



Pathogenesis of Skin Carcinomas and a Stem Cell as Focal Origin

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UV radiation in sunlight has long been recognized as the main exogenous cause of skin carcinomas. We present a brief historical perspective on the progress in understanding the pathogenesis of skin carcinomas, and recent advances. Sun-exposed skin carries numerous UV-related mutations, and skin carcinomas rank among the tumors with the highest mutational loads. In this multitude of mutations only a few are crucial in driving the tumor. Some are known from hereditary (skin) cancer syndromes and other recurrent ones have been validated in transgenic mice. Considering the continuous renewal of the epidermis, the question arises whether the lifelong residing stem cells are the main targets in skin carcinogenesis, a multistep process that would require ample time to evolve. Therefore, classic quiescent stem cells have been studied as potential tumor-initiating cells, as well as more recently discovered actively dividing stem cells (either Lgr5+ or Lgr6+). Interesting differences have emerged between experimental UV and two-stage chemical carcinogenesis, e.g., the latter appears to originate from follicular stem cells, in contrast to the former.

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INTRODUCTION

Skin cancers had already been linked to excessive sun exposure in the nineteenth century, specifically skin carcinomas were found predominantly in people with outdoor jobs. Genotoxicity, mutagenesis, and carcinogenesis by UV radiation, as present in sunlight, were experimentally established in the early decades of the twentieth century. Before the 2nd World War spectral analyses showed that DNA was the target of UV radiation for cell death and mutations (1, 2): i.e., well before Watson and Crick published the correct model of the structure of DNA, explaining how genes made up of DNA carried the genetic code which could be straightforwardly copied for daughter cells. Miscopies would introduce mutations. Consequently, replication of damaged DNA, hampering correct copying, for cell division was identified as the most prominent cause of mutagenesis. Carcinogenesis is considered to evolve primarily as a “multi-hit” process in which mutations accumulate in cells until a combination of mutations (and possibly other genetic defects and epigenetic modulatory effects) emerges which drive a cell to malignancy. As such a cell destined for malignancy requires time and cell divisions to transform, the most likely candidates would appear to be adult stem cells that constitute the very basis of tissue renewal. This premise was evidenced by a correlation that Tomasetti and Vogelstein (3) found between rate of stem cell division in various tissues and the risk of cancer. This led them to the controversial statement that most cancers are “bad luck” arising from an inherent risk of mutation in cell division. UV

irradiation is known to cause epidermal hyperproliferation and hyperplasia. This would increase the UV-related risk of carcinomas originating from the epidermis (4), in addition to the risk derived from the genotoxicity of UV radiation.

HUMAN SKIN CARCINOMAS AND SUN EXPOSURE

The skin is an evident frontier of the body in interactions with its environment. UV radiation in sunlight poses a recurrent (geno-) toxic challenge to skin, and like all life dwelling on the Earth's surface, it has powerful defense mechanisms, among which very importantly Nucleotide Excision Repair (NER) to maintain the integrity of the genome. A defect in NER increases the risk of skin cancer dramatically to the point that 50% of patients with Xeroderma pigmentosum succumb to multiple skin cancers before the age of 30 (5). NER eliminates the dominant UV-induced DNA damage (cyclobutane pyrimidine dimers, CPDs, and 6–4 photoproducts, 6–4 PPs) by a “cut-and-paste” action: cut out an oligo with the damage and fill in the gap using the complementary strand. As this UV-induced DNA damage occurs predominantly at neighboring pyrimidines in a DNA strand, the resulting mutations (mainly C > T) are located at dipyrimidine sites, and referred to as UV signature mutations. Strikingly, mutations in the *P53* tumor suppressor gene of skin carcinomas show predominantly this UV signature (6). Microscopic clusters of cells (clones) overexpressing mutant *P53* are present in chronically exposed skin, and presumed to be potential precursors of skin carcinomas (7). More recently, deep sequencing of 74 cancer-related genes (incl. *P53*) has shown that sun-exposed skin (from eye lid resections) is full of mutations (2–6/Mb), with a majority of UV signature mutations and an estimated average of 140 small clones/cm² with a mutation in one of these 74 genes (8). Strikingly, another recent study found SCC-related mutations to be restricted to *P53*-overexpressing cell clusters (9).

