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Modeling Cutaneous Squamous Carcinoma Development in the Mouse

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Cutaneous squamous cell carcinoma (SCC) is one of the most common cancers in Caucasian populations and is associated with a significant risk of morbidity and mortality. The classic mouse model for studying SCC involves two-stage chemical carcinogenesis, which has been instrumental in the evolution of the concept of multistage carcinogenesis, as widely applied to both human and mouse cancers. Much is now known about the sequence of biological and genetic events that occur in this skin carcinogenesis model and the factors that can influence the course of tumor development, such as perturbations in the oncogene/tumor-suppressor signaling pathways involved, the nature of the target cell that acquires the first genetic hit, and the role of inflammation. Increasingly, studies of tumor-initiating cells, malignant progression, and metastasis in mouse skin cancer models will have the potential to inform future approaches to treatment and chemoprevention of human squamous malignancies.

Nonmelanoma skin cancer, comprising SCC and basal cell carcinoma (BCC), is by far the most common cancer among Caucasian people, with a recorded incidence in 2006 of more than 3 million in the U.S. alone (Rogers et al. 2010). Of the two subtypes, SCC is the more aggressive with a significant risk of metastasis, and is associated with greater morbidity and mortality.

SCC most commonly arises on sun-exposed areas of the skin, but is also a feature of several hereditary disorders, including multiple self-healing squamous epithelioma (MSSE), which afflicts individuals with mutations in the *TGFBR1* gene coupled with rare variants in an adjacent region of Chromosome 9q22.3 (Goudie et al. 2011; Kang et al. 2013); a form of XX-male sex reversal that is caused by mutations in the *RSPO1* gene (Parma et al. 2006); and a number of diseases characterized by genome instability, such as dyskeratosis congenita (DKC), where SCC may arise as a consequence of elevated genome mutation rates.

Recent somatic mutation analyses of sporadic skin SCCs have revealed frequent mutations in *NOTCH1*, *NOTCH2*, *TP53*, *CDKN2A*, *HRAS*, and *KRAS* (Durinck et al. 2011; Mauerer et al. 2011; Wang et al. 2011). These new studies emphasize the strong similarities between the mutation spectra in human squamous tumors at different sites including the skin, head and neck, and lung (Agrawal et al. 2011; Stransky et al. 2011; The Cancer Genome Atlas Research Network 2012). Although most nonmelanoma

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skin cancer can be successfully treated with surgery and/or chemotherapy, metastatic SCC is associated with an extremely poor long-term prognosis, with a 10-yr survival rate of <20% (Alam and Ratner 2001). To develop better clinical treatments and chemoprevention strategies for SCC, there is a need to achieve a better understanding of the biology of the disease through animal models.

In this review, we describe the mouse models that have been developed to study cutaneous SCC, in particular the DMBA/TPA chemical carcinogenesis model. We discuss how this model has been used to address the central question of target cell for tumor initiation, an effort that has been greatly aided over the years by the development of transgenic mouse technology. Additionally, we discuss how the model has been used to dissect the functions of Ras effectors and to investigate tumor-suppressor pathways that operate to inhibit SCC development. Finally, we describe how the DMBA/TPA model has been used to examine the role of inflammation in skin carcinogenesis.

UV RADIATION CARCINOGENESIS

Although the majority of mouse skin cancer models involving carcinogens have used chemical mutagens and promoters, it is nevertheless the case that 90% of nonmelanoma skin cancer is estimated to be attributable to excessive exposure to UV radiation (Grossbart and Lew 1996). To examine whether UV radiation can induce SCC in mice, several groups used hairless but immunocompetent *SKH-1* mice, which are well suited for this purpose because they lack the dense, UV-impenetrable hair coat of wild-type mice. Together, these studies showed that UV exposure is capable of inducing SCC in *SKH-1* mice, in a dose-, exposure time-, and wavelength-dependent manner (for review, see Van Kranen and De Gruijl 1999). The SCCs that arise commonly have *Trp53* mutations, which closely recapitulate the *TP53* mutations seen in sporadic human SCCs (van Kranen et al. 1995).

However, it remains unclear what other mutations arise in this model to cooperate with *Trp53* mutations in driving tumorigenesis. For instance, while *RAS* mutations are seen in 3%–25% of human SCC cases (Khavari 2006; Durinck et al. 2011; Mauerer et al. 2011), they are extremely rare in this model (van Kranen et al. 1995). Work in this area is hampered by the fact that *SKH-1* mice have a nonfunctional *Hairless* (*Hr*) gene, and although this confers a practical advantage for experiments involving controlled exposure to UV light on a daily basis, the *Hr* gene plays an important role in skin metabolism (Kumpf et al. 2012), and its absence may influence the pathways by which tumors develop in this model. The remainder of this review will focus on chemical/genetic models of SCC development.

CHEMICAL INDUCTION OF SKIN TUMORS

The first links between chemical exposure and the development of skin cancers (Pott 1775) prompted early attempts to develop tractable models for the study of chemically induced skin cancers. Chemical carcinogenesis of the skin has since emerged as the workhorse model of SCC, having been used to test hundreds of individual hypotheses across a wide range of topics in cancer biology. It has played a pivotal role in the evolution of the concept of multistage carcinogenesis, as commonly applied to both human and mouse cancers, and has given rise to the operational definitions of the key tumor processes of initiation, promotion and malignant progression (for reviews, see Boutwell et al. 1982; Yuspa 2000).

In the most commonly used model, a typical treatment regimen involves first a single topical dose of the carcinogen dimethylbenzanthracene (DMBA), which introduces the initiating mutation to certain cells in the skin. This is then followed by repeated administration of a pro-inflammatory phorbol ester such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which promotes the selection and growth of initiated cells into benign tumors known as papillomas (Fig. 1). With time, some of the papillomas will progress to malignant SCCs, which can further disseminate to distant sites via metastasis. Some SCCs can also convert to a more aggressive form of tumor known as spindle carcinoma,

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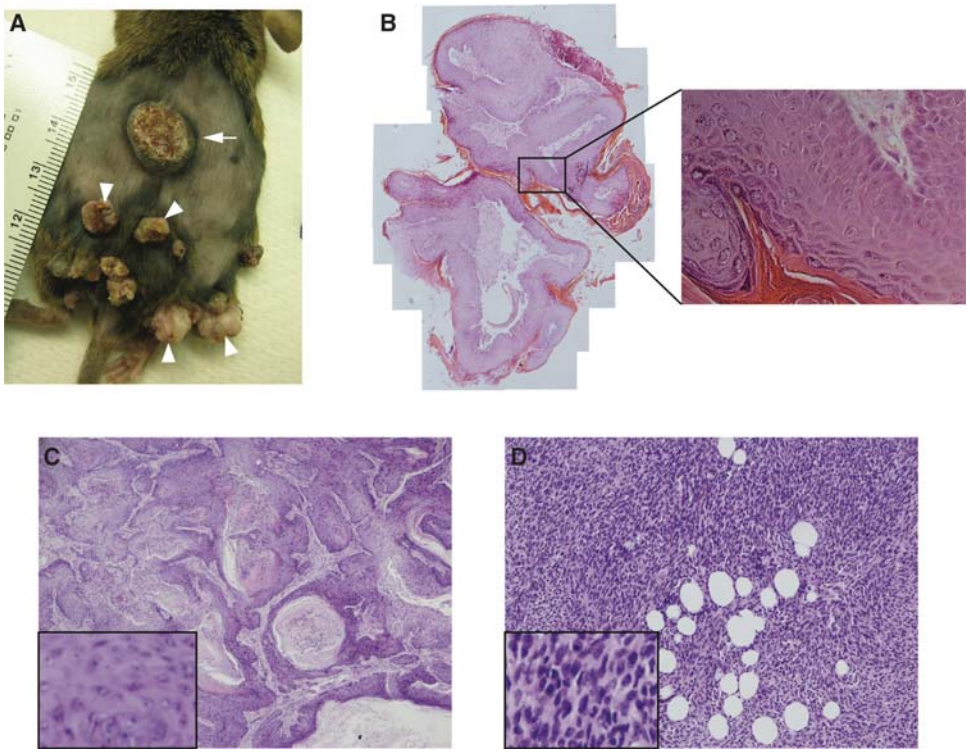

