEMMs in the two-stage skin carcinogenesis protocol to investigate the roles of specific genes in epithelial carcinogenesis[16–23]. Importantly, gene function can be studied *in vivo* and throughout the process of carcinogenesis. Also, the development of methods for targeting genetic modifications to the skin has allowed gene expression or gene deletion in specific compartments of the skin, avoiding complications such as embryonic lethality or systemic effects[79]. Two-stage skin carcinogenesis studies using GEMMs can reveal the roles of potential cancer risk modifier genes, proto-oncogenes, and tumor suppressor genes in tumor initiation, promotion, and progression. In addition, chemical inhibitors may also be used to test the role of specific signaling pathways during carcinogenesis. For example, recent studies from our lab using a PI3K inhibitor, LY294002, in a transgenic mouse model overexpressing human IGF-1, underscored the role of PI3 Kinase and Akt-mediated signaling in epithelial carcinogenesis[14].

The mouse multi-stage skin carcinogenesis model is particularly suited for evaluating the effects of dietary factors/dietary manipulations and other chemicals (both natural and

synthetic) on tumor development. Potential inhibitors of carcinogenesis can be evaluated for their effects on initiation, promotion, and/or progression[11–13]. For example, studies from our lab showed that delivery of the citrus coumarin, isopimpinellin, prior to DMBA exposure significantly inhibited tumor initiation[80]. In contrast, sulforaphane was most effective at inhibiting the promotion stage[81] while silymarin was effective at blocking tumor formation as well as inducing regression of established tumors depending on the sequence of delivery[82]. Although not yet widely examined, the possibility exists that this model may also be useful for evaluating the efficacy of therapeutic agents. Recent results showing regression of papillomas injected with a Stat3 decoy oligonucleotide[21] or treatment of existing tumors with rapamycin[15] support the feasibility of this application of the model. Importantly, topical application of chemopreventive agents as well as initiating and promoting agents precludes or reduces systemic effects.

The effect of dietary manipulation on carcinogenesis can also be studied in this model system. Since calorie restriction in known to inhibit the tumor promotion phase, this model is ideally suited for investigating the mechanistic basis for the link between negative energy balance and cancer risk[54,83]. Tissue specific alterations, as well as systemic effects of altered energy balance, may be examined[84,85]. In any chemoprevention or dietary intervention study, specific attention to study design is necessary to properly assess efficacy and draw rational conclusions.

While the two-stage skin carcinogenesis model has a variety of applications and has been extensively utilized to address of a number of questions about the fundamental biology of epithelial cancers, some limitations exist. In this protocol, mice develop primarily papillomas, of which there is no direct human equivalent; however, the SCCs that develop following malignant conversion are histologically very similar to human SCCs. Another limitation of the model is that *Hras* is the primary target for chemical initiation in mice, whereas *Tp53* appears to be a more important target for gene mutation in human non-melanoma skin cancer[86]. The gene targets for initiation by chemicals in mouse skin more closely resemble those found in other human epithelial cancers (e.g., lung, colon, and pancreatic cancers)[87]. Additionally, the two-stage skin carcinogenesis protocol is of limited utility for studying metastasis. As is true for a number of other mouse models of cancer, the rate of metastasis of skin tumors is quite low[53].

Experimental Design Considerations

The physical application of tumor initiating and promoting agents to mouse skin as well as the palpation of papillomas and SCCs is not technically challenging. As will be described, a solution of DMBA is applied to the shaved dorsal skin of mice to accomplish the initiation phase. Subsequently, TPA is applied to the skin twice weekly until the tumor response reaches a plateau. Any palpable mass greater than 1 mm in size can be considered a papilloma and recorded. Despite the technical ease of performing this protocol, the proper design and execution of a two-stage skin carcinogenesis study requires thoughtful planning and diligent attention to detail. In this section, a number of experimental design and execution considerations will be discussed.

A thorough understanding of chemically-induced carcinogenesis as well as sound hypothesis development are necessary for the proper design of two-stage skin carcinogenesis studies in mice. In developing a testable hypothesis, consideration should be given to the specific goal of the study. Is the goal to examine gain or loss of gene function or the effect of a chemopreventive agent on initiation, promotion, or progression? Existing literature concerning the role of the gene or agent can be very useful in developing a biologically plausible hypothesis. If the anticipated effect is on carcinogen metabolism, DNA repair, mutation induction, or cell survival, then more attention should be given to the initiation

stage, although factors or agents that affect maintenance of genomic stability and/or cell survival may also play a role in tumor progression in this model. In this respect, examination of short-term makers for effects on tumor initiation (i.e., DNA adduct formation and carcinogen-induced apoptosis) can guide the design of tumor experiments[21,80]. For agents or genes that affect cell proliferation, oxidative stress, or inflammation, a role in the tumor promotion stage may be hypothesized. Agents or genes with known effects on EMT, vascularization, or cell motility may be hypothesized to affect tumor progression[50,88–92]. As with initiation, preliminary studies of short-term markers of tumor promotion (i.e., epidermal proliferative response or dermal inflammation) or tumor progression (i.e., keratinocyte migration or invasion) may be performed prior to the design of tumor experiments[20,50,93,94]. Ideally, a set of well-designed experiments addresses all stages, although practical considerations may dictate focus on only one of the stages (e.g., initiation and/or promotion).

After development of a testable hypothesis, among the most critical next decisions are the choice of initiating and promoting agents as well as the dose of each to be applied. These parameters not only dictate the conclusions that may be drawn from the study but also the anticipated tumor burden and the health and welfare of the animals. Also, the choice of initiating and promoting doses may determine whether or not the effect of a chemopreventive agent or genetic manipulation can be accurately determined. For instance, the use of very high doses of these agents may overwhelm a potential chemopreventive effect or prevent accurate assessment of tumor progression. Depending on the anticipated effect, experiments that incorporate dose-response analyses are highly recommended, wherein a range of tumor initiator or tumor promoter doses is examined or, alternatively, a range of potential inhibitor doses is used.

An additional consideration for chemoprevention (or gene modification) experiments is the timing of agent delivery. By altering the time at which chemopreventive agent is delivered or altered gene expression occurs, effects on tumor initiation, promotion or progression may be monitored. From laboratory to laboratory, variation in the preferred waiting period between the initiation and promotion stages exists; our laboratory routinely utilizes a 2-week interval. It is important to note, however, that the time following initiation can be extended for many weeks without a negative impact on tumor yield[reviewed in 9]. Therefore, this waiting period can be adjusted to fit the goals of the study. For instance, when studying the effects of calorie restriction, we routinely initiate, and then place the mice on a calorie restricted diet for up to 8 weeks prior to beginning the promotion phase (unpublished data).