The authors (8) noticed that the sun-exposed skin appeared clinically normal despite the high mutation load, and that the clones remained restricted in size. Apparently, the skin is inherently able to cope with a multitude of mutated clones. In experiments with Wnt-activated clones, it was shown that in signal exchange the normal cells were stimulated to outcompete the mutated cells (10). Much earlier, it was reported that low grade malignant keratinocytes were kept in check to contribute to epidermal homeostasis by surrounding normal keratinocytes (11). Hence, the outgrowth of cells into a tumor would appear to require the collapse of growth control by surrounding normal cells.

Considering the high mutation load in sun-exposed skin, it is no surprise that skin carcinomas belong to the absolute top of cancers with high mutation loads (10,000–100,000 per cell). Mutation load was found to be proportional to the immunogenicity of a tumor (12) and consequently proportional to the success of immunotherapy by check-point inhibition (13). In immunosuppressed organ transplant recipients the risk of skin cancer is raised, most dramatically the risk of squamous cell

carcinoma, SCC (14) which correlated with preceding cutaneous HPV infections (15).

DRIVER MUTATIONS

With an overwhelming load of mutations it would appear impossible to separate the driver mutations from passenger mutations. However, recurrent mutations within this multitude could be considered drivers, and earlier on, potential drivers were identified from syndromes with an inherited pre-disposition to develop cancers. A textbook example of the latter is the Gorlin syndrome (Basal Cell Nevus Syndrome, BCNS) where mutations in the tumor suppressor *PTCH* gene predisposes to activation of the Hedgehog pathway (e.g., by loss of the wt allele by UV radiation) and subsequent formation of multiple basal cell carcinomas, BCCs (16). Also, most sporadic BCCs turned out to be driven by an activated Hedgehog pathway commonly involving mutations in *PTCH* or *SMO* (17). Activation of the Hedgehog pathway or ectopic expression of its downstream transcription factor, *Gli1*, in mouse skin gives rise to BCCs (18, 19).

In malignant progression of SCCs the RAS pathway was found to be activated (20, 21), however, apparently without any relevant recurrent mutations, notably rarely mutations in (*Ha-*)*RAS* genes (22). Next to a predominance of UV signature mutations in *P53*, nearly all SCCs were found to bear such mutations in one or more of the *NOTCH* (1–4) genes (23). *NOTCH1* mutations were already present in early stages of SCC development (24). Transgenic mice in which epidermal Notch signaling was blocked developed SCCs (25).

WHAT CELL DRIVES THE OUTGROWTH OF HUMAN SKIN CARCINOMA?

It is notoriously difficult to propagate skin carcinoma cells *in vitro* and establish cell lines. Our group could only maintain fresh SCCs intact as explants (26). Others were successful in culturing SCC cells on fibroblasts (3T3) as feeder layers (27). In contrast to normal fibroblast, the cancer-associated fibroblasts (CAFs) appear to harbor a special class of fibroblasts facilitating invasion of SCC into the dermis (28). SCCs show a clear heterogeneity with differentiated keratinocytes (around keratin “pearls,” horny layer-like deposits) enclosed by germinative basal cell layers of keratinocytes bordering and infiltrating the stroma. Like in normal epidermis, the stem cells that drive SCC, the tumor-initiating cells (TICs), are logically expected to reside in the germinative compartment of the tumor. CD133 (prominin-1) is a tumor stem cell marker (e.g., in lung cancer), and not detectable in normal epidermal keratinocytes (proteinatlas.com). But some cells in germinative outer rim of SCCs are CD133-positive, about 1% of the tumor cells (27). Transferring as few as 100–1,000 of these CD133+ cells in combination with a million of human fibroblasts in matrigel into a pre-created subcutaneous space resulted in a 50% chance to spawn a new SCC in immune compromised mice (not capable of rejecting the human SCC). Evidently, the human SCC TICs needed the

microenvironment of human fibroblast to support the outgrowth (generating appropriate CAFs?). It is not clear whether or how the CD133+ cells are related to stem cells of the normal human epidermis.

Similar results using the subcutaneous transplant assay have been obtained with CD200+ cells from BCCs, about 1–2% of the tumor cells (29). In contrast to CD133, CD200+ cells are present in normal skin: specifically in hair follicles in the region (the bulge) where stem cells reside in mice. However, this does not necessarily imply that the BCCs originate from these cells in hair follicles [although tracing mitochondrial DNA mutations by COX-deficiency would support this (30)]. Activation of the Hedgehog pathway and further transformation could conceivably lead to CD200 expression in the TICs. As monotherapies with SMO antagonists (e.g., vismodegib) inhibiting the Hedgehog pathway are not curative, the authors suggest to target the CD200+ cells instead for a permanent elimination of the tumor.