Figure 1. Macroscopic and histological views of cutaneous papillomas and SCCs. (*A*) Picture of the back skin of a DMBA/TPA-treated mouse showing papillomas (arrowheads) and an SCC (arrow). Note the exophytic, nodular appearance of the papillomas, and the crater-like architecture of the SCC. (*B*) Papillomas do not breach the underlying epithelial basement membrane and show a high degree of keratinization. (*C*) Class A tumors include pure squamous cell carcinomas and squamous tumors with a small spindle component. This particular class A tumor is well-differentiated with heavy keratinization. (*D*) Class B tumors are mostly pure spindle cell carcinomas, which are poorly differentiated with spindle-shaped cells that resemble fibroblasts. (*C* and *D* are based on data from Wong et al. 2013.)

via epithelial-mesenchymal transition (EMT) (Klein-Szanto et al. 1989). Besides DMBA, other chemical carcinogens that have been used to initiate skin cells include methylnitrosourea (MNU) and 3-methylcholanthrene (MCA), each of which induces carcinogen-specific mutations in *Hras* (Brown et al. 1990).

Although the two-stage DMBA/TPA model does not mimic the precise sequence of events that occur in spontaneous human SCC, it is highly reproducible and can be easily adapted to test various genetic and environmental factors that may affect tumor development. Moreover, it offers the advantage that the distinct tumor stages can be easily discerned and separately studied in the context of multistage carcinogenesis. Accordingly, the DMBA/TPA model has been extensively characterized in terms of the series of biological and genetic events that occur during tumor growth.

For instance, a specific mutation in codon 61 of *Hras* (*Q61L*) was identified as the initiating mutation in the two-stage mouse skin model (Balmain and Pragnell 1983; Balmain et al. 1984; Quintanilla et al. 1986). Evidence for this includes the demonstration that a viral version of activated *Hras* is sufficient to induce tumors in infected, TPA-promoted skin (Brown et al. 1986), and the observation that different mutagens produce different activating mutations in *Hras,* but can all effect tumor initiation (Brown et al. 1990). Interestingly, although *RAS*

mutations in general are relatively rare in human SCCs, the recent flurry of whole genome and exome sequencing studies of human cancers have identified the *Q61L* mutation in *HRAS* as the most common *RAS* mutation in squamous cancers of the skin, head and neck, and lung (Agrawal et al. 2011; Durinck et al. 2011; Mauerer et al. 2011; Stransky et al. 2011; Wang et al. 2011).

During the promotion phase, the papillomas that arise frequently show trisomy of chromosome 7 (Aldaz et al. 1989), where the *Hras* gene is located. Further, the duplicated chromosome is invariably that which bears the mutant *Hras* allele, which thus suggests a strong preference for amplification of the mutant *Hras* allele (Bremner and Balmain 1990). SCCs, on the other hand, often show mutation and loss of heterozygosity (LOH) of the tumor suppressor *Trp53* that are not seen in papillomas, which indicates a role for *Trp53* mutation or loss in malignant progression (Buchmann et al. 1991; Burns et al. 1991).

Apart from the classical route to squamous carcinoma (henceforth, class A carcinoma) formation outlined above, recent work in our laboratory has uncovered an alternative route to invasive carcinoma (henceforth, class B carcinoma) development (Wong et al. 2013). A genetically heterogeneous population of mice was treated with the DMBA/TPA protocol, and unsupervised hierarchical clustering of the gene expression profiles obtained from >60 carcinomas collected from these mice identified two distinct molecular categories. Class A carcinomas include pure squamous cell carcinomas and squamous tumors with a small spindle component, whereas class B carcinomas are the most aggressive carcinomas that develop in the DMBA/TPA model, composed primarily of pure spindle cell carcinomas. As expected from their spindle morphology, class B carcinomas express EMT markers, such as *Snai1*, *Zeb1,* and *Vimentin*; however, unlike class A carcinomas, class B carcinomas are characterized by loss of the *Ink4/Arf* locus and have, paradoxically, down-regulated components of the canonical HRas signaling pathway despite their increased invasiveness (although Mapk and Akt signaling remain elevated). Class B carcinomas are also less dependent on inflammation for their formation (discussed in detail later), and thus may represent a different category of tumors that diverge from the class A pathway soon after initiation or arise from a separate target cell altogether (Fig. 2).

The vast body of scientific work utilizing the DMBA/TPA model gives us the unique opportunity to compare and dissect the influence of many biological and genetic factors on tumor development. Nevertheless, when attempting to interpret the results of various DMBA/TPA studies, one should bear in mind the important caveat that mouse strain background has a considerable influence on tumor susceptibility and outcome. For instance, *Mus spretus* and C57BL/6 mice are known to be tumor-resistant, whereas FVB/N mice have been shown to be skin tumor-susceptible (Hennings et al. 1993). The difference in sensitivity between C57BL/6 and FVB/N mice to Ras-induced skin carcinogenesis, for instance, has been mapped to a polymorphism in the *Ptch* gene, which affects binding to the tumor suppressor Tid1 and consequently Ras-induced apoptosis (Wakabayashi et al. 2007). Hence, without prior knowledge of the mouse genetic backgrounds involved, one should exercise some caution when comparing the effects observed in different studies.

TARGET CELL FOR INITIATION

The identity and nature of the target cell that acquires the first genetic hit leading to initiation is a central question in cancer biology (for review, see Perez-Losada and Balmain 2003). Many tumors contain rare cells that express stem cell markers and are capable of long-term self-renewal; these cells have also been shown to initiate secondary tumors in limiting dilution transplantation assays and even generate large parts of the tumor in situ (Chen et al. 2012; Driessens et al. 2012; Lapouge et al. 2012; Schepers et al. 2012). Together, these observations are compatible with the notion of so-called "cancer stem cells," which act as tumor cells of origin and fuel the growth of the tumor (Lapidot et al. 1994). Further, it has been hypothesized

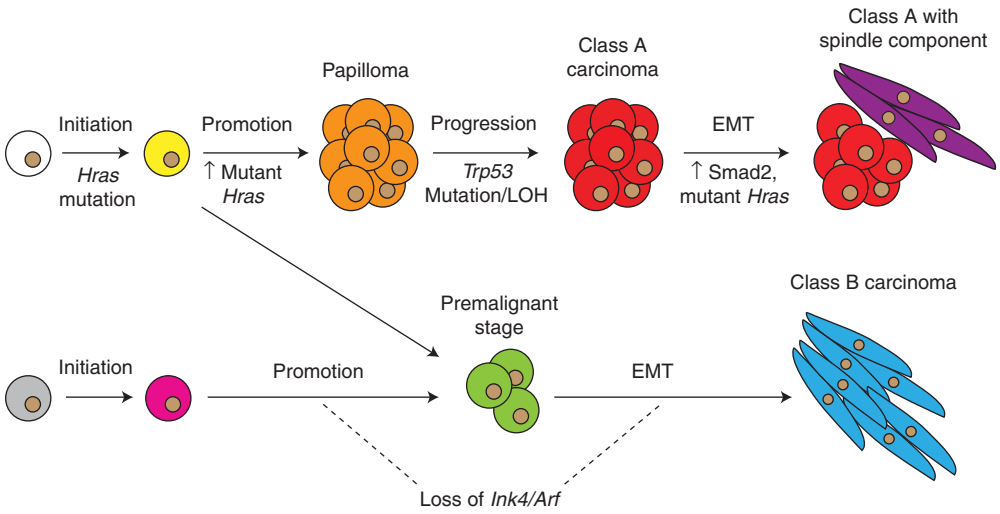

Figure 2. Genetic and molecular events during multistage skin carcinogenesis. Class A and class B carcinomas may arise from the same target cell, but diverge soon after initiation resulting in different premalignant stages. Alternatively, class A and class B carcinomas may come from distinct target cells, with possibly different initiating mutations in these cells. Spindle carcinoma formation via the class A route is thought to involve increased mutant *Hras* levels and nuclear accumulation of Smad2 (Oft et al. 2002); in contrast, in class B spindle carcinomas, mutant *Hras* levels are down-regulated instead.

that they arise from normal adult stem cells that become transformed during the tumor initiation phase, because both cell populations have a high capacity for self-renewal and clonogenic growth. However, it remains possible that cancer stem cells may actually originate from the initiation of more committed progenitor cells, which only acquire stem cell–like characteristics after undergoing oncogenic insult or exposure to inflammatory stimuli (for review, see Gupta et al. 2009; Gonzalez-Suarez et al. 2010; Schramek et al. 2010).

