A cellular model reflecting the phenotypic heterogeneity of mutant HRAS driven squamous cell carcinoma

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Squamous cell carcinomas have a range of histopathological manifestations. The parameters that determine this clinically observed heterogeneity are not fully understood. Here, we report the generation of a cell culture model that reflects part of this heterogeneity. We have used the catalytic subunit of human telomerase hTERT and large T to immortalize primary UV-unexposed keratinocytes. Then, mutant HRAS G12V has been introduced to transform these immortal keratinocytes. When injected into immunosuppressed mice, transformed cells grew as xenografts with distinct histopathological characteristics. We observed three major tissue architectures: solid, sarcomatoid and cystic growth types, which were primarily composed of pleomorphic and basaloid cells but in some cases displayed focal apocrine differentiation. We demonstrate that the cells generated represent different stages of skin cancerogenesis and as such can be used to identify novel tumor-promoting alterations such as the overexpression of the PADI2 oncogene in solid-type SCC. Importantly, the cultured cells maintain the characteristics from the xenograft they were derived from while being amenable to manipulation and analysis. The availability of cell lines representing different clinical manifestations opens a new tool to study the stochastic and deterministic factors that cause case-to-case heterogeneity despite departing from the same set of oncogenes and the same genetic background.

Key words: squamous cell carcinoma, tumour heterogeneity, cell culture and xenograft model

Abbreviations: IHC: immunohistochemistry; IM: immortalized keratinocytes; SCC: squamous cell carcinoma; T: transformed IM cells; Ts: in vivo selected transformed cells; hTERT: human telomerase reverse transcriptase; F-HRASV12: flag epitope-tagged HRAS G12V mutant; n.d.: not detected; s.d.: standard variation.

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Keratinocyte carcinomas are by far the most common malignancies worldwide and their incidence and morbidity continuously increase, specially in light-skinned populations.1,2 The two main subtypes are basal cell carcinomas and squamous cell carcinomas (SCC). Basal cell carcinomas are the most frequent among all cancers. They are slow-growing, largely non-invasive tumours that are caused by an aberrant sonic hedgehog pathway.3 SCC are tumours with interfollicular epidermal differentiation which are more prone to spread and can be originated by a wide range of genetic mechanisms.4 Solar UV radiation is the main risk factor for SCC.4 Additional risk factors include infection with human papillomavirus, iatrogenic immunosuppression after solid-organ transplantation and several different chemicals such as the nitrosamines found in tobacco smoke.2 SCCs evolve particularly fast as a consequence of targeted melanoma therapy.5 Almost half of patients with metastatic melanoma patients carry the BRAFV600E mutation and are candidates for treatment with the BRAF inhibitors vemurafenib and dabrafenib.5,7 SCC and related keratoacanthoma appear in up to 26% of treated melanoma patients as a sudden and adverse secondary effect.5,7 It is thought that these tumours arise through the fast clonal expansion of premalignant keratinocytes carrying mutant
What's new?
The histopathological heterogeneity encountered in non-melanoma skin tumours and even within the same tumour class such as squamous cell carcinoma (SCC) is still poorly understood. Here, the authors report the generation of a set of cell lines that reflect part of SCC heterogeneity and represent different stages of skin carcinogenesis. The model has the potential to be used as a unique tool for the study of genetic and non-genetic mechanisms underlying case-to-case heterogeneity. It also offers a linear model for the discovery of novel cancer driver genes and the development of drugs targeting Ras proteins or their downstream signaling components.

RAS, whose signaling is boosted by cooperation with inhibited BRAF.8–10

The best characterized genetic alteration in skin cancer is the inactivation of TP53 tumor suppressor gene.4 The gene product p53 has a major role in the DNA damage response by mediating cell cycle arrest and the induction of apoptosis.11 Inactivation of p53 is considered an early event in skin carcinogenesis that facilitates the accumulation of further cytogenetic aberrations.12 Indeed, over 90% of SCC cases present mutations in TP53, being the UV-induced C to T transitions the most common type of mutation.13 The function of p53 can also be impaired by Beta subtype HPV infection, increasing the susceptibility to cutaneous SCC, particularly in organ transplant and epidermodysplasia verruciformis patients.14 Beta HPV may disrupt host cell cycle in a similar way than high-risk alpha HPV in cervical, anal or oropharynx mucosa. In this regard, it was shown that the Beta HPV-encoded E6 and E7 proteins can increase the lifespan of host keratinocytes by reactivating the telomerase and functionally compromising p53 and members of the retinoblastoma family, which tightly regulate entry into cell cycle S phase.14