HISTORICAL PRELUDE TO EXPERIMENTAL SKIN CARCINOGENESIS

Present day research on experimental skin carcinogenesis employs two basic mouse models, chemically or UV driven, which stem from historical observations on skin cancer in man. First of all, the surgeon Sir Percival Pott (a founder of orthopedics) reported in 1775 on the frequent occurrence of scrotal cancer (SCCs) among chimney sweeps in London, and recognized soot (coal tar) as the evident culprit (31). And secondly, skin carcinomas were linked to sun exposure at end of the nineteenth century. In Hamburg the dermatologist Unna (32) stated in his book on skin diseases that degenerative changes in the sun-exposed skin of sailors (“Seemannshaut”) were associated with skin carcinomas. In Bordeaux Dubreuilh (33) noticed that vineyard workers contracted remarkably more skin carcinomas than people living in the city. Further detailed observations on body locations of the carcinomas indicated that they were most likely caused by sunlight.

CHEMICAL CARCINOGENESIS

Just before the First World War, the first experimental proof of tumor formation by coal tar was provided by the Japanese pathologist prof Yamagiwa. It was done in rabbits by repeated applications of coal tar to the ears. Yamagiwa had visited the Virchow Institute in Berlin where he learned about Virchow’s irritation theory (“Reiztheorie”) of carcinogenesis (34). The experiment was modified in mice to include “cocarcinogens” (35), such as the “irritant” croton oil which “promoted” tumor outgrowth (reminiscent of the “Reiztheorie”). From these early experiments the standard classic two-stage protocol evolved in which a single genotoxic challenge with, for example, coal tar [or one of its ingredients like benzo(a)pyrene] irreversibly “initiated” tumors after which tumor development was “promoted” by a regimen of repeated applications of an “irritant” like croton

oil (or its active ingredient 12-O-tetra- decanoyl-phorbol-13-acetate, TPA, activating PKC) (36). Tumor promotion was reversible in that tumors would not develop or regressed up on early termination of this regimen. This protocol yielded exophytically growing, wart-like, benign tumors (papillomas), and at a later stage some SCCs. *Ha-ras* mutations were commonly present in these tumors, notably already at the earliest tumor stages in hyperplastic foci in hair follicles (37). And even earlier, *Ha-ras* mutations could be detected by nested PCR from expanding clones in the in normal looking skin that had been subjected to the two-stage protocol (38). In contrast to *Ha-ras*, *p53* mutations occurred late in tumor progression and were linked to malignant conversion (39). Over a period of 80 years chemical carcinogenesis took central stage because of experimental convenience and because of its versatility in analysing the biology of carcinogenesis and in characterizing (anti-) carcinogenic substances and their interactions.

UV CARCINOGENESIS

Experimental proof of tumor induction by UV radiation was first published in 1928 by Findlay (40) who had chronically irradiated depilated albino mice for 8 months with a quartz mercury lamp. Interestingly, he also found that painting the animals with coal tar before irradiation speeded up the development of tumors (<3 months). Next, the prolific Brazilian professor of pathology Angel Roffo—who also pioneered in showing benzpyrene from tobacco to be carcinogenic—showed in the 1930s that the UV part in sunlight blocked by window glass (“UVB”) to be carcinogenic on rats (41, 42). The exact wavelength dependence (action spectrum) was determined much later in the 1990s for SCCs in hairless mice (43). The early experiments were done on the ears (and tails), or shaven backs of haired mice, but in the 1960s the more convenient and sensitive hairless mouse model was introduced which has become a standard in experimental UV carcinogenesis (44). In contrast to hairless mice, haired mice were reported to developed fibrosarcomas next to SCCs under chronic UV exposure (with substantially higher UV dosages than used on hairless mice). However, this was corrected by showing that the tumors were keratinocyte-derived (i.e., exclusively epidermal) and ranged from well differentiated to spindle cell carcinomas (45). The tumor progression in hairless mice was very similar to that in humans starting with endophytically growing actinic keratosis as benign precursor lesions (majority of tumors <2 mm across) of which a fraction progressed to malignant SCCs (majority > 3 mm) (44), and with a majority throughout bearing UV signature mutations in the tumorsuppressor *p53* (46); even before tumors appeared, microscopic clusters overexpressing mutant-*p53* could be detected in the chronically sub-sunburn UV-exposed skin (47). *Ras* mutations were virtually lacking in the tumors: only 1 tumor with a *Ki-ras* mutation out of 32 tumors, none with a *Ha-ras* mutation (48). Only with a NER defect, in XPA mice, did *Ha-ras* mutations occur in UV-induced tumors which notably were benign papillomas as found in chemocarcinogenesis (49). The repair defect impaired removal of CPDs from the transcribed strand of *Ha-ras*. This introduced