One of the first studies to shed light on this question of the target cell for initiation in the DMBA/TPA model was performed by Berenblum and Shubik (1949). The authors initiated mouse skin tumors with DMBA but allowed 1 yr to elapse before treating the mice repeatedly with croton oil (from which TPA was eventually isolated). They observed that papillomas developed with approximately the same latency and yield as they did in control mice that were treated immediately with the promoter, indicating that the initiated cells remained in the skin for most of the murine lifespan. This central finding of the "permanence" of the initiating event was subsequently corroborated by other groups, who accounted for confounding factors such as age at time of promotion (Van Duuren et al. 1975) and used intragastric DMBA administration to avoid topical DMBA-induced wound healing, which is thought to possess promoting activity (Loehrke et al. 1983). Importantly, these demonstrations that initiated cells can reside in the skin for long periods of time without giving rise to visible lesions until selection and growth through promotion are consistent with stem cells being the initiated target cells, as only stem cells have the self-renewal potential to allow for such long-term persistence (although, as noted above, it remains possible that stem cell properties may be induced by oncogenic events).

Another early study to address the question of target cell uses the process of epidermal abrasion, which involves using a tool, such as a motorized felt wheel, to remove the interfollicular epidermis (IFE) while leaving the hair follicles intact (Argyris 1985). This process will remove all the terminally differentiating cells in the IFE, while sparing the various stem cell populations

that have been shown to reside in the hair follicle (for review, see Jaks et al. 2010). One week after initiation with DMBA, mice were either abraded as the experimental group or left unabraded as the control group; TPA treatment was then started 4 wk later, when the IFE had regenerated from cells in the hair follicles and returned to normal (Morris et al. 2000). In this study, the authors observed that abraded mice developed far fewer papillomas than unabraded mice; however, the carcinoma responses of the two groups were similar. One interpretation of these data is that benign papillomas arise from initiated cells in the IFE, while most malignant SCCs arise from initiated cells in the hair follicle (including possibly stem cells). Together, these early studies point to a role for the identity of the target cell in influencing the course of tumor development.

TRANSGENIC MICE AND SCC DEVELOPMENT

With the advent of transgenic mouse technology (for reviews, see Palmiter and Brinster 1986; Hanahan et al. 2007) and the cloning of keratin promoters capable of directing transgene expression to specific skin compartments (Vassar et al. 1989; Fuchs et al. 1992; Byrne and Fuchs 1993), it soon became possible to target oncogenes to subpopulations of cells in the skin, to test the ability of these cells to initiate tumor formation. Early examples of this approach were the demonstrations that targeting of mutant *Hras* to even terminally differentiating cells in the skin (using the *K10* or *K1* promoter) could give rise to papillomas, which, however, did not progress to malignancy (Bailleul et al. 1990; Greenhalgh et al. 1993). In contrast, when mutant *Hras* was expressed in the outer root sheath (ORS) of the hair follicle (using a truncated version of the *K5* promoter), where stem cells have been shown to reside, malignant SCCs and spindle carcinomas arose (Brown et al. 1998).

One common criticism of these early transgenic mouse studies is that the oncogene of interest was vastly overexpressed in subpopulations of cells. This problem can now be circumvented through knock-in of mutant alleles into their endogenous loci, which allows the mutant alleles to be subsequently activated in subsets of cells via removal of a *Stop* cassette by a tissue-specific Cre recombinase (Jackson et al. 2001). Using this approach, several groups targeted the expression of an activated form of *Kras* (*KrasG12D*) to different cell populations within the skin (Caulin et al. 2007; Lapouge et al. 2011; White et al. 2011). When oncogenic *Kras* was expressed in terminally differentiating suprabasal cells of the IFE (using *Involucrin-Cre*), or cell populations with stem cell characteristics such as basal keratinocytes (using *K5-Cre*) and hair follicle bulge stem cells (using *K15-Cre* or *K19-Cre*), papillomas developed. However, when oncogenic *Kras* was expressed in transit amplifying hair matrix cells (using *Shh-Cre*) (Lapouge et al. 2011; White et al. 2011), no papillomas arose. More recently, expression of *KrasG12D* in hair follicle junctional zone stem cells (Jensen et al. 2009) using *Lrig1-Cre* has also been shown to be capable of giving rise to papillomas when combined with full-thickness back wounding (Page et al. 2013). Importantly, although these studies reinforce the idea that the identity of the target cell has a role to play in determining the course of tumor development, they also indicate a certain degree of plasticity among different stem cell populations during tumorigenesis, such that many of them are capable of giving rise to tumors when engineered to express an appropriate oncogene.

An interesting feature of these oncogene-targeting studies is that activation of Ras gives rise to tumors generally in the dorsal skin, but not usually in the tail skin, despite the activity of promoters of genes such as *K14* and *Involucrin* in tail epidermis (Youssef et al. 2010; Lapouge et al. 2011; White et al. 2011; Gomez et al. 2013). These data are compatible with previous observations of resistance of tail skin to DMBA/TPA carcinogenesis (Schweizer and Marks 1977). The reasons for this discrepancy between tail skin and back skin may give us additional information about the nature of the target cells for carcinogenesis, particularly as activation of the *Smoothened* (*Smo*) gene using the same *K14* promoter gives rise to basal cell carcinomas (BCCs)

predominantly in tail but not in dorsal epidermis (Youssef et al. 2010).

A final point of contention with transgenic mouse studies is that oncogene activation generally occurs in whole populations of cells in the targeted cell compartment, rather than in the clonal fashion that presumably characterizes spontaneous or carcinogen-induced tumors. The fact that a particular cell compartment is capable of giving rise to tumors when engineered to express an oncogene is not synonymous with those cells being bona fide target cells during carcinogen-induced tumor development. Single initiated cells carrying *Ras* mutations may have to "outcompete" their neighbors to expand within the stem cell niche (Vermeulen et al. 2013) and this restriction is presumably absent after coordinated activation of a *Ras* oncogene in multiple cells within a compartment. A better approach to study the target cell for initiation may be to use neutral labeling methods to mark the potential target cell a priori, followed by carcinogen treatment and lineage tracing of progeny cells that carry the permanent label during subsequent tumor development.