Irrespective of the etiology, cells with such pre-cancerous lesions are prone to undergo malignant progression through acquisition of additional molecular and cytogenetic changes including proto-oncogene activation.4 Oncogenic mutations in genes encoding members of the Ras family of GTPases have been found in 10–20% of keratinocyte carcinomas.5 In HRAS, the most common mutation occurs in the codons 12, 13 and 61. All three mutations render the GTPase less sensitive to GAP-assisted hydrolysis locking the GTPase in its active GTP-bound state.15 Consequential constitutive signaling through MAPK pathway promotes proliferation and carcinogenesis. In patients with melanoma treated with vemurafenib, the frequency of mutations in Ras protein encoding genes in SCC rises up to 21–57% with HRAS being the most prevalently mutated gene of the family.16,17

In order to enable the study of mutant HRAS driven tumorigenesis in skin, we have introduced a mutant HRAS G12V transgene in immortalized UV-unexposed primary human keratinocytes (PHK). We found a surprisingly high histological heterogeneity in the derived tumour xenografts that reflected part of the clinical spectrum of manifestations of both UV-exposed and vemurafenib-related SCC. After reintroduction in culture, cells maintain the characteristics of their tumour xenograft of origin making them amenable for manipulation and analysis. We demonstrate that these engineered cells provide a valuable tool to identify new drivers of skin carcinogenesis by identifying the overexpression of the oncogene PADI2 in solid-type SCC. Taken together, we describe a panel of cell lines representing discrete steps of skin carcinogenesis and different histopathological manifestations. These cell lines provide a versatile toolkit for the analysis of the parameters determining histopathological heterogeneity in culture.

Materials and Methods
Antibodies
We used the antibody anti-SV40 T Ag (Santa Cruz Biotechnology, Dallas, TX; Pab 419), Flag M2 (Sigma-Aldrich, Spain; F3165), H3 C-terminal (Abcam, UK; ab204964); P63 (Biocare Medical, Concord, CA; CM 163 A, B, C), cytookeratin CAM5.2 (Becton Dickinson, Franklin Lakes, NJ; 345779), PADI2 (Proteintech, UK; 12110–1–AP), Human Anti-Modified Citrulline (Modiquest, Netherlands; clone C4; MQR2.601), anti-mouse and anti-rabbit HRP secondary antibodies (Dako Diagnosticos SA, Spain; P0447 and P0448) and HRP-coupled anti-human IgG (H + L) (Life Technologies, Spain; 31410).

Maintenance of cell cultures
The here described IM, T, Ts1 and T4 cells have been deposited in the German tissue culture collection and are available under the name MBU-IM, MBU-T, MBU-Ts1 and MBU-Ts4, respectively (reference number definition is pending). They were grown in keratinocyte serum free medium (Keratinocyte-SFM, liquid with L-Glutamine, Life Technologies) supplemented with 15 µg/mL Bovine Pituitary Extract and 0.2 ng/mL EGF (Keratinocyte Supplement Kit, Life Technologies) and 1% penicillin-streptomycin (Life Technologies). Cells were propagated with a split ratio of 1:10 every 2–3 days.

Karyotyping
Karyotypes were analyzed by the institutional laboratory of the Hospital Germans Trias i Pujol essentially as described.18 For each cell line, 10–20 metaphases were analyzed.

Gene transduction, plasmids and generation of cell lines. PHK from a healthy neonatal foreskin were obtained from a routine circumcision. Immediately after removal, the
tissue was immediately transferred to the laboratory dry in a sterile container and sterilized by immersion in iodine antiseptic for 30 sec and twice in 70% alcohol for 10 sec. After rinsing with PBS, the tissue was transferred to a 100 mm diameter petri dish, epidermis down and the connective tissue was removed using sterile curved scissors. The epidermis was transferred to a fresh dish and chopped into fine 1–3 mm² pieces. The dish was flooded with 20 mL 0.05% Trypsin-EDTA (Life Technologies, Spain; 25300062), and the solution containing pieces of skin was transferred with a wide-bore pipette to a sterile CelStir and incubated at 37°C for 30 min on a magnetic stirrer. After removing it from the incubator, the lumps of tissue were allowed to settle down. The supernatant was collected in a new tube and fresh trypsin-EDTA was added to the remaining tissue to repeat the procedure five times. To stop the trypsinization reaction, an equal volume of cold medium was added in each collected supernatant. After centrifuging, the PHKs were pooled and seeded onto a 3T3 J2 fibroblast feeder layer as previously described.