The choice of mouse strain and the number of mice utilized per group are additional factors to consider in designing a successful two-stage skin carcinogenesis experiment. Moreover, given the well-described variation in sensitivity to two-stage skin carcinogenesis among mouse stocks and strains, the choice of dose of the promoting agent and mouse strain are interdependent. It is important to recognize that the development of both papillomas and SCCs is under independent genetic control[95]. Therefore, selection of an appropriate genetic background is critical for the hypothesis to be tested. For many years, outbred CD-1 and SENCAR mice have been used for two-stage skin carcinogenesis studies[96–99]. In more recent years, two-stage skin carcinogenesis experiments have used a variety of genetic backgrounds due, in part, to the generation of gain and loss of function GEMMs on diverse genetic backgrounds. FVB mice have emerged as an appropriate inbred mouse strain for two-stage skin carcinogenesis studies[20,42]. The use of an inbred mouse strain reduces variability in tumor response, and FVB mice represent a strain that is moderately sensitive to tumor promotion by TPA, which facilitates analysis of modifying factors (both positive and negative). In some cases, it is not possible to use FVB mice or even an inbred strain. Therefore, as a guideline in these instances, recommended dosing regimens as well as

expected outcomes for various mouse stocks and strains are listed in Table 2. Since repeated wounding due to fighting or irritation of the skin can act to promote skin tumor formation, aggressive mice should not be group housed. For this reason, female mice are preferred due to less aggressive behavior, which permits reduction in animal housing costs. It is often useful and even necessary to identify individual mice in each group housing by ear punch pattern, tail tattoo, or other identification methods.

In studies of GEMMs, it is important to use wild-type littermates as control mice to insure that genetic background does not confound results. It is also recommended that null alleles or transgenes be backcrossed at least 10 generations to an inbred mouse strain that is moderately sensitive to skin carcinogenesis before performing two-stage skin carcinogenesis experiments. Alternatively, a process commonly called "speed congenics" can be employed, wherein marker assisted selective breeding is used to transfer the genetic modification to an inbred background in less time, using fewer mice[100]. These are critical considerations in studies of bi-transgenic or inducible gene deletion models since very high levels of heterogeneity will result if transgenic mouse strains of discrepant genetic background are crossed. It is also especially important to include additional control groups in the study design when characterizing a novel GEMM or potential carcinogen. As appropriate to the goal of the study, vehicle only control groups for the initiation and/or promotion stages should be included when using either non-transgenic mice or GEMMs. These controls may reveal potential endogenous initiating or promoting activity associated with genetic modification. Sample size calculations can be performed during the experimental design phase to predict the number of mice needed per group to allow ample statistical power to compare the response of control and test groups[101,102]. (Additional information concerning sample size determinations can be found at http://web.ncifcrf.gov/rtp/lasp/intra/acuc/fred/animal_number.asp). Power calculations should take into account that tumor response data is not normally distributed; therefore, use of normative tests will underestimate the required sample size.

The mice used in two-stage skin carcinogenesis studies should be age- and gender-matched and then randomly assigned to treatment groups (using a random number generator). Although some variation exists, most mice are in telogen (resting phase) at 7–9 weeks of age. When possible, this is the target age for beginning a two-stage skin carcinogenesis experiment. The majority of the dorsal skin of the mice should be shaved 48 hr prior to application of the initiating agent. (Waiting 48 hours before applying the initiating agent allows any inflammation generated during shaving to dissipate.) Once shaved, the skin of the mice should be examined to determine the approximate phase of the hair cycle. The skin of FVB mice in the anagen phase of the hair cycle appears thickened and white compared to skin in the resting phase due to extended hair follicle length. Similarly, the skin of pigmented mice in anagen is much darker. In addition, mice still in anagen may display partial hair regrowth after shaving. Any mice that are not found to be in the resting phase of the hair cycle should be eliminated from the study.

Another important consideration is the choice of diet. In this regard, specific dietary constituents and chow composition may also influence tumor response. A semi-purified chow, such as AIN- 76 diet, is often preferred for two-stage skin carcinogenesis experiments since the composition of natural-ingredient chow is likely to fluctuate depending on the cultivation, harvest, and storage conditions of the plant or fish components[103]. Consequently, it is imperative that control and test groups have equal ability to access identical diets. Factors that affect food consumption, such as digestive tract malformations, extreme tumor burden, or unpalatable chow, may confound results. Therefore, body weight should be monitored regularly throughout the study to insure that groups maintain similar rates of weight gain.

Finally, if one of the goals of the two-stage skin carcinogenesis study is to examine the conversion of papillomas to SCCs, additional considerations are needed. To achieve the maximum number of conversion events, treatment with the tumor promoting agent should be continued for the duration of the experiment (e.g., up to 52 weeks for SCC development)[43,44]. Papillomas can be expected to convert to SCCs after ~20 weeks of promotion in most wild-type strains. The presence of SCC should be confirmed histologically at the conclusion of the studies; however certain macroscopic characteristics indicate the presence of SCC. Tumors with flattened and downward appearing growth as well as attachment to the underlying muscle layer can be considered SCCs. As noted above, significant variation in the rate of conversion of papillomas to SCCs exists among various stocks and strains of mouse. A genetic background that confers sensitivity to tumor progression, such as SENCAR or FVB, should be selected. Also, as is true for papilloma formation, the doses of initiating and promoting agent will determine the ultimate response[43]. Use of high doses is not recommended both to insure that the mice do not become morbid due to tumor burden and because the rate of conversion to SCCs is limited by papilloma burden and biological constraints of the skin[44]. In general, the use of excessive doses of initiating and/or promoting doses is discouraged since as the tumor burden increases, lesions may coalesce and become difficult to track.

In this article, a standard two-stage skin carcinogenesis protocol using FVB mice is presented. As noted above, FVB mice are moderately sensitive to tumor promotion by TPA and are susceptible to development of SCCs[42]. Therefore, relatively low doses of DMBA (25 nmol) and TPA (1.7, 3.4, and 6.8 nmol) can be utilized. Similar treatment regimens have been extensively used by numerous groups (see Table 2), demonstrating the reliability and reproducibility of this model.