An early study used a neutral labeling approach to address the question of whether chemically induced skin papillomas have a polyclonal origin (Winton et al. 1989). The authors first generated chimeric mice by aggregating embryos of two different strains. These mice show mosaicism in their tissues, including skin. By subjecting these mice to the DMBA/TPA protocol and performing immunohistochemical analyses, the authors were able to show that about 30% of the papillomas that arose have a mixture of cells from both parent embryos. Hence, they conclude that a significant proportion of papillomas are polyclonal in origin. A similar approach was adopted by Arwert et al. (2010) to investigate the contribution of terminally differentiating cells to tumorigenesis in InvEE mice, which overexpress activated Mek1 in the suprabasal layer of the IFE. Here, the authors generated chimeras by aggregating InvEE embryos and GFP-positive embryos, and showed that proliferative, transgene-negative (but GFP-positive) cells are fully incorporated into the tumors that develop on wounding; these transgene-negative cells thus actively contribute to tumor formation, and are stimulated to do so by non-cell-autonomous signals from the Mek1-expressing, terminally differentiating cells.

More recently, a neutral labeling method was also used to determine if bulge stem cells are the cells of origin of chemically induced skin tumors (Li et al. 2012). In this study, the authors initiated *Krt1-15CrePR1;R26R* mice with DMBA, then activated Cre recombinase with the drug RU486 1 wk later; finally, tumor promotion was performed with biweekly administration of TPA for 20 wk. When the authors collected tumors from the mice and performed LacZ staining to trace the progeny of the $K15^+$ bulge stem cells, they observed that all papillomas after 20 wk of TPA treatment contained a mixture of nonblue and blue cells, with the latter making up, on average, ~30% of papilloma cross-section area. This suggests that $K15^+$ stem cells may have the potential to contribute long-term to papillomas in the DMBA/TPA model. However, there are a few caveats to this study. One, there is a significant level of promoter leakiness in the *Krt1-15CrePR1;R26R* mice, which makes it difficult to determine the true contribution of the $K15^+$ stem cells to papillomas. Second, Cre recombinase was activated only after DMBA treatment, in which case initiated progenitor cells may acquire stem cell properties and start expressing K15 de novo. Finally, microdissection suggested that even in papillomas containing blue marked cells, only 30% of these labeled cell populations carried the known *Hras* initiating mutation. It is presently unclear whether this interesting heterogeneity is caused by technical issues or has a real biological basis.

Another recent study that uses a similar but distinct approach was performed by Driessens et al. (2012). They treated *K14CreER/Rosa-YFP* mice with the DMBA/TPA protocol and then initiated lineage tracing in the resulting papillomas and SCCs at clonal density, by administering a low dose of tamoxifen (Driessens et al. 2012). Through 3D reconstruction of whole clones from serial sections, they report the existence of two proliferative cell compartments in papillomas, mirroring the hierarchy seen in

normal tissues—a slower-cycling "progenitor cell" fraction that is shorter-lived and gives rise to smaller clones, and a faster-cycling "stem cell" fraction that persists longer and gives rise to larger clones. In contrast, in SCCs, the authors report the existence of a single proliferative cell population that expands geometrically and has a low probability of terminal differentiation. One caveat to this study is that the lineage tracing results reported are for relatively short periods of time (9 d for SCCs, and up to 7 wk for papillomas). It remains to be seen if the same tracing patterns will hold up over longer periods of time and qualify the observed proliferative cell compartments as arising from bona fide cancer stem cells. Moreover, because lineage tracing is only initiated after tumor formation, several important questions remain outstanding. For example, what is the relationship of the $K14^+$ proliferative cell populations reported in this study to stem cell compartments in normal skin? Also, what is the nature of the target cells that lead to benign or malignant tumors and how may these differ?

Taking into account all the studies described in this and the preceding section, a picture emerges of the existence of a continuum of target cells amenable to initiation. Here, the course of tumor development, in terms of cell fate decisions and malignant potential, is likely a function of both the nature of the initiating mutation and the identity of the target cell. Hence, although initiated terminally differentiating cells are capable of giving rise to papillomas, these often lack the propensity to progress to malignancy; ultimately, the promotion of initiated multipotent stem cells may be required to induce malignant SCCs.

RAS EFFECTORS IN SKIN CARCINOGENESIS

The highly reproducible mutational activation of *Hras* in the DMBA/TPA model, together with the demonstration of the causality of this event in initiating carcinogenesis, has led to the widespread use of this model for testing the functional roles of a wide variety of components of the Ras signaling pathway. Besides mutant *Hras*, transgenic expression of a number of upstream activators of Ras in the skin has also been reported to be capable of inducing tumors. In particular, mice that overexpress *TGF-α* in their epidermis develop papillomas on wounding or TPA treatment (Vassar et al. 1992; Dominey et al. 1993). TGF-α is the ligand for the EGF receptor, which activates Ras through the guanine nucleotide exchange factor SOS (Fig. 3). Importantly, the papillomas that arise in *TGF-α* transgenic mice do not have a mutation in *Hras*, which suggests that activation of the Ras pathway through *TGF-α* overexpression is sufficient to induce skin tumor initiation in the absence of *Hras* mutational activation. Consistent with this idea, most sporadic human SCCs show activated Ras signaling, despite *Ras* being mutationally activated in only a small subset of these tumors (references above). Interestingly, the papillomas from the *TGF-α* transgenic mice also have the tendency to regress and were never observed to progress to malignancy. More recently, *K5-SOS-F* transgenic mice that overexpress a dominant form of *SOS* in basal keratinocytes were generated and these develop spontaneous papillomas with 100% penetrance (Sibilia et al. 2000).

The roles of Raf-MAPK and PI3K-Akt signaling downstream from Ras have been extensively characterized. Raf-1 is the first identified and most intensively studied Ras effector. It activates mitogenic MAPK signaling, leading to induction of the Ets family of transcription factors and Cyclin D1. PI3K-Akt signaling, on the other hand, activates mTOR, with downstream consequences for protein synthesis and cell growth. Apart from Raf-1 and PI3K, Ras has a number of other known effectors (Fig. 3; Table 1) whose in vivo functions remain relatively poorly understood. Here, use of the DMBA/TPA model has again been instructive.

For instance, Ras binds and activates PLC-ε, which produces the second messengers diacylglycerol and inositol 1,4,5-triphosphate. These, in turn, activate protein kinase C (PKC) and mobilize intracellular calcium, respectively. PKC is known to have a role in skin tumor promotion, because TPA is known to bind and regulate PKC (Castagna et al. 1982; Fournier and Murray

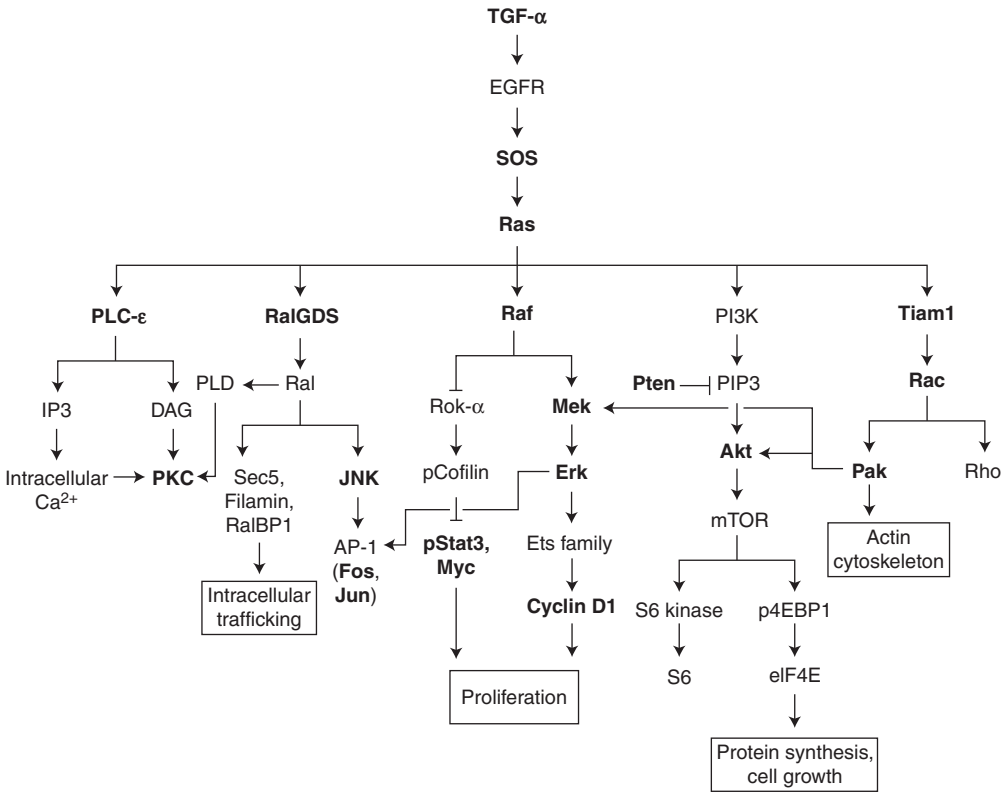

Figure 3. Ras effector pathways in skin carcinogenesis. Signaling components in bold have been directly investigated for their in vivo functions during skin tumor development. The roles of TGF-α, SOS, Ras, PLC-ε, RalGDS, Raf-1, Stat3, Pten, and Tiam1 are described in detail in the text. The rest are summarized in Table 1.