To immortalize and transform the PHK, we used the following retroviral constructs: pBabe-hygromycin with human telomerase reverse transcriptase (hTERT) (Addgene #1773), pBabe-neomycin with SV40 early region (Addgene #1780) and pMSCV2.2 (Addgene #60206) with FLAG epitope-tagged HRASV12-IRESGFP. Constructs were either obtained from Addgene or generated by subcloning the coding sequences of interest from plasmids generated by the Weinberg lab into the specified backbones using standard cloning techniques. To create immortalized keratinocytes (IM), cells were transduced with hTERT and SV40 early region using Phoenix-AMPHO packaging cells. To do this, Phoenix-AMPHO cells were seeded at 2–3 × 10⁶ cells/well. Transfection medium was 10 mM KCl, 12 mM D-glucose, 1.5 mM Na₂PO₄ adjusted to 1108°C. Tissue remnants were removed by filtration through a 70 μm cell strainer. Cells were pooled at the end of the protocol and seeded onto a P10 plate. Finally, the medium was removed and fresh keratinocyte medium was added. The infection procedure was repeated twice at 24-hr intervals. IM cells were selected with 500 μg/mL neomycin in the absence of feeder cells. For transformation, IM cells were transduced with the HRASV12 encoding vector following the same infection protocol. T cells were selected by flow cytometric sorting of GFP+ cells. In vivo selected cells (Ts) were derived from T cell xenografted tumours (see below).

Samples of primary oral SCC from 11 patients were collected and processed as described previously. Pellets of cultured primary cell lines from these samples were kindly provided by Fiona Watt (King’s College London).

**Functional assays and xenograft growth**

For the proliferation assay, exponentially growing cells were reiteratively collected and 250,000 cells were re-seeded at 0.25 × 10⁶ cells/P10 plate. For the soft agar, 10,000 cells were resuspended in 500 μL 0.3% agar and seeded on 500 μL solidified 0.5% agar. Both layers were done with FAF medium. Colonies were stained with crystal violet and counted using ImageJ software. To establish the Ti culture, the colonies of T cells from three different wells were pooled together in one P10 dish after mechanic disaggregation of agar by pipetting. Xenograft experiments were performed as described using Swiss nude mice, SPF-housing conditions, and procedures approved by the local Ethical Committee for Animal Experimentation. T cells were injected subcutaneously in both lateral flanks of the animals and they animals were sacrificed when tumours reached a diameter of >1 cm or 6 weeks after case animals had been sacrificed. Tumour specimens were split in two parts for their inclusion in paraffin and tissue homogenization followed by cell culture (Ts cells). For the later, tumours were disaggregated by repeated incubation in 0.1% trypsin plus EDTA (Life Technologies) for 45 min at 37°C. Tissue remnants were removed by filtration through a 70 μm cell strainer. Cells were pooled at the end of the protocol and seeded onto a P10 plate.

**Protein and RNA analysis**

Cell lysis, Western blot and RNA extraction were performed as previously described. Immunohistochemistry (IHC) and hematoxilin-eosin staining on formalin-fixed paraffin-embedded tumour sections were performed as described. Cultured cells were resuspended in agar and then embedded in paraffin blocks. Human samples were obtained after written, informed consent following the guideline of the Declaration of Helsinki. RNA levels were quantified by RT-qPCR and normalized to two reference genes (HTRI and GAPDH). All oligo sequences are available on request. For comparative gene expression analysis, we extracted the RNA from IM, T and Ts4 cell in biological replicates. The samples were then prepared and hybridized to SurePrint Human 8 × 60K (Agilent) following the supplier’s instructions. The data were essentially analyzed as described before. In brief, a probe is
considered differentially expressed if it displays a fold change $\geq 2$ and a Benjamini–Hochberg corrected $p$ values (FDR) less than or equal to 0.05 between any of two of the categories (IM, T or Ts4).

Detection of citrullination
Citrullinated proteins were detected by immunoblotting after chemical modification following an established protocol described elsewhere.24

Data file
Microarray data have been deposited in the GEO database under accession number GSE68930.