novel mutational targets for UV radiation corresponding with the oncogenic *Ha-ras* mutations. Overall, the mutational spectrum of UV SCCs in hairless mice resembled that of human SCCs, including Notch 1–4 mutations (50). In the 1970s it was discovered that UV-induced tumors were antigenic and that UV irradiation raised a specific immune tolerance toward these tumors (51). Recently, cutaneous papilloma virus infection was shown to enhance UV carcinogenesis (52). In all, experimental UV carcinogenesis shows striking parallels with human SCCs supporting the validity of the model.

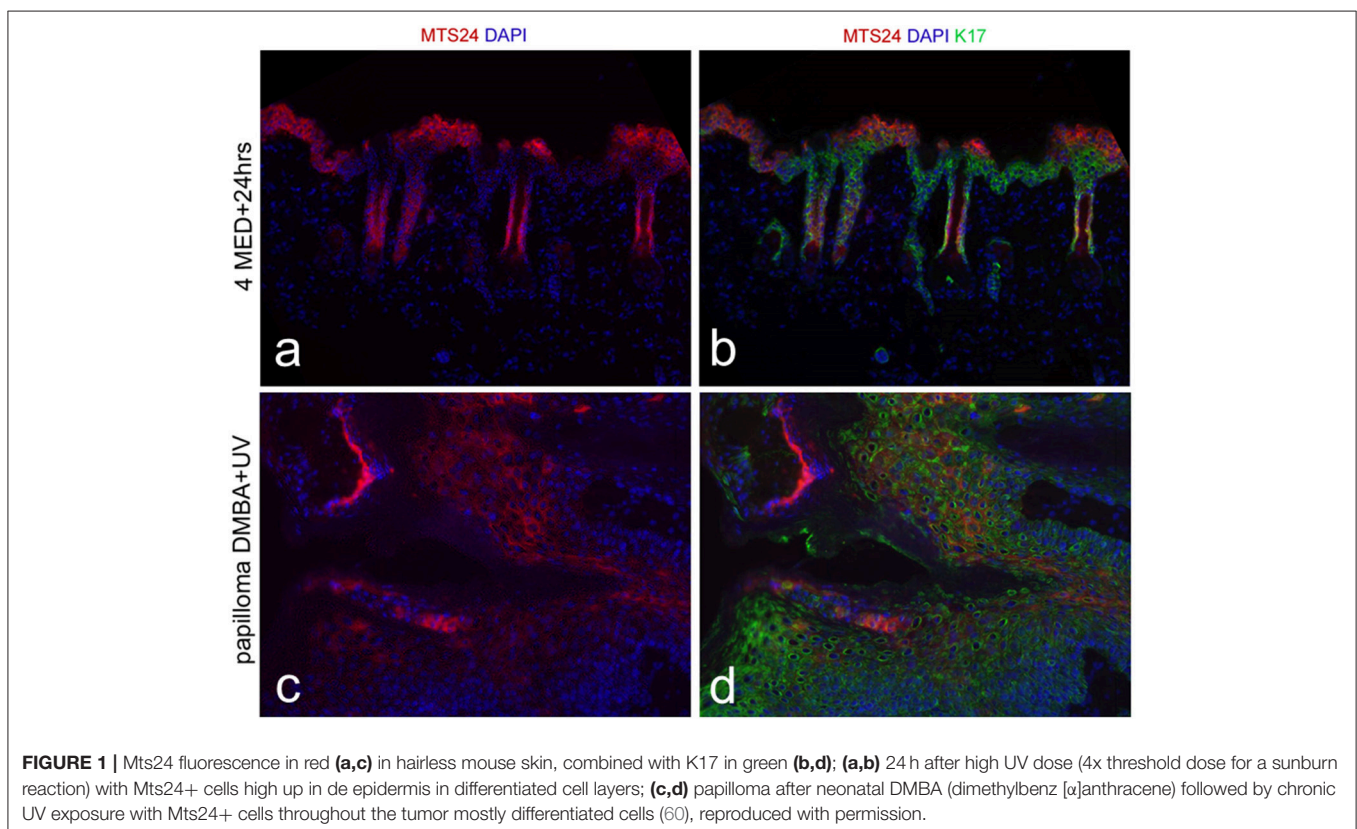
STEM CELLS

A remarkable difference between chemical and UV carcinogenesis appears to be the origin of the SCCs. After initiation, abrasion of the interfollicular (IF) epidermis did not affect development of chemo-SCCs, indicating that they originated from the hair follicles (53). In contrast, our group showed that apoptotic elimination of the IF basal layer by a single UV overdose nullified the UV carcinogenic regimen up to that point and carcinogenesis had to restart afterwards, indicating that UV-SCCs originated from the IF epidermis (54).

The observation that the interval between tumor initiation and promotion could be extended to months demonstrated that the initiated cells were not shed in epidermal turnover and were therefore likely to be stem cells. This was confirmed by radioactive tracing of the initiating substance, benz(a)pyrene,

which was retained in hair follicles and interfollicular epidermis in label-retaining cells, i.e., in quiescent stem cells (55). CD34+ cells located in the bulge of hair follicles were found to harbor such quiescent cells (56) and they were identified as tumor stem cells, or tumor-initiating cells (TICs), in chemically induced skin tumors (57). We similarly found that IF quiescent cells retaining CPDs from a low level UV regimen were linked to the development of non-regressing *in situ* carcinomas after TPA tumor promotion (58). There is, however, no established reliable protein marker for IF quiescent cells (resting or activated) by which to identify these cells in a tumor mass; putative stem cell markers (*Wif-1*, *Lrig1*, *Dll1*) did not label the CPD-retaining quiescent cells. Our group earlier identified *Mts24/Plet1* as a stem cell marker (59) but later found this marker expressed in differentiated cells after UV exposure (**Figures 1a,b**) and in papillomas (**Figures 1c,d**) but absent in SCCs (not shown) (60). This demonstrated that a stem cell marker in homeostasis need not be one under (UV) stress, in hyperplasia or in tumors.