MATERIALS

REAGENTS

- Female FVB mice 7–9 weeks of age with dorsal skin in the resting phase of the hair cycle.

  !CAUTION—All experiments involving animals should be undertaken in compliance with national and institutional regulations.

- DMBA, ≥ 95% pure (25 nmol/0.2 ml acetone) (D3254, Sigma-Aldrich, St. Louis, MO)

  !CAUTION—Hazardous mutagenic compound. Avoid contact.

  !CRITICAL—DMBA is light sensitive.

- TPA, ≥ 99% pure (1.7, 3.4, and 6.8 nmol/0.2 ml acetone) (P-1680, LC Laboratories, Woborn, MA)

  !CAUTION—Potentially hazardous compound. Avoid contact.

- Acetone (HPLC grade)

  CAUTION Flammable liquid, Handle as hazardous waste and keep away from open flames.

EQUIPMENT

- Surgical Clippers
- Disposable and conventional cages

- Disposable glass vials to contain at least 15 ml volume
- Foil
- Disposable laboratory apron
- Mask
- Hair net
- Shoecovers
- Goggles
- Shoulder length gloves
- Standard gloves
- Absorbent pads
- Yellow light source
- Disposable spatulas

REAGENT SETUP

2500 nmol/0.2 ml acetone DMBA stock solution (100X)—Under yellow or subdued light and inside a dedicated carcinogen weighing room, measure 15 mg of DMBA into a glass vial using a disposable spatula. Add 4.68 ml acetone and mix by gentle agitation. Prior to use, dilute the 100X stock solution to 1X (25 nmol/0.2 mL acetone final concentration) by adding 150 µl of the 100X stock solution to 14.85 ml of acetone. Protect all DMBA solutions from light by wrapping vials in foil or using brown glass. The DMBA solution should be made fresh on the day of initiation. !CAUTION—DMBA is carcinogenic. Wear protective clothing (hair net, mask, disposable gown, goggles, and shoulder-length gloves layered with standard gloves) when handling solid DMBA or DMBA-containing solutions. All items that may have come into contact with DMBA as well as excess DMBA solutions should be disposed of as hazardous waste. Ideally, an isolated area in the laboratory should be designated for handling DMBA.

27.2 nmol/0.2 ml concentrated TPA stock solution—To make a stock solution of 27.2 nmol TPA/0.2 ml of acetone, dissolve 25 mg of TPA in acetone for a final volume of 298 ml. Make serial dilutions in acetone to achieve final working solutions of 1.7, 3.4, and 6.8 nmol TPA in 0.2 ml of acetone. For example, to make 120 ml of 6.8 nmol TPA/0.2 ml acetone, dilute 30 ml of the 27.2 nmol TPA/0.2 ml acetone concentrated stock in 90 ml of acetone. Subsequently, dilute 50 ml of the 6.8 nmol TPA/0.2 ml acetone stock in 50 ml of acetone to achieve a 3.4 nmol TPA/0.2 ml acetone solution. Finally, dilute 30 ml of the 3.4 nmol TPA/0.2 ml acetone solution in 30 ml of acetone to generate the 1.7 nmol TPA/0.2 ml of acetone working solution. The TPA solutions may be stored at −20 °C for at least one month.

EQUIPMENT SETUP

Disposable caging—Mice should be housed in disposable caging in a room equipped with yellow light prior to initiation with DMBA. !CAUTION--Following application of DMBA, avoid unnecessary handling of the mice for a period of two weeks. At the end of two weeks, mice may be rehoused in conventional caging and the DMBA-contaminated cages and bedding disposed as hazardous waste.

DMBA treatment hood—The application of DMBA should occur in a Class IIB2 hood. The floor of the hood should be covered with absorbent pads that are secured by laboratory or masking tape. !CAUTION--All items that may have come into contact with DMBA should be disposed as biohazardous waste or decontaminated as described below.

Pipettes for delivery of DMBA—!CAUTION—Pipettes used for the application of 0.2 mL 1X DMBA solution should not be used for any other purposes. Following application of DMBA the surfaces of the pipette should be decontaminated by wiping with ethanol. The pipette tips and tissues used for cleaning the pipettes should be disposed as biohazardous waste.

PROCEDURE

Tumor initiation TIMING ~1–2 hr for 50 mice followed by 1–2 week waiting period

1 Obtain a sufficient number of age-matched mice per test group.

!CAUTION—All experiments involving animals should be undertaken in compliance with national and institutional regulations.

?TROUBLESHOOTING

2 Shave the dorsal skin of the mice using surgical clippers. Gently restrain mice by the tail for no longer than 1–2 min while hair is removed.

!CRITICAL STEP-- Hair regrowth may interfere with uniform application of the initiating agent and the hair cycle should be synchronized as much as possible before treatment.

3 Randomize the mice into treatment groups.

4 Rehouse mice in disposable biohazard cages and relocate cages to a carcinogen treatment room equipped with a yellow light source.

5 Two days after shaving, the mice should be 'initiated'. Place a freshly prepared stock of 25 nmol/0.2 ml DMBA (1X), a 20–200 microliter pipette and 300 microliter filter tips in the treatment hood under yellow light.

6 Place a single cage of mice in the hood along with an extra cage bottom with fresh bedding.

7 Gently restrain a mouse by the tail and apply 0.2 mL of 1X DMBA solution to the dorsal skin. Restrain the mouse for an additional 5 to 10 seconds to allow the acetone solution to evaporate. Release the treated mouse into the extra empty cage bottom. Repeat for the remaining mice. Control mice receive 0.2 ml acetone only.

!CAUTION— DMBA is a hazardous mutagenic compound. Avoid contact and dispose of any unused DMBA solution as hazardous waste.

8 Isolate the mice in disposable biohazard caging for 1–2 weeks following application of DMBA.

!CAUTION-The mice should be handled as infrequently as possible during this period to avoid personal exposure to DMBA.

Tumor promotion TIMING ~0.5–1 hr for 50 mice, twice weekly for up to 52 weeks

9 After two weeks have passed, rehouse the mice in conventional caging.

10 Begin twice weekly applications of 1.7, 3.4, and 6.8 nmol TPA in 0.2 ml of acetone. Maintain either a Monday/Thursday or Tuesday/Friday treatment schedule for the duration of the study.