Results
Sequential immortalization and transformation of PHK
We have used a modification of the well-established protocol initially introduced by the Weinberg lab25 to generate pools of cells representing discrete steps from normal keratinocytes to mutant HRAS-driven SCC (Fig. 1a). As mutant HRAS-driven tumors, in particular those related to BRAF inhibitor treatment, occur in both UV-exposed and unexposed areas, we decided to use UV-unexposed keratinocytes from neonatal foreskin. After circumcision, the tissue from a healthy male individual was immediately homogenized in the lab to isolate the keratinocytes as described in the Material and Methods section. Primary keratinocytes were initially grown on a layer of fibroblast feeders that were not required after immortalization with transgenes encoding the catalytic subunit of human telomerase hTERT and the Simian Virus 40 proteins small and large T. While hTERT allows to avoid replicative senescence, the viral proteins small and large T inhibit p53 and retinoblastoma in a comparable manner to the E6 and E7 proteins of beta HPV.14 We confirmed that after neomycin selection only those cells that received both transgenes were able to grow, while control primary keratinocytes stopped proliferating after seven passages (Fig. 1b).

All transductions have been performed with pools of cells and we decided to select the transformed keratinocytes further for their malignant phenotype. For this, we tested two approaches in parallel based on the ability of transformed cells to grow in attachment-free conditions (left) or as xenografts (right). The fraction of xenografts and total injections are given above the bars. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 1. Immortalization, transformation and selection of primary human keratinocytes. (a) Scheme depicting the genetic elements used for immortalization (IM) and subsequent transformation of primary keratinocytes (N). Icons represent affected cancer parameters according to.40 Transformed cells (T) were selected in vitro by attachment-free growth in soft agar (Ti) or in vivo by xenograft growth (Ts). (b) Growth curves comparing different cell populations with the established SCC13 cell line. (c) Western blot and RT-qPCR analysis of transgene expression. The Ts sample shown here is later referred to Ts1 (see Fig. 3). (d) To assess the tumorigenic potential of generated cells, we assayed their capacity to grow in attachment-free conditions (left) or as xenografts (right). The fraction of xenografts and total injections are given above the bars. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
cells in soft agar and eventually pooled all those clones that were able to grow to spheric colonies for further expansion. We refer to in vitro selected cells as Ti. The expression levels of hTERT mRNA, large T and small t protein were comparable in transformed and selected cells, while protein levels of mutant Ras were increased (Fig. 1c). When replated in soft agar, selected cells were tendentially more potent to grow in attachment-free conditions than the parental transformed population of cells (Fig. 1d). However, when injected subcutaneously into athymic mice, in vitro selected Ti cells failed to grow as xenografts (Fig. 1d). These results reiterate that soft agar assays do not necessarily reflect the tumorigenic potential of cells.

**Transformed keratinocytes give rise to heterogeneous xenografts**

As the in vitro selection had failed to produce cell populations with an increased tumorigenic capacity, we decided to directly select cells in vivo. For this, transformed cells were grown as xenografts (Fig. 2a). In total, 14 tumours were resected and processed for histopathological analysis. While all lesions were diagnosed as SCC by a dermatopathologist on the basis of histopathological features, they presented three main architectural patterns, which were cystic, solid or sarcomatoid (Figs. 2b and 2c). Six of the xenograft tumours formed cystic lesions with a wall of small atypical keratinocytes that tend to acquire larger cytoplasms on the upper layers and contain keratin-filled central spaces (Fig. 2b). These cystic lesions had some resemblance with the vemurafenib-induced keratoacanthomatous proliferations (compare to Fig. 2d and Ref. 27), which frequently contain HRAS mutations.16,17 About one third of the xenografts corresponded to solid masses of poorly differentiated cells. One case showed a mixed solid-cystic pattern. Finally, two lesions were classified as pleomorphic SCCs with large sarcomatoid...
areas (Fig. 2b). As summarized in Table 1, all 14 tumor xenografts showed some overlapping histologic features such as pleomorphic, basaloid and poorly differentiated cell populations and the presence of central keratine (Fig. 2e and Table1).

Taken together, we found that primary cells from the same donor and transformed with the same set of oncogenic elements grow as tumour xenografts that displayed part of the heterogeneity found in the clinical spectrum of SCCs.

**In culture, cells retain the histopathologic traits from the parental tumour**

Whenever the size of xenografts permitted, half of the tumor mass was homogenized individually and cells were re-expanded in culture. We refer to these *in vivo* selected cells as Ts cells and number them according to the xenograft they were derived from. We confirmed that virtually all cells in culture were GFP positive indicating the expression of the mutant *HRAS* transgene. Next, we selected a sarcomatoid and solid xenograft and the derived cell cultures Ts1 and Ts4, respectively, for further analysis. Both cell lines present genomic abnormalities of which the gain of a third copy of chromosome 20 is a shared feature (Table 1). In addition, both cell lines present additional variations such as a translocation involving chromosomes 8 and 5 in Ts1 and the partial loss of one copy of chromosome 13 in Ts4 cells.

