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An in vitro three-dimensional model of primary human cutaneous squamous cell carcinoma

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Abstract

Squamous cell carcinomas (SCC) represent a substantial clinical problem because of increases, frequent recurrences and successive de novo tumors, especially in organ transplant recipients. To improve upon the current surgical and other non-selective therapies, a validated organotypic in vitro model of primary human SCC needs to be developed. Such a model will have obvious advantages over current cell line and animal based approaches, and may render the latter partly obsolete. In a first approach, an explant technique of primary SCC biopsies onto dermal constructs was used to emulate tumor expansion in an in vitro model. Histological analysis revealed the formation of nests of squamous cells, mimicking an invasive morphological feature of primary SCC. Immunohistochemical analysis comprised an array of markers characteristic of keratinocyte (hyper) proliferation (K6, K16, K17 and Ki67), differentiation (K1, K10 and involucrin), basement membrane (collagen types IV and VII, integrins a6 and b4 and laminin 332) and SCC (K4, K13 and Axl). The generated human SCC models displayed disturbed differentiation and keratins associated with hyperproliferation, but a low frequency of Ki67 positive cells. Basement membrane composition of the in vitro SCC model resembled that of normal skin. These results show for the first time that in vitro modelling of three-dimensional growth of primary cutaneous human SCC is feasible. This model may provide a platform to develop refined preventive and curative treatments and thereby gain understanding of SCC pathogenesis.
Introduction

Cutaneous squamous cell carcinoma (SCC) is a malignant tumor of epidermal keratinocytes characterized by invasive growth into the dermis. After basal cell carcinoma, SCC is the most common malignancy in white populations with epidemic incidence rates currently ranging from 18 per 100,000 person years in the Netherlands up to 395 per 100,000 person years in Australia, and predicted to have increased by 80% in the Netherlands in 2015.\(^1\),\(^2\) Actinic keratoses (AK) are precursor lesions of SCC and precede the biological continuum ranging from carcinoma in situ to invasive SCC. The clinical problem of SCC is particularly dramatic in immunosuppressed individuals: in a population of Australian immune suppressed kidney transplant recipients, the cumulative incidences of SCC after 5, 11 and 20 years have increased to 3%, 16% and 41%, respectively.\(^3\) Our clinic closely monitors and treats organ transplant patients presenting with skin carcinomas. The current study focuses on SCCs from this group of patients.

Since medical intervention is always required for treatment of primary, successive and recurrent cutaneous SCCs, refined therapies focused on precursor lesions are highly desirable. Well-targeted specific therapeutic agents could serve this purpose, based on essential knowledge on cutaneous SCC carcinogenesis. The development of a representative \textit{in vitro} skin carcinoma model system allows for a better understanding of fundamental carcinogenesis mechanisms and may serve as a validated pre-screening platform for candidate drugs. Traditional \textit{in vivo} models rely on the use of chemical, genetic or mechanical induction or propagation of carcinogenesis in mice.\(^4\)\(^-\)\(^8\) Current \textit{in vitro} approaches are limited to the use of cell lines, which have commonly undergone multiple adaptations to long-term culture, resulting in a clonal selection of a subpopulation of cells from primary heterogeneous tumors. This may compromise a true representation of primary skin cancer. \textit{In vitro} SCC progression models involve the transformed HaCaT cell line, the MET1 to MET4 cell lines derived from primary and recurrent cutaneous SCC and metastases thereof and the benign Ha-Ras mutated murine keratinocyte cell line 308 that is capable of transformation \textit{in vitro}.\(^7\),\(^9\)\(^-\)\(^11\) More refined approaches are based on human skin equivalents, comprising three-dimensional systems in which a natural skin tissue context is reconstructed. These model systems have the advantage of harboring different cell types in a local environment that is highly similar to that of the \textit{in vivo} tissue. Thus, these model systems will more closely emulate cellular processes than conventional monolayer cultures.\(^12\),\(^13\) But again, SCC cell line based human skin equivalents harbor the drawback of an artificially selected clone of tumor cells, rendering primary SCC biopsies more suitable for SCC modeling \textit{in vitro}. Earlier studies show that phenotypic defects in genetic skin disorders persist in organotypic skin cultures,
e.g. with the hereditary blister disease epidermolysis bullosa simplex. Based on these findings, we reasoned that three-dimensional modeling of human cutaneous SCC of immunocompromised organ-transplant recipients should be possible by using cells from primary human cutaneous SCCs. Here we present the generation of such a model, which was subsequently characterized for the presence of putative SCC markers as well as markers specific for keratinocyte activation, proliferation and differentiation and basement membrane (BM) formation.