11 Continue twice weekly application of the TPA doses in 0.2 ml of acetone for up to 52 weeks.

Data acquisition TIMING ~1 hr for 50 mice, once weekly for up to 52 weeks

12 Beginning at approximately 6 weeks of tumor promotion with TPA, palpate the back of each mouse once-weekly to detect tumor formation. Each week record the number of tumors detected as well as the number of mice remaining in the study; document any palpable mass ≥1 mm in size. Tumor volume may also be estimated and recorded periodically[104,105].

?TROUBLESHOOTING

13 Monitor body weight at regular intervals to insure that test and control mice maintain approximately equal rates of weight gain.

?TROUBLESHOOTING

14 Monitor mice for the conversion of papillomas to SCCs and notate findings.

15 Note date and circumstance of death for any mice that die during the study. When possible, kill any mice where death appears imminent, and harvest tumor tissues for histological verification.

16 Kill the mice 2 weeks after the final TPA application according to Institutional Animal Care and Use Committee (IACUC) guidelines. Harvest tumors for histological verification and further study. Tumors and adjacent skin may be snap frozen, cryopreserved in optimum cutting temperature (OCT) compound or fixed in ethanol or formalin depending upon predicted experimental needs.

TIMING

See Figure 1 for protocol timeline including approximate anticipated time of appearance for papillomas and SCCs.

Steps 1–8 Tumor initiation 1–2 hr for 50 mice and 1–2 week waiting period

Steps 9–11 Tumor promotion 0.5–1 hr dosing twice weekly

Steps 12–16 Data Acquisition 1 hr tumor palpation once weekly

TROUBLESHOOTING

See Table 3 for troubleshooting advice.

ANTICIPATED RESULTS

During the promotion stage, repeated TPA treatment results in sustained hyperplasia of the epidermis and, eventually, the selective clonal expansion of initiated cells into premalignant papillomas (Figs. 1 and 2). Papillomas can be expected to appear on the dorsal skin of FVB mice by ~6–8 weeks of promotion (for example data see Fig. 3). The predicted tumor incidence and tumor multiplicity for various stocks and strains of mice, including FVB, are shown in Table 2. By approximately 20 weeks of promotion, a fraction of papillomas will begin to convert to SCCs by becoming increasingly invasive and penetrating deeper into the dermis. As SCCs evolve, cellular architecture may become disorganized and cells become anaplastic, losing normal polarity as well as markers of differentiation (tumors with

disorganized cells are referred to as poorly differentiated SCCs as opposed to well-differentiated SCCs (Fig. 2)). A small percentage of SCCs may convert into poorly differentiated spindle cell carcinomas that are composed of fibroblast-like carcinoma cells (Fig. 2). A number of proteins exhibit distinctive expression patterns during the promotion and progression of skin tumors in mice and can serve as biomarkers for the pathologic changes associated with the later stages of skin carcinogenesis in mice. Figure 2 shows the representative expression of several marker proteins (K1, loricrin, E-cadherin, K8 and GGT) in each sequential stage of mouse skin carcinogenesis (normal epidermis, hyperplastic epidermis, papilloma, SCC (differentiated), SCC (poorly differentiated), and spindle cell carcinoma). Staining for these markers is also shown for a regional lymph node metastasis that was harvested from the same mouse bearing the differentiated SCC in this figure.

The histologic and cytogenetic abnormalities that occur during mouse skin carcinogenesis have been shown to occur in tandem with changes in the expression patterns of select keratin genes. Keratin 14 and keratin 5 (K14 and K5, respectively) are primarily expressed in the basal cells of the proliferative compartment of the epidermis. As keratinocytes differentiate and migrate to the suprabasal layer of the epidermis, the expression of K14 and K5 decreases while the expression of keratin 1 (K1) and its partner, keratin 10 (K10), increases. Loss of K1 and K10 expression (Fig. 2b) combined with an accompanying increase in aberrantly expressed K13 (not pictured) are characteristic gene expression patterns during the progression of mouse skin papillomas to SCCs[106–109]. As the expression of K1 and K10 is suppressed, expression of keratin 8 (K8) is then observed in SCCs (Fig. 2e)[110]. Aberrant expression of K8 has been noted in SCCs, but not in papillomas, generated by the DMBA-TPA two-stage skin carcinogenesis protocol[111]. While K13-positive foci are primarily found in well-differentiated regions, K8-positive cells in SCCs are found primarily in anaplastic areas, suggesting that the expression of K8 can serve as a useful marker of the later stages of tumor progression in this model.

Loricrin expression is an additional marker for differentiation in mouse skin and skin tumors. Loricrin is expressed during the late terminal differentiation of keratinocytes and is the major protein of the epidermal cornified cell envelope (Fig. 2c)[112]. Loricrin is expressed in the granular layer of the epidermis where it accumulates in granules before it is integrated into the developing cornified envelope[113,114]. Whereas cornified cells and some granular cells within hyperplastic epidermis and papillomas are positive for loricrin, expression is abruptly decreased in SCCs and spindle cell carcinomas (Fig. 2c).

The expression of gamma-glutamyltransferase (GGT) and E-cadherin are additional markers of tumor progression in this model. GGT, a cell surface enzyme involved in cellular glutathione homeostasis, is present in the plasma membrane. It has been reported that GGT expression is detectable in papillomas while only a percentage of SCCs express GGT[24,115]. In Figure 2, some of the cells in the suprabasal layer are positive for GGT. GGT expression is widespread in papillomas but becomes more focal in SCCs (Fig. 2f). Spindle cell carcinomas show widespread positive staining for GGT (Fig. 2f). E-cadherin is a calcium-dependent cell adhesion molecule that is expressed primarily on the surface of epithelial cells. E-cadherin plays a major role in cell-cell interactions in epithelial tissues and it is well established that decreased E-cadherin function plays a critical role in the progression of SCCs[51,116,117]. A sequential loss of E-cadherin in mouse skin treated with both the two-stage carcinogenesis protocol and complete carcinogenesis with UV has been shown as lesions progress from dysplasia to SCCs and spindle cell carcinomas (Fig. 2d)[51,118,119]. In Figure 2, E-cadherin staining intensity is markedly reduced from normal skin epidermis to hyperplastic epidermis, papillomas, and differentiated SCCs. In poorly differentiated SCCs, E-cadherin staining is scattered in small isolated areas and its expression is significantly decreased in spindle cell carcinomas. Additionally, expression of markers of differentiation

as well as GGT is also quite low in poorly differentiated SCCs, while K8 expression is high (Fig. 2b, 2c, 2e, 2f).