The sarcomatoid tumour contained pleomorphic cells and some of them presented large cytoplasmic vacuoles, giving them the form of signet ring cells, which were also present in the subsequent cell culture (Fig. 3a). Signet ring cells are typically found in highly malignant de-differentiated adenocarcinomas, but have been reported to occur sporadically in SCC. We next confirmed the squamous epithelial origin by detecting p63 expression in some nuclei in both the tumour tissue and cultured Ts1 cells (Fig. 3a). These *in vivo* selected cells were more oncogenic than the parental population as shown by an increased capacity to grow colonies under attachment free conditions (Fig. 3b). Furthermore, injected subcutaneously in athymic mice, Ts1 cells grew faster tumours (Fig. 3c).

The solid tumour presented masses of poorly differentiated carcinoma cells at a first glance (Fig. 4a). However, when we analysed it more carefully, we could observe features of focal apocrine differentiation with glandular spaces indicative of apical secretion. In total, we confirmed presence of focal apocrine differentiation in three xenografts (Table 1, a second example shown in Fig. 4b). Similar focal apocrine differentiation can occasionally be observed in human biopsies of poorly differentiated SCCs that were not related to sun exposure (see Fig. 4b for an example). The appearance of such aberrant apical secretory differentiation most likely reflects the plasticity of neoplastic cells. Interestingly, in culture Ts4 cells seemed to retain this apocrine component as judged by the rosetoid gland-like disposition of the cells (Fig. 4a). Apocrine areas in both the xenograft and the culture were highlighted using the CAM5.2 antibody against low molecular weight keratins (Fig. 4a). It is generally accepted that this type of keratins is most abundant in secretory cells.

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**Table 1. Histopathologic features of tumour xenografts and established cell lines**

| Tumour | Primary histopathologic features | Secondary histologic features | Poor epidermal differentiation | Established cell culture |
|--------|---------------------------------|------------------------------|--------------------------------|-------------------------|
|        | Pleomorphic                     | Apocrine differentiation     | Central keratine              | Name    | Karyotype                        |
| X1     | Sarcomatoid                     | ++                            | +                              | Ts1     | 47, XY, der(8)t(5;8)(q13;p21), +20 [20] |
| X2     | Cystic                          | ++                            | +                              | Ts2     |                                |
| X3     | Cystic                          | +                             | +                              | Ts4     | 47, XY, +20 [5]/46, XY, −13, +20 [3]/47, XY, −13, +20, +mar [3] |
| X4     | Solid                           | +                             | +                              | Ts5     |                                |
| X5     | Cystic                          | ++                            | +                              | Ts6     |                                |
| X6     | Solid                           | ++                            | +                              | Ts7     |                                |
| X7     | Cystic                          | ++                            | +                              | Ts8     |                                |
| X8     | Solid-cystic                    | ++                            | +                              | Ts9     |                                |
| X9     | Cystic                          | +                             | +                              | Ts10    |                                |
| X10    | Sarcomatoid                     | ++                            | +                              | Ts11    |                                |
| X11    | Cystic                          | ++                            | +                              | Ts12    |                                |
| X13    | Solid                           | ++                            |                                | Ts13    |                                |
| X14    | Solid                           | +                             |                                | Ts14    |                                |

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and their visualization with CAM5.2 antibody is the gold standard for the detection of secretory glands. The stronger signal of CAM5.2 in Ts4 cells compared to the tumour may also reflect a change in keratine composition after seeding them in a non-tissue environment (Fig. 4a). Again, cultured Ts4 cells were more aggressive than the parental population of transformed keratinocytes since they were able to grow tumours in a shorter period of time (Fig. 4d).

Taken together, the results summarized in Figures 3 and 4 make two important points. First, even in culture, cells maintain the distinctive traits of the xenografts they have been derived from. Second, in vivo selected Ts1 and Ts4 cells have clearly higher tumorigenic potential than the parental population of transformed cells.