Methods

Patient material
Three primary excisional well-differentiated cutaneous SCC biopsies from different renal transplant patients were freshly collected. Of these biopsies, one part was processed for diagnosis while the remainder was used for explant culture. Two native skin biopsies originating from the buttock of immunocompromised renal transplant patients and two native skin biopsies originating from mamma reduction surplus skin of immunocompetent individuals were freshly collected and served as control explants. In addition, three archived well-differentiated SCC samples from the institutional Department of Pathology were used as reference material for immunohistochemical analyses. All samples used were collected with written informed consent of the patient according to the Declaration of Helsinki after approval of the medical ethical committee of the Leiden University Medical Center. Furthermore, two SCC cell lines representing the tumorigenic populations of cutaneous SCCs (SCC-12B2 and SCC-13) were included. These cell lines were kindly provided by Dr. J.G. Rheinwald. Clinical specifications of all starting materials are provided in Table 1.

Fibroblast culture
For isolation of normal human dermal fibroblasts (NHDFs), dermis was obtained through overnight incubation of fresh mamma reduction surplus skin with dispase II (Roche Diagnostics, Almere, the Netherlands). Fibroblasts were isolated from the dermis through incubation with a solution consisting of collagenase II (Invitrogen, Breda, the Netherlands) and dispase II (ratio 1:3 and 3 ml/g dermis) at 37°C for 2 hours. The cells were filtered using a 70 μm cell strainer (BD Biosciences, Breda, the Netherlands), and cultured in fibroblast medium at 37°C and 5% CO₂ until subconfluency. Fibroblast medium consisted of Dulbecco’s modified Eagle’s medium (DMEM, Gibco/Invitrogen, Breda, the Netherlands) supplemented with 5% fetal bovine serum (FBS, HyClone/Greiner, Nurtingen, Germany), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Invitrogen,
**Table 1 | Clinical specifications of starting materials.**

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Gender</th>
<th>Age</th>
<th>Localization</th>
<th>Diagnosis</th>
<th>Background</th>
<th>Treatment</th>
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<td>Pred / Aza</td>
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<td>Pred / Aza</td>
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F, female; M, male; SCC, squamous cell carcinoma; Pred, prednisone; Aza, azathioprine; Immune suppr, unspecified immune suppression.
Breda, the Netherlands). Culture medium was refreshed every three days. Fibroblast passages two to four have been used for the experiments.

**SCC cell line culture**

SCC cell lines were cultured on lethally irradiated murine 3T3 fibroblasts in standard keratinocyte medium at 37°C and 7.3% CO₂ until subconfluency. Standard keratinocyte medium consisted of DMEM and Ham’s F12 medium in a 3:1 ratio, supplemented with 5% FBS, 0.5 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin (Sigma-Aldrich, Zwijndrecht, the Netherlands), 100 U/ml penicillin and 100 μg/ml streptomycin. Culture medium was refreshed every three days.

**Explant cultures**

Upon arrival, the three fresh SCC biopsies from immune-suppressed individuals were rinsed three times in sterile phosphate buffered saline (PBS). Thereafter the biopsies were incubated overnight at 4°C in PBS supplemented with penicillin-streptomycin solution (300 U/ml) and amphotericin B (Fungizone, 5 μL/ml, Gibco/Invitrogen). Next, the biopsies were rinsed three times with sterile PBS. For generation of explant cultures, dermal equivalents were constructed as described earlier. In brief, rat tail collagen was seeded with 1.25x10⁵ NHDF per milliliter and incubated for a period of one week under submerged conditions. Thereafter, the SCC biopsies were placed onto the collagen matrices. All explants (skin cancer and healthy skin biopsies) were cultured at the air-liquid interface under serum-free conditions. The SCC models were cultured for three weeks in serum-free keratinocyte medium as described above, supplemented with 53 μM selenious acid, 10 mM L-serine, 10 μM L-carnitine, 7 μg/ml β-dextrin, 1 μM dl-α-tocopherol-acetate, 100 μg/ml ascorbic acid phosphate and a lipid supplement containing 25 μM palmitic acid, 30 μM linoleic acid, 7 μM arachidonic acid and 2.4x10⁻⁵ M bovine serum albumin (Sigma-Aldrich, Zwijndrecht, The Netherlands).