Tumor response data from a two-stage skin carcinogenesis study may be displayed in a number of ways. To reflect the longitudinal nature of the experiment, tumor incidence may be plotted as either the percentage of mice bearing any papilloma over time or as fractional tumor-free survival over time[20]. Either plot highlights differences in tumor latency between groups. Additionally, overall tumor burden is displayed as tumor multiplicity over time (calculated as the total number of papillomas detected in a group divided by the total number of mice in the group). As an example, please see our previously published findings presented in Figure 3[120]. In this study, the effect of targeted deletion of *Bcl2l1* (which encodes Bcl-x_L) on skin tumor development in FVB mice was analyzed[120]. In this case, we utilized mice with floxed alleles for Bcl-x_L and targeted deletion of Bcl-x_L to the basal layer of the epidermis using the bovine keratin 5 (BK5) promoter. The mice were initiated with 25 nmol DMBA and promoted twice weekly with 6.8 nmol TPA. Bcl-x_L deficiency did not significantly affect tumor incidence (Fig. 3a) but resulted in significantly reduced tumor multiplicity (i.e., average number of papillomas per mouse) (Fig. 3b, $P < 0.05$ by Mann-Whitney U test). Similar plots may be constructed for the incidence and multiplicity of SCCs[20].

Mice are unlikely to experience adverse health effects due to papilloma burden during the analysis of tumor promotion. Less than 10% of the mice are expected to die during the course of the study; therefore, papilloma incidence and multiplicity are typically calculated by dividing either the number of tumor bearing mice or total number of papillomas by the number of mice alive at each week. In contrast, mice will likely die or require sacrifice due to the presence of SCCs. In this case, SCC incidence and multiplicity are calculated cumulatively. Any SCCs that appear are carried forward, even after tumor bearing mice are sacrificed or die, and the total number of SCCs is divided by the number of mice alive at the time that the first SCC was macroscopically observed. In some instances it may be desirable to score papilloma data cumulatively; in this case the data are handled similarly to that for SCCs. In addition to tumor incidence and multiplicity, a plot of the average tumor volume per mouse at an interim time point as well as at the conclusion of the tumor study can be a useful way to communicate alteration in tumor size that occurs in the absence of an effect on tumor incidence or multiplicity. Statistical analysis of differences in tumor multiplicity should be performed using non-parametric methods since tumor burden data is non-normative[20]. The Mann-Whitney *U* test is recommended. The χ^2 test is appropriate for comparing tumor incidence between groups.

VARIATIONS

Modifications of the traditional two-stage skin carcinogenesis protocol have been developed for a number of purposes. One purpose is to facilitate the study of tumor progression. For instance, it has been reported that papillomas that are at "high risk" for conversion to SCC can be generated and distinguished by gene expression profile from "low risk" papillomas[121,122]. In these protocols, SENCAR mice are initiated with 5 µg DMBA and then promoted once weekly with 2 µg TPA for varying amounts of time. Those papillomas that arise after only 5–10 weeks of TPA treatment are considered to be at high risk of conversion to SCC. Low risk papillomas are those papillomas that are present after 14–15 weeks of treatment with TPA.

Additionally, a "three stage" model has been suggested wherein tumor progression is accelerated by application of initiating agents to papillomas generated in the initiation-promotion protocol[53,123,124]. MNNG, 4-nitroquinoline-*N*-oxide (4-NQO), cisplatin, and

urethane have all been shown to enhance malignant progression of papillomas. In this protocol, SENCAR mice are initiated with 20 µg DMBA and then promoted weekly with 2.5 µg TPA for 10 weeks. During weeks 11–40, mice are either treated topically with 250 µg 4-NQO or injected intraperitoneally with 20 mg urethane. Under these conditions, more than 60% of the SENCAR mice will develop SCC by the end of the study Alternatively, CD-1 mice may be initiated with 50 µg DMBA and then promoted weekly with 10 µg TPA for 12 weeks. At week 13, the mice are given either a single intraperitoneal injection of 100 µg cisplatin or weekly treatments with the doses of mutating agents listed above. In CD-1 mice, these treatment protocols significantly increase the conversion rate but the overall number of carcinomas/mouse is lower since fewer papillomas form during the second stage, tumor promotion.