Cells with progressively transformed phenotype can be used as model to identify drivers of skin carcinogenesis

Next, we decided to exploit the fact that immortal, transformed and in vivo selected cells reflect discrete steps with incremental tumorigenic potential, in order to identify potential drivers of skin carcinogenesis. For this, we have compared the global gene expression pattern of the Ts4 cells derived from a solid-type xenograft with parental transformed T and immortal IM cell populations. As shown in Figure 5a, gene expression patterns are clearly altered and biological replicates group together. In total we found that comparing IM and Ts4 samples 1250 genes were differentially expressed at least 2-fold. Among the differentially expressed genes were several known cancer genes such as...
DKK3 and SOX9. DKK3 is an inhibitor of the Wnt signalling pathway and has previously shown to be down-regulated in both mammary and thyroid carcinoma samples.\textsuperscript{31,32} Conversely, SOX9 was found upregulated in hyperproliferative skin diseases including SCC, basal cell carcinoma and keratoacanthoma.\textsuperscript{33} We could validate both the upregulation of SOX9 as well as the downregulation of DKK3 in independent replicates of Ts4 samples analyzed by semi-quantitative RT-PCR (Fig. 5\textbf{b}).

Interestingly, we found that two members of a family of peptidyl arginine deiminases (PADI) termed PADI1 and PADI2 were similarly strongly up-regulated in Ts4 cells as was SOX9 (Fig. 5\textbf{a}). PADI enzymes have recently attracted much attention when it was shown that a member of the family PADI4 regulated the pluripotent state of stem cells.\textsuperscript{34} PADI2 has further been implicated in the development of breast cancer.\textsuperscript{35} We confirmed by PCR the pronounced increase of PADI1 and PADI2 transcripts in transformed \textit{in vivo} selected Ts4 cells (Fig. 5\textbf{b}). We could not detect the expression of other PADI family members including PADI3 which is known to be expressed in the most differentiated upper layer of the epidermis.\textsuperscript{36} Next we took advantage of the availability of an anti-PADI2 antibody to confirm its overexpression on the protein level by immunoblotting (Fig. 5\textbf{c}). PADI are enzymes that convert positively charged arginine residues into neutral citrullines, which has a direct impact on regulating the protein-protein interaction of its substrates. To assess whether PADI1 and PADI2 are enzymatically functional, we tested whether we can detect citrullination in Ts4 cells. Indeed, we observed a strong increase of citrullinated proteins after calcium-induced enzyme activation in total cell extracts from Ts4 but not IM cells (Fig. 5\textbf{d}). As both cytoplasmic as well as nuclear functions have been proposed for PADIs,\textsuperscript{37} we analyzed the subcellular localization of PADI2 in the xenograft and cultured cells by IHC. We found that PADI2 was primarily detected in the cytoplasm (Fig. 5\textbf{c}).

Next, we analyzed the level of PADI1 and PADI2 mRNA in 11 primary cell lines extracted from tumor samples collected from patients with oral SCC. These samples were kindly provided by Fiona Watts (King’s College London). Two of these 11 samples had a clear overexpression of PADI2 and/or
PADI1 suggesting that this is recurrent event occurring in a subpopulation of patients with SCC (Fig. 1f). While performing these studies, the Coonrod lab reported that mice overexpressing PADI2 spontaneously develop skin tumour lesions. This provides strong evidence for a tumour-promoting role of PADI2 in non-melanoma skin cancer and demonstrates that engineered cells provide a valuable tool to identify new drivers of skin carcinogenesis.

**Discussion**

The histopathological heterogeneity encountered in non-melanoma skin tumours and even within the same tumour class such as SCC is still poorly understood. Here, we report the establishment and characterization of a panel of model cell lines that represent part of the clinically observed heterogeneity of SCC and that makes its mechanistic analysis feasible. Immortalized and initially transformed keratinocyte cultures can be combined with two in vivo selected sub-lines that represent two different clinical manifestations of SCC (Fig. 5g). These cell lines can serve (i) as linear models for two different manifestations of non-melanoma skin carcinogenesis, (ii) as tool to identify the factors determining cell and tissue phenotype and (iii) for complementing cell culture based approaches for drug development.

The identification of the minimal genetic requirements to transform human primary cells has been a milestone for modern cancer research. Since then, several groups have applied the Weinberg protocol or modifications of it to genetically engineer cell culture models of cancer. Here, we describe the generation of a set of cell cultures that reflect progressive steps of skin carcinogenesis. These include the initial immortalization of primary keratinocytes, transformation by mutant Ras and in vivo selection by xenograft growth. Interestingly, xenografts manifested different histopathological phenotypes that reflected part of the diversity usually observed in the clinical routine by dermatopathologists. Importantly, cells maintained the characteristics from the xenograft after reintroduction in culture. These cells now provide an accessible cell culture model fully amenable for genetic and pharmacological manipulation allowing us to dissect in vitro the molecular parameters