**Human skin equivalents**

Human skin equivalents (HSEs) using keratinocytes from two SCC cell lines were generated as described earlier. Briefly, dermal equivalents containing NHDFs were seeded with 5x10⁴ keratinocytes cm⁻² from cell lines SCC-12B2 and SCC-13. The HSEs were incubated overnight under submerged conditions in standard keratinocyte medium. The next day, cultures were lifted to the air-liquid interface and medium was replaced by the same keratinocyte medium as described above for the explant cultures, but with reduced FBS (1%). From the second day, serum was omitted and HSEs were cultured for three to four weeks while medium was refreshed every three days.
Immunohistochemical analysis
After the culture period, skin equivalents were split in two fragments for immunohistochemical analyses. One part was snap-frozen in liquid nitrogen while the other part was fixed in 4% paraformaldehyde, dehydrated and paraffin embedded. Formalin fixed, paraffin embedded primary SCC archive material was obtained from the Department of Pathology of the Leiden University Medical Center. Global histological analysis was performed on 5 μm sections through staining with haematoxylin and eosin (HE). Immunohistochemistry was performed on 5 μm sections and all samples were analyzed for the presence of a set of markers specific for keratinocyte proliferation, differentiation and BM composition. Immunohistochemical analysis of collagen types IV (clone PHM12, Chemicon, Temecula CA) and VII (clone LH7.2, Dr. I.M. Leigh, London, UK), integrin subunits α5 (clone JEB5, Dr. A Sonnenberg, Amsterdam, The Netherlands) and β4 (clone 3E1, Telios Pharmaceuticals, San Diego CA), involucrin (clone SY5, Santa Cruz Biotechnology, Santa Cruz CA), Ki67 (clone MIB1, DAKO, Glostrup, Germany), laminin 332 (clone BM165, Dr. A. Aumailley, Cologne, Germany), keratin 1 (clone LHK1, Dr. I.M. Leigh, London, UK), keratin 4 (clone 6B10, Dr. G.N.P. van Muijen, Leiden, The Netherlands), keratin 6 (clone Ks6.KA12, Sanbio BV, Uden, The Netherlands), keratin 13 (clone 1C7, Dr. G.N.P. van Muijen, Leiden, The Netherlands) and keratin 17 (clone CKE3, Sigma, Saint Louis MO) was performed on 5 μm frozen sections that were air-dried overnight and fixed in acetone for 10 min. Paraffin embedded cultures were sectioned and 5 μm sections were deparaffinized in ethanol and rehydrated in preparation of immunohistochemical analysis of Axl (clone C20, Santa Cruz Biotechnology, Santa Cruz CA), keratin 10 (clone DEK10, Neomarkers, Fremont CA) and keratin 16 (clone LL025, Dr. I.M. Leigh, London, UK). After incubation with secondary antibodies, sections were stained with streptavidin-biotinylated horseradish peroxidase complex (ABCComplex/HRP, DakoCytomation, Glostrup, Denmark), as described by the supplier. All sections were counterstained with haematoxylin.

Estimation of proliferation index
To determine the proliferation index, the number of Ki67 positive nuclei from the total number of basal cells (x100%) was used. A minimum of 100 basal cells was counted in sections of two different samples at a magnification of 200×. The resulting data are expressed as the mean of the two independent experiments ± SD (error bars).
Results

Invasive behavior in biopsy and cell line based skin cancer models
Fresh skin biopsies of both healthy and SCC origin were cultured on fibroblast-seeded collagen matrices at the air-liquid interface under serum-free conditions. During a four-week culture period, biopsy-associated keratinocytes migrated and proliferated over the dermal equivalent in a lateral fashion (Figure 1a). Cross sections of all explant cultures revealed spreading of the epidermis with viable cell-layers including the stratum basale, stratum spinosum, stratum granulosum and the stratum corneum. Furthermore, invasive behavior of a subpopulation of expanded SCC-associated keratinocytes was observed in two of three SCC explant models. These keratinocytes were capable of forming invasive nests in the dermal equivalent, originating from observed epidermal protrusions (Figure 1b–e). These invasive nests were seen in the direct vicinity of the original tumor biopsy as well as in dermal parts more distant from the original tumor biopsy. Skin equivalents generated with cell line SCC-12B2 showed extensive invasion into the dermal equivalent (Figure 1f), while cell line SCC-13 showed disturbed attachment of the epidermal keratinocytes to the dermal equivalent (Figure 1g). None of these features were observed in explant cultures of healthy skin biopsies of both immunocompetent and immunocompromised individuals.