Acknowledgments

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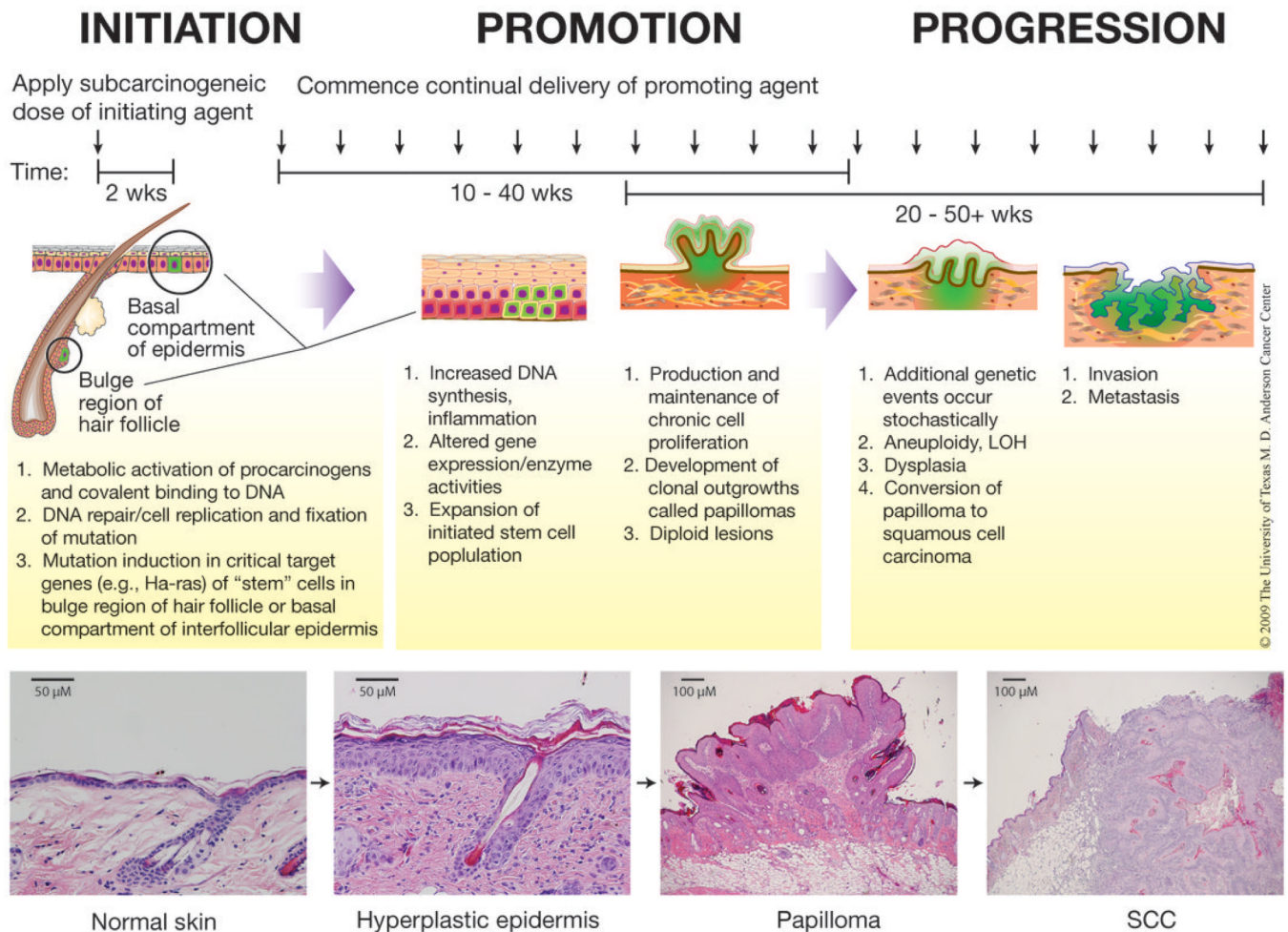
Figure 1. Two-stage model of skin carcinogenesis in mice
During initiation, topical application of a sub-carcinogenic dose of a mutating agent induces mutations in target genes in keratinocyte stem cells. Repeated topical application of a promoting agent begins two weeks after initiation and continues for the duration of the study. Papillomas begin to arise after approximately 6–12 weeks of promotion and a fraction begin to convert to SCC after approximately 20 weeks. Representative H&E stained sections of normal skin, hyperplastic skin, a papilloma, and a SCC are presented. All mice were handled in accordance with institutional and national regulations. This figure is a modification of a previously published figure[133].
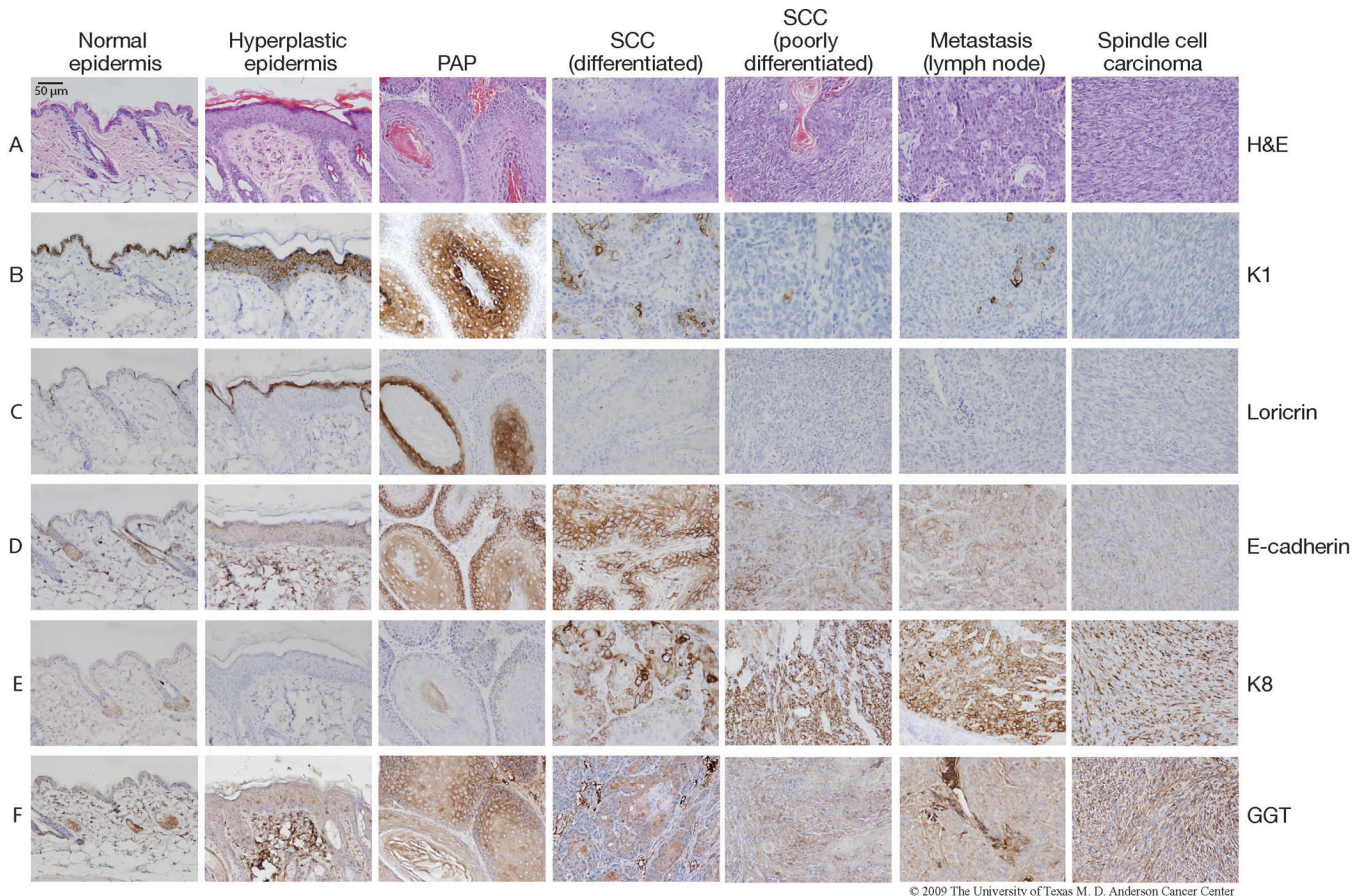
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Figure 2. Expression of several marker proteins in each sequential stage of skin carcinogenesis in mice