**Figure 5.** The enzyme PADI2 is overexpressed in cells reflecting solid-type SCC. (a) Unsupervised cluster analysis of genes differentially expressed in immortal (IM), transformed (T) and in vivo selected cells Ts4 derived from a solid-type xenograft. Up and down refers to Ts4 compared to IM (P < 0.05 and |logFC| > 2). (b) Semi-quantitative RT-qPCR data are represented as mean and error bars indicate SD, n = 3. n.d., not detected. (c) Western blot for PADI2. (d) Visualization of Ca+ induced citrullination of proteins in whole cell lysates using a chemical reaction-coupled immunoblotting described elsewhere. (e) IHC for PADI2 in xenograft and derived Ts4 cells. (f) Semi-quantitative RT-qPCR analysis of PADI1 and PADI2 mRNA levels in oral SCC samples. Patient-derived tumor cell pellets were kindly provided by Fiona Watt (King’s College London). IM, Ts1 and Ts4 cells have been included as reference. (g) Scheme of engineered model cell lines reflecting disease progression and two distinct histopathological manifestations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
determining histopathological heterogeneity. This intrinsic heterogeneity distinguishes the here described model cell lines from previously skin cancer cell lines engineered by similar controlled genetic transformation of primary keratinocytes. The factors that could be causative for this heterogeneity include transformation of different cell types and stages in the initial primary culture, differential genome insertion and epigenetic plasticity. In any case, the different histopathological traits found in xenografts are matched by reported observations in primary human biopsies, emphasizing the value of our model in successfully recapitulating SCC.

Two major advantages of SCC models generated by in vitro transformation are the availability of control cells of the same genetic background and exact knowledge about the introduced oncogenic elements. Thus engineered cells overcome a major limitation caused by comparing established SCC cell lines with spontaneously IM that differ in genetic background, donor age and degree of UV exposure correlating with the accumulation of genetic variation. As such, the cells described here can be used as a discovery tool for alterations co-occurring or contributing with disease progression to two different clinical manifestations (Fig. 1f). Indeed, using our model we were able to identify a novel SCC disease gene by comparing the global expression pattern of in vivo selected transformed cells corresponding to the solid SCC phenotype with the parental transformed and immortal cells. We found that the two genes encoding the citrullinating enzymes PADI1 and PADI2 were most pronouncedly overexpressed. Others recently reported that overexpression of a PADI2 transgene in several tissues of mice was sufficient to selectively cause tumours in skin. This provides strong evidence for an oncogenic function of PADI2 in non-melanoma skin cancer and demonstrates that engineered cells provide a valuable tool to identify new drivers of the carcinogenic process.

Concerning the underlying molecular mechanism in skin cancer, PADI2 was suggested to induce neoplasias by upregulating on the transcriptional level inflammatory cytokines and mediators of the epithelial-to-mesenchymal transition. Although we cannot exclude a nuclear function of PADI2, immunohistochemical analysis of a solid-type xenograft and derived cells demonstrated that the vast majority of the PADI2 protein localized to the cytoplasm. This supports the idea that PADI2-mediated citrullination of cytoplasmic proteins could contribute to the increased tumorigenic phenotype. Future work will be required to determine the substrates and citrullination events that are relevant for the tumour-promoting function of PADI2 in skin.

Taken together, here we report the generation of a set of cell lines that stand out as they represent part of the clinically observed heterogeneity of SCC histopathology. These cells enable the functional analysis of factors that determine phenotypic differences. They are further a valuable complement to our toolkit for the analysis of non-melanoma skin cancer and for the development of drugs targeting Ras proteins or their downstream signalling.