Immunohistochemical SCC model characterization
To further characterize the SCC explant cultures, immunohistochemical analyses were performed on its epidermal outgrowth, targeting various protein specific markers for keratinocyte activation, proliferation and differentiation and BM formation. Results of all immunohistochemical analyses are summarized in Table 2.

Generated SCC models display hyperproliferative features
To assess the activation and proliferation status of keratinocytes in the cultures, expression of activation and hyperproliferation-specific keratins K6, K16 and K17 and proliferation-specific nuclear protein Ki67 was analyzed. In normal epidermis, none of the activation-specific keratins were detected (Figure 2a) and 13% ± 0.7% of basal cells stained positive for Ki67. Explant models of normal skin of immunocompetent individuals showed suprabasal staining of K6 and slight suprabasal staining of K16, while K17 was not detected and 15% ± 3% of basal cells stained positive for Ki67. Explant models of normal skin from immunocompromised patients showed slight suprabasal staining of K6 and subcorneal staining of K16 and K17 (Figure 2b). Epidermis, but not squamous cell nests of primary SCC stained strongly positive for K6, K16, K17 (Figure 2e) and Ki67 (62% ± 25%), lining the
Figure 1 | Haematoxylin–eosin (HE) staining of a skin cancer explant model, generated by culturing a squamous cell carcinoma (SCC) biopsy on a fibroblast-seeded collagen matrix (a). Explant models exhibit formation of squamous nests in the dermal compartment (a, indicated with arrows and b), originating from epidermal protrusions (c–e). Human skin equivalents were generated with SCC cell lines SCC-12B2 (f) and SCC-13 (g). Scale bars indicate 100 µm.
Table 2 | Summarized results of all immunohistochemical stainings on squamous cell carcinoma (SCC) explant models, primary SCC, normal skin explant models of organ transplant recipients (NS-OTR), primary normal skin of healthy individuals (NS-H), normal skin explant models of healthy individuals and SCC-12B2 and SCC-13 human skin equivalents (HSE). SC, stratum corneum; SS, stratum spinosum; SB, stratum basale; DEJ, dermal-epidermal junction.
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**Figure 2** | Immunohistochemical staining of epidermal hyperproliferation marker keratin 17 (K17) in normal skin of immunocompetent individuals (a) and immunocompromised individuals (b), squamous cell carcinoma (SCC) explant model (c, d), primary SCC (e) and human skin equivalents generated with SCC cell lines SCC-12B2 (f) and SCC-13 (g). Counterstained with haematoxylin. Scale bars indicate 100 µm.

Proliferative edges of the invasive SCCs. The staining pattern of the SCC explant cultures resembled that of primary SCC with a strong K6 and K17 (Figure 2 c,d) staining throughout the epidermis and suprabasal staining of K16. Ki67 staining was present in 12% ± 2% of basal cells of SCC explant models. Skin equivalents generated with the SCC cell lines SCC-12B2 and SCC-13 stained positive for K17, which was present throughout all epidermal layers. SSC-13 showed a more pronounced expression of K17 in the basal cell layers (Figure 2f,g). Ki67 was present throughout all cell layers of SCC-12B2 and SCC-13 in 39% ± 8% and 82% ± 3% of cells, respectively.
Disturbed differentiation in SCC models