Tumor tissue was harvested from FVB mice that had undergone two-stage skin carcinogenesis initiated by DMBA and promoted by TPA. The tumors as well as hyperplastic dorsal skin from between tumors and untreated ventral skin were fixed in formalin and embedded in paraffin for immunohistochemical analyses. Representative images of normal epidermis, hyperplastic epidermis, papilloma (PAP), differentiated SCC, poorly differentiated SCC, lymph node metastasis and spindle cell carcinoma are shown. The lymph node metastasis was harvested from the same mouse bearing the poorly differentiated SCC. Immunostaining using the following antibodies was performed at the Histology Core at M.D. Anderson Cancer Center Science Park-Research Division as previously described[24]: Loricrin (Covance, Princeton, NJ), K1 (Covance), K8 (Origene, Rockville, MD), K15 (Covance), E-cadherin (Santa Cruz), and GTT (Abcam, Cambridge, MA). All mice were handled in accordance with institutional and national regulations.
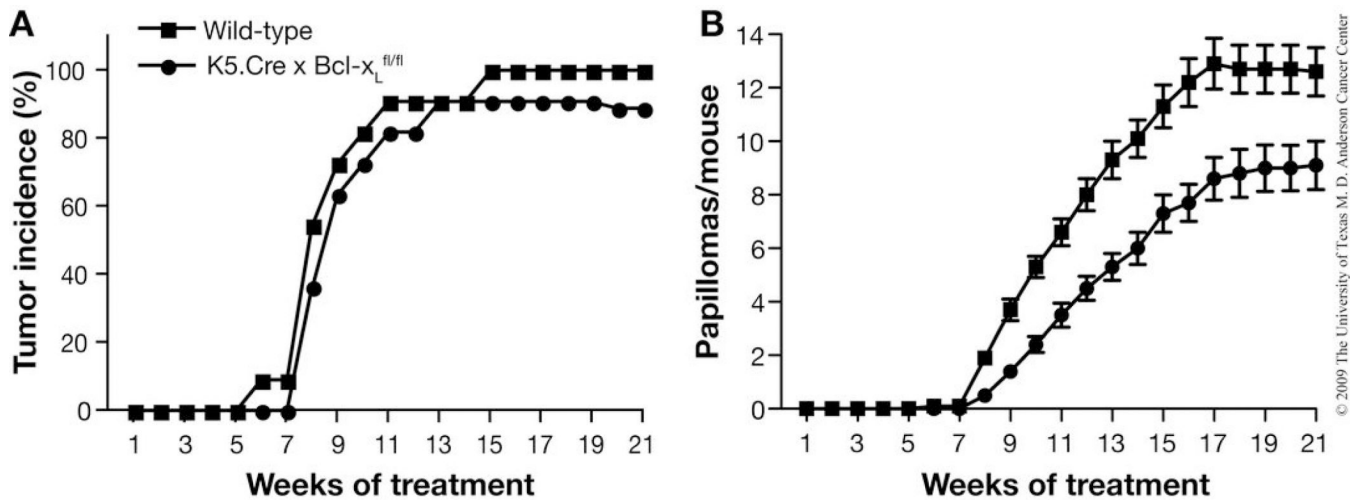
Figure 3.
Representative data from a previously-published two-stage skin carcinogenesis study in
FVB mice[120]. This study was designed to test the effect of Bcl-x_L deficiency on tumor
development in the two-stage skin carcinogenesis model. BK5.Cre × Bcl-x_L mice (lack Bcl-
x_L expression in the basal layer of the epidermis) and wild-type mice (n=11 per group) were
initiated with 25 nmol DMBA and promoted twice weekly with 6.8 nmol TPA. Tumor
incidence (A) and multiplicity (B) were monitored until the maximum papilloma response
was achieved (21 weeks). In panel B, the average number (mean ±SEM) of papillomas per
mouse is presented. Bcl-x_L deficiency significantly reduced tumor multiplicity ($P < 0.05$ by
Mann-Whitney U test). All mice were handled in accordance with institutional and national
regulations.

Table 1

Examples of chemical or physical agents that can serve as initiating or promoting agents and their primary molecular target or event.

Initiating agents	Genetic Target[a]	Promoting agents	Initial molecular target or event associated with tumor promotion
DMBA	*Hras1, Kras*	TPA	Protein Kinase C
Benzo[a]pyrene (B[a]P)	*Hras1*	Telocidin	Protein Kinase C
MNNG	*Hras1, Kras*	Okadaic acid	Protein Phosphatases -1 and -2A
N-methyl-*N*-nitrosourea (MNU)	*Hras1*	Chrysarobin	Generates oxidative stress
Bis(chloromethyl)ether	Unknown	Benzoyl peroxide	Generates oxidative stress
Ultraviolet radiation	*Tp53, Nras*	Ultraviolet radiation	Protein Kinase C, EGFR
Cisplatinum	*Hras1*	Wounding	Stimluation of EGF receptor
β-propiolactone	*Hras1*		

[a]Primary target based on use of TPA as the promoting agent or, in the case of UV, the use of a complete carcinogenesis regimen

Table 2

Expected tumor response of various mouse stocks and strains subjected to two-stage skin carcinogenesis using various initiating and promoting agents