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References

1. Rogers HW, Weinstock MA, Harris AR, et al. Incidence estimate of nonmelanoma skin cancer in the United States, 2006. Arch Dermatol 2010; 146:283–7.
2. Madan V, Lear JT, Szemies R-M. Non-melanoma skin cancer. Lancet 2010; 375:673–85.
3. Arwert EN, Hoste E, Watt FM. Epithelial stem cells, wound healing and cancer. Nat Rev Cancer 2012; 12:170–80.
4. Boukamp P. Non-melanoma skin cancer. Drives tumor development and progression? Carcinogenesis 2005; 26:1657–67.
5. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. Nature 2010; 464:427–30.
6. Hatzivassiliou G, Song K, Yen I, et al. RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. Nature 2010; 464:431–5.
7. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000; 408:307–10.
8. Ziegler A, Jonason AS, Leffell DJ, et al. Sunburn and p53 in the onset of skin cancer. Nature 1994; 372:773–6.
9. Brash DE. Roles of the transcription factor p53 in keratinocyte carcinomas. Br J Dermatol 2006; 154(Suppl 1):8–10.
10. Quinit KD, Genders RE, de Koning MN, et al. Human Beta-papillomavirusinfection and keratocytic carcinomas. J Pathol 2015; 235:342–54.
11. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 2007; 7:295–308.
12. Oberhofer PA, Kee D, Dziunycz P, et al. RAS mutations are associated with the development of cutaneous squamous cell tumors in patients treated with RAF inhibitors. J Clin Oncol 2012; 30:316–21.
13. Suf F, Viros A, Milagre C, et al. RAS mutations in cutaneous squamous-cell carcinomas in patients treated with RAF inhibitors. N Engl J Med 2012; 366:207–15.
14. Mollón C, Ribera J-M, Morgades M, et al. Prognostic significance of complex karyotype and monosomal karyotype in adult patients with acute lymphoblastic leukemia treated with risk-adapted protocols. Cancer 2014; 120:3958–64.
15. Creppe C, Janisch P, Cantarino N, et al. Macrophage alloactivation on BRAF inhibitor treated with BRAF inhibitors. N Engl J Med 2012; 366:207–15.
23. Uribesalgo I, Buschbeck M, Gutiérrez A, et al. E-box-independent regulation of transcription and differentiation by MYC. Nat Cell Biol 2011; 13: 1443–9.
24. Raats JMH, Wijnen EM, Pruijn GJM, et al. Recombinant human monoclonal autoantibodies specific for citrulline-containing peptides from phage display libraries derived from patients with rheumatoid arthritis. J Rheumatol 2003; 30:1696–711.
25. Hahn WC, Counter CM, Lundberg AS, et al. Cre-ation of human tumour cells with defined genetic elements. Nature 1999; 400:464–8.
26. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144:646–74.
27. Sufficool KE, Hepper DM, Linette GP, et al. Histopathologic characteristics of therapy-associated cutaneous neoplasms with vemurafenib, a selective BRAF kinase inhibitor, used in the treatment of melanoma. J Cutan Pathol 2014; 41:568–75.
28. Fuku Y. Mechanisms behind signet ring cell carcinoma formation. Biochem Biophys Res Commun 2014; 450:1231–3.
29. Demellawy DE, Onuma K, Alowami S. Signet ring squamous cell carcinoma—the forgotten variant: case report and review of the literature. J Cutan Pathol 2011; 38:306–8.
30. Wasserman J, Maddox J, Racz M, et al. Update on immunohistochemical methods relevant to dermatopathology. Arch Pathol Lab Med 2009; 133:1053–61.
31. Veeck J, Bektas N, Hartmann A, et al. Wnt signalling in human breast cancer: expression of the putative Wnt inhibitor Dickkopf-3 (DKK3) is frequently suppressed by promoter hypermethylation in mammary tumours. Breast Cancer Res 2008; 10:R82
32. Yin DT, Wu W, Li M, et al. DKK3 is a potential tumor suppressor gene in papillary thyroid carcinoma. Endocr Relat Cancer 2013; 20:507–14.
33. Shi G, Sohn K-C, Li Z, et al. Expression and Functional Role of Sox9 in Human Epidermal Keratinocytes. PLoS One 2013; 8:e54355.
34. Christophorou MA, Castelo-Branco G, et al. Citrullination regulates pluripotency and histone H1 binding to chromatin. Nature 2014; 507:104–8.
35. McElwee JL, Mohanan S, Griffith OL, et al. Identification of PAD2 as a potential breast cancer biomarker and therapeutic target. BMC Cancer 2012; 12:500.
36. Nachat R, Méchin M-C, Takahara H, et al. Peptidylarginine deiminase isoforms 1-3 are expressed in the epidermis and involved in the deimination of K1 and filaggrin. J Invest Dermatol 2005; 124: 384–93.
37. Bicker KL, Thompson PR. The protein arginine deiminases: structure, function, inhibition, and disease. Biopolymers 2013; 99:155–63.
38. McElwee JL, Mohanan S, Horibata S, et al. PAD2 Overexpression in Transgenic Mice Promotes Spontaneous Skin Neoplasia. Cancer Res 2014; 74:6306–17.
39. Lazarov M, Kubo Y, Cai T, et al. CDK4 coexpression with Ras generates malignant human epidermal tumorigenesis. Nat Med 2002; 8:1105–14.
40. Vaughan MB, Ramirez RD, Andrews CM, et al. H-Ras expression in immortalized keratinocytes produces an invasive epithelium in cultured skin equivalents. PLoS One 2009; 4:e7908.