Recently, a new class of proliferating stem cells (either *Lgr5+* or *Lgr6+*) was studied as possible TICs in chemical and UV carcinogenesis; this was done by “lineage tracing” to identify the progeny of these stem cells in tumors. *Lgr5+* cells and progeny were not detected in either chemically or UV-induced tumors (61, 62). Our group could not detect any appreciable presence of *Lgr6+* cells in tumors and only some sporadic remnants of progeny deep into the differentiated compartments, i.e., no indication that *Lgr6+* cells were TICs or drove tumor growth (63). In contrast, Huang et al. (62) reported the presence of



Lgr6+ cells in chemically induced tumors in a different mouse strain than we used, and with a different protocol for lineage tracing. However, these Lgr6+ cells did not exclusively reside in the germinative compartment of the tumors, showed a lack of expression of K14 (marker of germinative basal cells) and some even appeared flattened out in terminal differentiation. Apparently Lgr6 was no longer a marker of stem cells in these tumors (reminiscent of what we found with Mts24/Plet1). Intriguingly, Huang et al. (62) concluded from experiments with Lgr6 knockout mice that Lgr6 in normal epidermis functioned as a tumor suppressor. Interestingly in this respect, we found that Lgr6+ cells and progeny were lost from IF epidermis under chronic UV exposure long before the occurrence of SCCs; in contrast, a TPA regimen caused a clear expansion of progeny in the IF epidermis (63).

CONCLUSION

From the present vantage point, UV carcinogenesis in mice appears to emulate SCCs in humans better than two-stage chemical carcinogenesis. And the quiescent stem cells appear to be the most likely target cells from which SCCs arise, either from quiescent cells in hair follicles in chemocarcinogenesis or

quiescent cells in the IF epidermis in UV carcinogenesis. Future research should be directed toward identifying the latter cells by reliable protein markers, which may subsequently serve to develop well targeted interventions to prevent or cure cutaneous SCCs.

As there is no robust mouse model available for the *de novo* induction of BCCs by exogenous agents, identification of the primary target cells requires further research.

AUTHOR CONTRIBUTIONS

FdG (biophysicist/photobiologist): outline of review, wrote draft, finalized text, and references. CT (molecular biologist): outline of review, brought in and checked molecular aspects, filled in some gaps, edited draft versions.

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