1987; Hansen et al. 1990), which in turn suggests a potential role for *PLC-ε* in tumorigenesis downstream from Ras activation. When treated with the DMBA/TPA protocol, *PLC-ε*-deficient mice developed papillomas with increased latency, reduced yield and a decreased propensity to undergo malignant conversion (Bai et al. 2004). This indicates that PLC-ε has far-ranging roles in skin carcinogenesis, impinging on both the promotion and malignant progression stages.

Ras also binds and activates Tiam1, which is a guanine nucleotide exchange factor that in turn activates Rac GTPases. In vitro, Rac GTPases are implicated in cell migration and cell-cycle progression (Olson et al. 1995, 1998; Hordijk et al. 1997; Keely et al. 1997). When treated with the DMBA/TPA protocol, *Tiam1*$^{-/-}$ mice were resistant to tumor development, and this was attributed to increased apoptosis during the initiation stage and reduced proliferation during the promotion stage (Malliri et al. 2002). However, *Tiam1*$^{-/-}$ mice also had a higher ratio of malignant to benign tumors, which thus indicates a biphasic role for Tiam1 in skin carcinogenesis. Interestingly, *Tiam1* is down-regulated in class B carcinomas (Wong et al. 2013). Hence, while papilloma formation via the classical route is impeded by *Tiam1* deficiency, the higher percentage of malignant tumors seen in DMBA/TPA-treated *Tiam1*$^{-/-}$ mice could be a reflection of their origin as class B carcinomas. Indeed, when the authors treated *Tiam1*$^{-/-}$ mice with an alternative carcinogenesis protocol that involved repeated treatment with DMBA in the absence of TPA (known as the complete carcinogenesis protocol), they obtained a higher percentage of

Table 1. Summary of the in vivo functions of select genes downstream from Ras during skin carcinogenesis

Gene	Mouse model	Phenotype	References
Akt	*Akt1* knockout	In the DMBA/TPA model, *Akt1*$^{-/-}$ mice develop tumors with reduced yield and size	Skeen et al. 2006
	Overexpression of wild-type and constitutively active *Akt1* in the basal layer of stratified epithelia using the bovine *K5* promoter	Epidermal hyperplasia and hyperkeratosis, and enhanced keratinocyte proliferation in response to TPA treatment; heightened tumor susceptibility in the DMBA/TPA model	Segrelles et al. 2007
Cyclin D1	*Cyclin D1* knockout	*Cyclin D1*$^{-/-}$ mice develop papillomas with increased latency and reduced incidence and yield in the DMBA/TPA model	Robles et al. 1998
Erk	*Erk1* knockout	*Erk1*$^{-/-}$ mice show reduced skin inflammation and proliferation in response to TPA treatment and are tumor-resistant in the DMBA/TPA model	Bourcier et al. 2006
Fos	*c-fos knockout*	*c-fos*-deficient papillomas quickly become dry and hyperkeratinized, and fail to progress to malignancy	Saez et al. 1995
	K5-driven overexpression of a dominant-negative form of *c-fos* (*A-Fos*)	Mild alopecia and sebaceous gland hyperplasia; when subjected to chemical carcinogenesis, mice develop predominantly sebaceous adenomas	Gerdes et al. 2006
Jnk	*Jnk1* and *Jnk2* knockouts	In the DMBA/TPA model, *Jnk1*$^{-/-}$ mice show enhanced tumor susceptibility while *Jnk2*$^{-/-}$ mice are tumor resistant	Chen et al. 2001; She et al. 2002
Jun	*c-jun* knockout in the epidermis using *K5-Cre*	In the *K5-SOS-F* skin tumor model, *c-jun* ablation leads to smaller papillomas that show increased differentiation, possibly caused by down-regulation of EGFR	Zenz et al. 2003
	Transgenic expression of a dominant-negative form of *c-jun* (*TAM67*) in the basal epidermis (using the *K14* promoter) or in the suprabasal epidermis (using the *Involucrin* promoter)	*K14-TAM67* mice have no apparent epidermal defect but *TAM67* expression in the suprabasal epidermis results in keratinocyte hyperproliferation and delayed differentiation; in the DMBA/TPA model, papillomagenesis is strongly inhibited in both transgenic mice	Young et al. 1999; Rorke et al. 2010
Mek	Overexpression of *Mek1* in basal keratinocytes and hair follicle ORS using the *K14* promoter	Epidermal hyperplasia and spontaneous skin tumor formation	Feith et al. 2005
	Mek2 knockout and conditional *Mek1* knockout using *K14-Cre*	In the DMBA/TPA model, *Mek1* knockout but not *Mek2* knockout impedes tumorigenesis; in a mouse model of oncogenic *Ras*-driven skin cancer; however, both *Mek1* and *Mek2* (or at least one copy of each) have to be deleted to impede carcinogenesis	Scholl et al. 2009a,b

Continued

Table 1. *Continued*

Gene	Mouse model	Phenotype	References
Myc	*K5-Myc* transgenic mice	Spontaneous papilloma and SCC development; mice are also more tumor susceptible in the DMBA/TPA model	Rounbehler et al. 2001
	K14-driven *Myc* overexpression	Epidermal hyperplasia, enlarged sebaceous glands, spontaneous skin lesions and stem cell loss; DMBA/TPA-treated *K14-Myc* mice develop tumors with reduced latency and increased yield, but these are predominantly sebaceous adenomas	Arnold and Watt 2001; Waikel et al. 2001; Honeycutt et al. 2010
Pak1	*Pak1* knockout	*Pak1* deficiency impedes tumor development and progression in a mouse model of *KrasG12D*-driven skin cancer	Chow et al. 2012
PKC	*PKC-η* knockout; *K5*-driven *PKC-α* overexpression; *K14*-driven *PKC-δ* or *PKC-ε* overexpression	In the DMBA/TPA model, *PKC-η*$^{-/-}$ and *K5- PKC-α* mice show enhanced tumor formation; *K14-PKC-δ* and *K14-PKC-ε* mice, on the other hand, are resistant to papilloma development; *K14-PKC-ε* mice also show increased de novo carcinoma formation	Reddig et al. 1999, 2000; Chida et al. 2003; Cataisson et al. 2009
Rac1	Keratinocyte-specific deletion using *K5-* and *K14-Cre*	Hair follicle (and epidermal) stem cell loss/impairment; *K5*-driven *Rac1* ablation leads to tumor-resistance in the DMBA/TPA model, associated with a decrease in keratinocyte proliferation	Keely et al. 1997; Benitah et al. 2005; Chrostek et al. 2006; Wang et al. 2010

high-grade, poorly differentiated SCCs, which may represent class B carcinomas.