Initiator Dose	Promoter Dose	Mouse Strain	% Papilloma Incidence	Papillomas/ Mouse	Reference
DMBA/TPA[a]					
25 nmol	1.7 nmol	SENCAR	100	21.7	125
0.1–100 nmol	8.5 nmol	SENCAR	20–100	0.6–24	126
0.1–100 nmol	8.5 nmol	CD-1	0–90	0–5.6	126
20–100 nmol	1–5 µg (1.6–8.1 nmol)	129/SvEv	100	10	127
10 nmol	6.8 nmol	DBA/2	94	5.7	128
400 nmol	1.7–6.8 nmol	DBA/2	15–100	0.25–8.67	129
25 µg (97.5 nmol)	4 µg (6.5 nmol)	DBA/2	83	3.6	130
100 µg (390 nmol)	2.5 µg (4 nmol)	FVB	97	12.7	131
97.4 nmol	32.4 nmol	FVB	100	32.5	59
100 nmol	6.8–13.6 nmol	FVB	100	20–25	132
100 nmol	3.4–6.8 nmol	FVB	54–100	1–10	20
25 nmol	5 nmol	FVB	100	10.9	23
97.4 nmol	32.4 nmol	PWK	0	0	59
10 nmol	6.8 nmol	C57BL/6	4	0.04	128
25 µg (97.5 nmol)	4 µg (6.5 nmol)	C57BL/6	42	2.2	130
25 µg (97.5 nmol)	4 µg (6.5 nmol)	I/LnJ	100	4.3	130
25 µg (97.5 nmol)	4 µg (6.5 nmol)	HRS	83	4.6	130
25 µg (97.5 nmol)	4 µg (6.5 nmol)	BALB/c	83	4.4	130
25 µg (97.5 nmol)	4 µg (6.5 nmol)	C3H/He	67	5.1	130
25 µg (97.5 nmol)	4 µg (6.5 nmol)	A/J	64	2.2	130
25 µg (97.5 nmol)	4 µg (6.5 nmol)	SJL	45	1.9	130
25 µg (97.5 nmol)	4 µg (6.5 nmol)	CBA	45	1.6	130
400 nmol	1.7–10 nmol	(C57BL6 × DBA/2)F$_1$ hybrid	0–100	0–11.8	129
DMBA/TPA[b]					
5–100 µg (19.5–390 nmol)	0.5–5 µg (0.8–8 nmol)	FVB	20–100	0–11.8	42

Initiator Dose	Promoter Dose	Mouse Strain	% Papilloma Incidence	Papillomas/ Mouse	Reference
25 µg (97.5 nmol)	2 µg (3.2 nmol)	SENCAR	100	18.8	42
25 µg (97.5 nmol)	2 µg (3.2 nmol)	CD-1	53	2.5	42
25 µg (97.5 nmol)	2 µg (3.2 nmol)	C57BL/6	0	0	42
25 µg (97.5 nmol)	2 µg (3.2 nmol)	BALB/c	17	< 1	42
DMBA/Chrysarobin[b]					
25 nmol	10–100 nmol	SENCAR	20–100	0.9–9.2	66
25 nmol	10–100 nmol	SSIn	4–79	0.04–1.83	66
25 nmol	100–880 nmol	DBA/2	33–58	0.7–1.5	66
25 nmol	100–880 nmol	C57BL/6	0–46	0–0.5	66
DMBA/Benzoyl peroxide[a]					
25 nmol	5–20 mg	SENCAR	60–88	4.4–7.2	66
25 nmol	5–20 mg	SSIn	96–100	11.7–14.3	66
25 nmol	10–40 mg	DBA/2	35–44	0.5–0.6	66
25 nmol	10–40 mg	C57BL/6	18–39	0.4–0.6	66
DMBA/Telocidin[a]					
25 nmol	0.43–1.7 nmol	SENCAR	4–31	0.24–088	67
25 nmol	0.85–3.4 nmol	C57BL/6	4–15	0.04–0.19	67
MNNG/TPA[a]					
2.5 µmol	6.8 nmol	SENCAR	83	3.3	94
2.5 µmol	1.7–10 nmol	CD-1	0–80	0–2.2	66
2.5 µmol	17.0 nmol	C3H/He	97	1.6	94
2.5 µmol	1.7–13.6 nmol	DBA/2	33–95	0.9–1.65	129
2.5 µmol	3.4–17.0 nmol	C57BL/6	0–10	0–0.1	129
2.5 µmol	1.7–13.6 nmol	(C57BL.6 × DBA/2)F$_1$ hybrid	5–90	0.5–1.3	129
MNNG/Chrysarobin[b]					
2.5 µmol	25–440 nmol	SENCAR	48–90	0.9–4.5	66
2.5 µmol	440–880 nmol	SSIn	8–21	0.08–0.2	66
2.5 µmol	25–440 nmol	CD-1	0–5	0–0.05	66

Initiator Dose	Promoter Dose	Mouse Strain	% Papilloma Incidence	Papillomas/ Mouse	Reference
2.5 µmol	440–880 nmol	DBA/2	25	0.25–0.32	66
2.5 µmol	440–880 nmol	C57BL/6	21–24	0.25	66
MNNG/Benzoyl Peroxide[a]					
2.5 µmol	10–20 mg	SENCAR	20–52	0.5–2.1	66
2.5 µmol	10–20 mg	SSIn	83–92	2.4–2.6	66

[a] Promoter applied twice weekly

[b] Promoter applied once weekly

Table 3

TROUBLESHOOTING

Step	Problem	Possible Reason	Solution
1–16	Wounds noted on dorsal skin	Mice are fighting	Separate the aggressor, individually house all males and aggressive females; Interpret results with caution since wounding promotes tumor development
13–16	Weight loss	Toxicity associated with chemotherapeutic or chemopreventive agent	Lower the dose of chemopreventive or chemotherapeutic agent; Interpret results with caution since negative energy balance inhibits tumor response
		Lack of food consumption; Dietary delivery of chemotherapeutic or chemopreventive agent has made chow less palatable	Change route of delivery; Interpret results with caution since negative energy balance inhibits tumor response
		Cachexia due to large tumor burden or malignant progression	Sacrifice individual mice and harvest tissue; terminate the experiment if indicated by IACUC regulations. Note: Papilloma counts may be unreliable after the appearance of SCC.
			In subsequent experiments reduce the dose of DMBA and/or TPA
12–16	No tumors arise in positive control group	Resistant strain was used as a positive control; Dose of initiating or promoting agent too low	Use a sensitive strain, backcross transgene or gene deficiency allele to sensitive strain such as FVB. Increase the doses of DMBA/TPA.
		Improper composition, storage, or handling of DMBA or TPA solutions	Verify solution composition; Prepare a fresh stock of DMBA for each initiation and protect from light; Store TPA solutions at −20°C.
12–16	Predicted effect of therapeutic or preventative agent or genetic manipulation is not detected	True negative result	Do nothing
		False negative results may occur if the doses of DMBA/TPA overwhelm the potential preventative or therapeutic effect of treatment or genetic manipulation	Lower the dose of DMBA/TPA
		Inadequate statistical power	Recalculate the necessary sample size based upon newly collected preliminary data
12–16	Abnormal lesions form	Transgene or inhibitor conferred unexpected gross appearance of papillomas	Harvest tumors tissues, cut sections and perform histologic investigation.
12–16	Papillomas arise but a portion regresses	Tumor regression is normal and the rate at which it occurs depends upon the strain utilized	Adjust tumor incidence and multiplicity calculations as necessary