Another guanine nucleotide exchange factor downstream from Ras is RalGDS, which activates the Ral GTPase. In vitro, Ral is involved in intracellular trafficking and regulating gene expression through transcription factors such as AP-1 (Kops et al. 1999; Nakashima et al. 1999; de Ruiter et al. 2000; Moskalenko et al. 2002). DMBA/TPA treated *RalGDS*-deficient mice had reduced tumor incidence, size and malignant progression (González-García et al. 2005). Further, RalGDS was reported to mediate survival through the JNK/SAPK signaling pathway, so that heightened apoptosis was observed in the papillomas that arose in *RalGDS*-deficient mice.

Finally, a novel function of Raf-1 was more recently characterized by studying mice with epidermis-specific *Raf-1* deficiency. These mice show hyperactivity of the ρ effector Rok-α in their skin, while Erk activation is unaffected by *Raf-1* ablation (Ehrenreiter et al. 2005). When treated with the DMBA/TPA protocol, *Raf-1*-deficient mice were resistant to tumor development; further, induction of *Raf-1* ablation only after tumors had already developed resulted in tumor regression (Ehrenreiter et al. 2009). This addiction of SCCs to Raf-1 was attributed to Raf-1's role in binding and inhibiting Rok-α, which prevents Rok-α from phosphorylating and inactivating cofilin; when active, cofilin is able to stimulate Stat3 phosphorylation and Myc expression, leading to proliferation. Hence, when *Raf-1* is ablated in mice, Stat3 phosphorylation and Myc expression will be turned off, so that differentiation predominates over proliferation and tumorigenesis is impeded.

Therefore, apart from its well-documented role in tumor initiation, Ras also has diverse and complex functions that range across multiple stages of skin carcinogenesis. Depending on the particular Ras effector ablated, specific arms of these Ras responses will be abrogated, leading

to varied, nuanced consequences for tumor development.

KNOCKOUT MICE AND TUMOR-SUPPRESSOR PATHWAYS

As with the marriage of transgenic mouse technology to oncogene research in the previous decade, the development of knockout mouse technology in the 1990s made it possible to assess the functions of tumor suppressors in vivo (for review, see Jacks 1996). A subsequent major advance in the field was the invention of Cre-Lox technology, which made it possible to target tumor-suppressor ablation to subpopulations of cells, by flanking the gene of interest with *LoxP* sites and activating its removal with Cre recombinase specifically in subsets of cells. Further development of an inducible form of the Cre recombinase allowed for postnatal gene deletion at any time of the researcher's choosing.

This knockout mouse technology has been extensively used to interrogate the functions of a number of tumor-suppressor pathways using the DMBA/TPA model. *TP53* is frequently mutated in spontaneous human SCC and, as described earlier, is also commonly mutated or lost in SCCs that arise in the DMBA/TPA model. When *Trp53*$^{+/-}$ mice were treated with the DMBA/TPA protocol, papillomas developed with approximately the same latency, yield and size as they did in wild-type mice (Kemp et al. 1993). However, these papillomas progressed more rapidly to SCCs, and malignant conversion was associated with loss of the remaining copy of wild-type *Trp53*; DMBA/TPA-treated *Trp53*$^{-/-}$ mice, on the other hand, had a reduced yield of papillomas but these progressed even more rapidly to malignancy. Hence, *Trp53* does not appear to have a major role in the initiation or promotion phase of tumor development; rather, loss of *Trp53* is associated with malignant progression. In line with these observations, restoration of p53 activity was shown to have no effect on early stage tumors but caused regression of late stage tumors in a mouse model of *KrasG12D*-driven lung adenocarcinoma (Feldser et al. 2010; Junttila et al. 2010). Additionally, in the *KrasG12D*-driven skin tumor mouse models described earlier, oncogenic *Kras* alone can only give rise to papillomas, with no evidence of malignant progression; instead, oncogenic *Kras* driven by *K5*, *K15,* or *K19* has to be coupled to *Trp53* deficiency to induce malignant SCCs.

Apart from promoting the malignant conversion of papillomas, *Trp53* loss can have other effects that are also consistent with an increased frequency of malignant tumors. For instance, *Trp53* loss may lead to increased numbers of stem cells, and hence an expansion of the pool of target cells that may specifically give rise to malignant tumors. In support of this hypothesis, *Trp53* loss has been shown to increase the self-renewal of both mammary and neural stem cells (Meletis et al. 2006; Cicalese et al. 2009). In particular, p53 regulates the polarity of cell division in mammary stem cells, with *Trp53* loss predisposing toward self-renewing symmetric cell divisions. Remarkably, mammary tumor stem cells also show an elevated frequency of symmetric cell divisions, and restoration of p53 activity rescues asymmetric stem cell divisions and results in tumor growth reduction. Importantly, stem cell numbers increase progressively in premalignant *Trp53*$^{-/-}$ murine mammary gland over time. In the context of the epidermis, various models have been proposed to account for how stem cells in the basal layer proliferate and self-renew in maintaining the tissue (Potten 1981; Potten et al. 1982; Clayton et al. 2007; Mascré et al. 2012). Like mammary stem cells, epidermal stem cells are capable of both asymmetric and symmetric cell divisions. If *Trp53* loss in the epidermis similarly favors self-renewing symmetric stem cell divisions at the expense of asymmetric stem cell divisions, then we might also expect stem cell numbers to increase in *Trp53*$^{-/-}$ skins.

Yet another area where *Trp53* loss can have an effect is cell competition. This phenomenon was first described in *Drosophila melanogaster*, where cells of two different genotypes within a common developmental niche were shown to compete with each other for tissue occupancy (Morata and Ripoli 1975); at the cell population level then, cell competition can be described as a process of natural selection of the fittest cells.

The same principle has since been shown to govern cell–cell interactions in many tissue types and organisms, including mammals. For instance, ionizing radiation (IR)-induced stress has been shown to be capable of eliciting cell competition within the hematopoietic stem cell (HSC) compartment (Bondar and Medzhitov 2010); in particular, by generating mice with a mosaic pattern of p53 deficiency within the HSC compartment, the authors of the study showed that in the presence of IR stress, p53-deficient HSCs have a selective proliferative advantage and induce a senescence-like phenotype in outcompeted HSCs with higher levels of p53 activity. More recently, a genome-wide screen in murine induced pluripotent stem cells similarly identified p53 as a key gene whose down-regulation created "cheater" cells that outcompete wild-type cells during pluripotent stem cell differentiation in vitro and in vivo (Dejosez et al. 2013).

In the context of the skin, UV exposure in both mice and humans gives rise to patches of normal-looking cells that contain mutant p53, which suggests that p53 mutation is an early event in the development of UV-induced SCC (Berg et al. 1996; Jonason et al. 1996; Ren et al. 1996). More recent work by Klein et al. indicates that such p53 mutant clones (PMCs) undergo stochastic exponential growth during periods of UV illumination (Klein et al. 2010); such a mode of growth is remarkably consistent with PMCs being derived from mutant committed progenitor (CP) cells (Clayton et al. 2007; Mascré et al. 2012) that show a stochastic cell fate tipped in favor of proliferation. Further, once UV exposure ceases, the data suggest that the proliferation and loss of p53 mutant cells become balanced so that the population of preneoplastic cells reaches a steady state. Notably, such a paradigm for the behavior of p53 mutant cells in the skin is fully compatible with the cell competition model described above—in the presence of UV-induced stress, p53 mutant CP cells have a proliferative/survival advantage more than wild-type CP cells, leading to the expansion of PMCs; on UV cessation, this competitive advantage disappears, so that the stochastic cell fate of p53 mutant CP cells is once again balanced between proliferation and loss. This interpretation is supported by a recent report describing the effect of p53 deletion on stem cell competition within the intestinal stem cell niche (Vermeulen et al. 2013)

Besides *TP53*, another tumor-suppressor gene commonly mutated in tumors is *PTEN* (Li et al. 1997; Steck et al. 1997), which negatively regulates the PI3K-Akt signaling pathway downstream from Ras. Although somatic *PTEN* mutations have not been reported in cutaneous SCC, germline *PTEN* mutations are the cause of Cowden Disease in humans, which is associated with an elevated risk of SCC (Liaw et al. 1997). Mice that are *Pten*-null in their epidermis develop spontaneous papillomas, many of which eventually progress to malignancy (Suzuki et al. 2003). Further, $Pten^{+/-}$ mice treated with the DMBA/TPA protocol have increased papilloma numbers and develop SCCs at a faster rate (Mao et al. 2004). Interestingly, most of the SCCs from these mice do not have an initiating *Hras* mutation; rather, these SCCs have lost their remaining copy of wild-type *Pten*, while the minority of SCCs that do have an *Hras* mutation retain their wild-type *Pten*. Hence, *Hras* activation and *Pten* LOH are mutually exclusive events in the DMBA/TPA model, which may be because of their redundant effects in activating Akt signaling. In support of this hypothesis, *Pten*-null SCCs have down-regulated pERK levels but similar pAkt levels compared with *Hras*-mutated SCCs, which suggests that Akt signaling, and not MAPK signaling, may be the critical Ras effector in this particular model of skin carcinogenesis.

TUMOR-SUPPRESSOR AND NON-CELL-AUTONOMOUS EFFECTS OF NOTCH SIGNALING IN SCC DEVELOPMENT

Notch signaling has been implicated in controlling the process of keratinocyte differentiation (Lowell et al. 2000; Rangarajan et al. 2001). In vitro, activated Notch1 in keratinocytes induces p21 expression, which leads to growth arrest, whereas in vivo, keratinocyte-specific deletion of *Notch1* results in epidermal hyperplasia and deregulated expression of differentiation mark-

ers. These observations suggest a role for Notch1 in limiting proliferation in the skin, in contrast to the situation in many other tissues where Notch signaling is involved in preventing differentiation and plays a positive oncogenic role (Jhappan et al. 1992; Zagouras et al. 1995; Capobianco et al. 1997). Indeed, loss-of-function mutations in *NOTCH1* or *NOTCH2* have been reported in 75% of human cutaneous SCCs (Wang et al. 2011), which indicates a tumor-suppressor function for Notch signaling in SCC development.

To investigate this, Nicholas et al. (2003) ablated *Notch1* postnatally in the epidermis of mice. These mice developed spontaneous BCC-like skin tumors, and were more susceptible to tumor development when subjected to the DMBA/TPA protocol. The chemically induced tumors were mostly papillomas, but also included some malignant SCCs and BCC-like tumors. The authors suggest that the increased tumor susceptibility may be caused by heightened Gli2 expression and derepressed β-catenin signaling in *Notch1*-deficient epidermis. In line with these observations, mice that overexpress the pan-Notch inhibitor *DNMAML1* in their skin also show enhanced epidermal β-catenin signaling and develop spontaneous skin tumors, although these were histologically characterized as SCCs and not BCCs (Proweller et al. 2006).

More recently, a non-cell-autonomous tumor-suppressor function has been reported for *Notch1* in the epidermis. Using mice with a chimeric pattern of *Notch1* deletion in the epidermis, Demehri et al. (2009) showed that both *Notch1*-deleted and *Notch1*-expressing keratinocytes readily formed papillomas in the DMBA/TPA model, which indicates that Notch1 does not suppress tumorigenesis in a cell autonomous fashion. Rather, *Notch1* loss leads to defective skin barrier formation and thus induces wound repair responses; the resulting stromal microenvironment is characterized by inflammation, dermal fibroplasia and increased angiogenesis, which the authors suggest to be responsible for promoting tumor formation in these mice. In support of this hypothesis, deletion of other *Notch* paralogs that also impaired skin barrier formation similarly led to spontaneous skin tumor development.

Further evidence for a non-cell-autonomous function for Notch signaling in the epidermis comes from Ambler and Watt (2010). Here, the authors activated Notch signaling aberrantly in the epidermis, by utilizing transgenic mice with 4-hydroxy-tamoxifen (4OHT)-inducible, K14-driven expression of the Notch intracellular domain (NICD) (Ambler and Watt 2010). On the 4OHT application, these mice developed blisters at the epidermal–dermal junction, along with dermal accumulation of T lymphocytes and stromal cells. The authors showed that this phenotype was dependent on up-regulation of the Notch ligand Jag-1 in the epidermis, which is associated with a concomitant induction of Jag-1 in the underlying dermis and activated NF-κB signaling. Hence, the authors concluded that Jag-1 is a key mediator of non-cell-autonomous epidermal Notch signaling.

Finally, a non-cell-autonomous role for Notch signaling in the dermis has also been reported. Hu et al. (2012) ablated *CSL/RBP-Jκ*, a key Notch effector, specifically in murine mesenchyme, which includes the dermal fibroblasts that underlie the epidermis. These mice developed dermal atrophy and inflammation, which preceded the spontaneous appearance of multifocal SCCs. *CSL*-deficient dermal fibroblasts were shown to show features of cancer-associated fibroblasts, such as the expression of growth factors and matrix metalloproteases, as well as the deposition of Periostin and Tenascin C—two extracellular matrix proteins that have been reported to foster cancer stem cell niches (Oskarsson et al. 2011; Malanchi et al. 2012). Importantly, in human skin samples, the stroma underlying premalignant lesions also showed reduced Notch signaling; moreover, UVA exposure was shown to be capable of inducing *NOTCH2* silencing by DNA methylation in human skin explants, which provides a potential mechanism for how dermal Notch signaling may be down-regulated in response to sun exposure that predisposes to SCC development.

Therefore, in the skin, Notch signaling may have both cell-autonomous and non-cell-autonomous tumor-suppressor activities, the

latter deriving from a crosstalk between the epidermis and the underlying dermis.

POSITIVE AND NEGATIVE ROLES OF INFLAMMATION IN SKIN CARCINOGENESIS

Inflammation is a common feature of the tumor microenvironment, with 25% of cancers estimated to be associated with chronic inflammation (Mantovani et al. 2008). Indeed, inflammation has been considered an "enabling characteristic" that facilitates the acquisition of cancer hallmarks, capable of nurturing nascent lesions into full-blown tumors by orchestrating the action of such players as growth and proangiogenic factors (Hanahan and Weinberg 2011). In the DMBA/TPA model, in particular, inflammation is thought to play a key role in promotion, as TPA induces the production of cytokines (such as TNF-α and IL-1α) and eicosanoids (such as prostaglandins) that are crucial mediators of inflammatory responses (Hursting et al. 1999).

To elucidate the role of inflammation in skin carcinogenesis, many groups again used transgenic and knockout mice in conjunction with the DMBA/TPA model. For instance, TNF-α is transiently but extensively induced in the epidermis on TPA treatment (Moore et al. 1999). To investigate the role of TNF-α in skin carcinogenesis, *TNF-α*$^{-/-}$ mice were subjected to the DMBA/TPA protocol (Moore et al. 1999; Suganuma et al. 1999). These mice showed resistance to papilloma development, and because TPA-induced keratinocyte hyperproliferation and inflammation was suppressed while malignant progression was unaffected, Moore et al. (1999) also concluded that keratinocyte-produced TNF-α is critical to the promotion phase but not later stages of skin carcinogenesis. More recently, the tumor resistance of *TNF-α*$^{-/-}$ mice has also been partly attributed to a defect in B cells (Schioppa et al. 2011). In the absence of TNF-α signaling, mice have lower levels of Il-10-producing B regulatory cells and this is associated with an increase in IFN-γ-producing, antitumor CD8^{+} T cells in the skin.

Apart from TNF-α, Stat3 is another key factor implicated in cancer-related inflammation (Mantovani et al. 2008). It is constitutively activated in both tumor and immune cells in the tumor microenvironment, and is thought to have a central role in mediating the suppression of antitumor immune responses (Yu et al. 2007). Accordingly, skin tumor promoters have been shown to activate Stat3 expression in the skin (Chan et al. 2004a), and epidermis-specific ablation of *Stat3* abrogates tumor formation in the DMBA/TPA model (Chan et al. 2004b). Also in line with a protumor role for inflammation, when mice lacking *COX-1* or *COX-2* (the key enzymes catalyzing the first committed step in prostaglandin synthesis) were treated with the DMBA/TPA protocol, tumor development was profoundly inhibited (Tiano et al. 2002). These mice showed suppressed prostaglandin production in TPA-treated skin and in papillomas, and tumor growth inhibition was attributed to enhanced keratinocyte differentiation.

A positive role for inflammation was also shown using transgenic mice that overexpress activated Mek1 in the suprabasal layer of the IFE (the InvEE mice described earlier). These animals develop epidermal hyperproliferation and skin inflammation (Hobbs et al. 2004), and papillomas form after wounding (Arwert et al. 2010). The authors show that the terminally differentiating cells expressing activated Mek1 contribute to tumorigenesis by stimulating neighboring, transgene-negative basal cells to proliferate, which then become incorporated into the developing tumor mass. Tumorigenesis is dependent on IL-1α production and infiltrating immune cells such as γδ-T cells and macrophages. This study shows that besides reacquiring the ability to divide, differentiating cells can also contribute to tumor development by providing non-cell-autonomous signals to proliferative cells, in this case, IL-1α, which fosters an inflammatory microenvironment.

However, not all proinflammatory factors have a tumor-promoting effect. For instance, *K14/IL-1α* transgenic mice that overexpress IL-1α in the basal layer of their epidermis are known to develop spontaneous skin inflammation, characterized by the expression of pro-

inflammatory cytokines and chemokines (Groves et al. 1995). However, when these mice were subjected to the DMBA/TPA protocol, tumor formation was, surprisingly, strongly inhibited (Murphy et al. 2013). Similarly, mice selectively bred to have maximal acute inflammatory responsiveness were shown to be resistant to DMBA/TPA-induced skin carcinogenesis (Biozzi et al. 1998). Additionally, anti-inflammatory agents were reported to have contradictory effects on mouse skin tumor promotion (Viaje et al. 1977).

Further support for a tumor-inhibiting role for inflammation came from analysis of the effects of germline polymorphisms on gene expression networks in skins from a genetically heterogeneous population of mice (Quigley et al. 2009). Gene expression networks associated with tumor susceptibility showed higher expression of the inflammation antagonists *Il-15f* and *Il-16f* and lower expression of proinflammatory *Pde4b,* consistent with an antitumor effect for inflammation in this context.

As illustrated by the above studies, many of the cytokines that mediate inflammatory responses in the skin are produced by the epithelial cells themselves and not solely by skin-resident or infiltrating immune cells. These epithelial-derived, secreted molecules and other molecules displayed on the surface of epithelial cells, collectively termed the "epimmunome" (Swamy et al. 2010), can play a key role in instructing immune cells and consequently impinge on immunocompetence and tumor immunosurveillance. For instance, the major histocompatibility complex class I protein Rae-1 is expressed on skin epithelial cells and engages the activating receptor NKG2D expressed on a variety of immune cell types, such as Natural Killer cells and $CD8^+$ T cells. Rae-1 expression in the skin is known to be upregulated within 24 h of carcinogen treatment, and is also sustained throughout papilloma and carcinoma development (Girardi et al. 2001). When Rae-1 is acutely induced in the epidermis of transgenic mice, the two resident immune cell populations in the skin, the dendritic epidermal T cells (DETCs) and antigen-presenting Langerhans cells, undergo rapid, simultaneous reorganization, followed soon after by the infiltration of T cells (Strid et al. 2008). The authors of this study also showed that whereas DETCs are tumor-inhibiting, Langerhans cells are tumor-promoting, which thus suggests a potential role for Rae-1 in the early stages of a complex, multidimensional tumor immunosurveillance program.

In summary, the role of inflammation in skin carcinogenesis is a highly complex one, with possibly different consequences depending on the balance among individual members of the immune cell and cytokine network present in the tumor. Another factor that plays into this dynamic is the category—whether class A or B—of skin carcinomas involved. Mice treated with a modified DMBA/TPA protocol in which the duration of TPA treatment was restricted to 5–10 wk, rather than the usual 20 wk, showed the highest relative proportion of Class B (spindle) carcinomas (Wong et al. 2013). This suggests that class B carcinomas arise through a pathway that is less dependent on TPA-induced inflammation, whereas class A squamous papillomas and carcinomas are highly dependent on this inflammatory stimulus. Previous data are also compatible with this notion of an alternative route to tumor formation that has different requirements for inflammation (Fig. 2). Although *K14/IL-1α* transgenic mice, as described above, are resistant to tumor development via the classical route when treated with the DMBA/TPA protocol, they nevertheless show increased susceptibility to de novo carcinoma formation when subjected to the complete carcinogenesis protocol (Groves et al. 1995). On the other hand, *K14-PKC-ε* transgenic mice that overexpress the *PKC* isoform *PKC-ε* in basal keratinocytes develop spontaneous skin inflammation; when treated with the DMBA/TPA protocol, these mice developed, on average, less than one papilloma per mouse via the classical route but nevertheless showed increased de novo carcinoma formation (Reddig et al. 2000).

Finally, to highlight the dynamic nature of the role of inflammation in skin carcinogenesis, two recent studies looked at TSLP-mediated inflammation in the skin (Demehri et al. 2012; Piazza et al. 2012). Ablation of both *Notch1* and

Notch2 or *RBP-Jκ* alone in the mouse skin led to a chronic inflammatory condition caused by heightened TSLP expression by keratinocytes. When TSLP receptor components were also genetically deleted in these mice, spontaneous skin tumors arose. The authors showed that this was caused by the depletion of antitumor T cells in the inflammatory infiltrate on loss of TSLP signaling; Piazza et al. (2012) also showed in their model that in place of T cells, protumor myeloid cells accumulated and supplied Wnt ligands, which were necessary for tumor development in this context.

The above examples illustrate how loss of signaling of one cytokine can induce a switch in the inflammatory profile from one that is antitumor to one that is protumor. In the future, studying how to tip the balance in favor of "good" inflammation in tumors may be a fruitful avenue for developing treatments for SCC in the clinic.

CONCLUDING REMARKS

When considering the vast literature on the DMBA/TPA model of SCC development, it is worthwhile bearing in mind a couple of caveats. First, many studies use constitutive, whole-body knockout mice, in which any phenotypes observed can be caused by developmental effects or non-cell-autonomous effects from gene deletion in tissues other than the skin. A good example of the latter phenomenon is how loss of Notch signaling in the dermis is capable of fostering skin tumor formation (Hu et al. 2012). In principle, the development of inducible, conditional knockout mice addresses many of these concerns, but one should still be wary of issues of promoter leakiness and recombination efficiency. Second, many studies look only at the early stages of carcinogenesis. Relatively few look at the clinically relevant stages of tumor development, that is, progression to malignancy and metastasis.

In conclusion, the DMBA/TPA model remains very much relevant to current research into the biology and genetics of SCC. Although *RAS* mutations are found in only 5%–10% of human SCCs, the prevalence of SCC development in Caucasians is very high, resulting in many thousands of patients yearly with *HRAS* or *KRAS* mutations, including the common mutations found in the DMBA/TPA mouse model. Because there are presently no treatments available generally for *RAS* mutant tumors in any tissue, this mouse model has the potential to provide crucial directions for the future development of novel drug targets, chemoprevention strategies and clinical treatments.

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