To gain more insight in the differentiation of keratinocytes in the cultures, immunohistochemical analyses were performed, focusing on differentiation-specific keratins K1, K4, K10 and K13, as well as on the terminal differentiation marker involucrin. In normal skin, K1 and K10 are expressed throughout the first suprabasal layers of the epidermis, indicating a normal proliferation program (Figure 3a,b). This staining pattern of K10, but not of K1, was also observed in explant models of normal skin of immunocompetent individuals (Figure 3e,f). In primary SCC however, both K1 and K10 were abundantly expressed in the cytoplasm of both basal and suprabasal keratinocytes (Figure 3j,k). The SCC explant model also showed strong basal expression of both K1 and K10, similar to primary SCC (Figure 3n,o). Furthermore, K1 was weakly detected in the suprabasal layers of the SCC explant model, whereas K10 was abundantly present in these layers of this model. K4 and K13 are both absent in normal skin and in normal skin explant models (Figure 3c,d,g,h). In all primary SCC and SCC explant skin models, both K4 and K13 were expressed in all cell layers (Figure 3l,m,p,q). In normal skin, involucrin is only expressed in the upper layers of the epidermis. In explanted normal skin of both immunocompetent and immunocompromised individuals, involucrin was also strongly expressed in the stratum corneum. In primary SCC, involucrin was abundant, without clear spatial restrictions, excluding the basal cell layers, but with greatest intensity in the cornified center of squamous cell nests. SCC explant models exhibited an involucrin staining pattern resembling that of primary SCC, with staining of all suprabasal layers as well as prominent staining in invasive squamous nests. Human skin equivalents of SCC cell lines SCC-12B2 and SCC-13 were only stained for K10 and involucrin. K10 was virtually absent in the model seeded with SCC-12B2 and present throughout the entire epidermis of the model seeded with SCC-13 (data not shown). Diffuse cytoplasmic staining of involucrin was shown in all epidermal layers of SCC-12B2 models and in focal areas of SCC-13 models (data not shown).

Normal basement membrane in SCC models

In order to assess the composition of the BM and associated cell adhesion molecules, immunohistochemical analysis was performed for collagen type IV, collagen type VII, laminin 332, integrin α6 and integrin β4 (data not shown). In normal skin of immunocompetent individuals and explants thereof, both collagen types IV and VII, laminin 332 and integrin α6β4 were confined to the epidermal-dermal junction (EDJ). Explant models of normal skin of immunocompromised individuals showed similar staining, but lacked collagen type VII staining. Primary SCC showed considerable variation in the presence of these molecules both within and between tumors. In some cases, the presence of collagen type IV along the EDJ was disrupted or completely absent, while in
Figure 3 | Immunohistochemical staining of differentiation-specific keratins K1, K10, K4 and K13 in normal skin (a–d), normal skin explant models of immunocompetent individuals (e–h), primary SCC (i–m) and SCC explant models (m–p). Rows indicate the various samples (from top to bottom: normal skin; NS, normal skin explant models; NSX, primary SCC and SCC explant models; SCCX) and columns indicate the various markers (from left to right: K1, K10, K4, K13). Explant models were cultured for 4 weeks under airexposed and serum-free conditions prior to processing for immunohistochemical analysis. Counterstained with haematoxylin. Scale bars indicate 100 µm.
other cases the immunolocalization and intensity were similar to that in normal skin. Collagen type VII staining was often absent in primary SCC, but focal expression of varying intensity in proliferating fields of tumorigenic keratinocytes was observed as well. Expression of laminin 332 was not evaluated in primary SCC. The integrin subunit α6 was not detected in primary SCC, whereas the integrin β4 subunit was highly abundant in most tumors, particularly lining squamous cell nests. In explant models of SCC, the detection of collagen types IV and VII, laminin 332 and integrin α6β4 was confined to the EDJ, indicating a fully formed BM. However, these BM molecules were also present at the circumference of the dermal squamous cell nests observed in SCC explant models. Models incorporated with the highly invasive SCC-12B2 cell line stained positive for collagen type IV lining the numerous areas of dermal invasion. Collagen type VII however was not detected in these cultures, whereas both laminin 332 and integrin subunit β4 were clearly present throughout all the epidermal compartments. In models seeded with SCC-13, lacking a proper dermal-epidermal attachment, collagen type IV was not detected, while collagen type VII was detected abundantly in the entire epidermal layer. Laminin 332 and integrin subunit β4 were both present along the EDJ and in the basal region.

**Axl is expressed both in SCC models and primary SCC**

As a candidate for involvement in SCC development and progression, we evaluated the presence of Axl in our current *in vitro* skin cancer model. In both native skin and explanted normal skin of both immunocompetent and immunocompromised individuals, no epidermal staining of Axl was observed (Figure 4a,b). Primary SCC and SCC explant models showed significant cytoplasmic staining of Axl in keratinocytes throughout the epidermis, particularly in more differentiated areas surrounding keratin pearls and in subcorneal layers (Figure 4c,d).

**Discussion**

In this study we used a skin explant method for the development and characterization of refined organotypic *in vitro* SCC models. Starting with primary human SCC biopsies of immune-suppressed individuals, we were able to create a three-dimensional skin equivalent harboring both keratinocytes and fibroblasts in a well-controlled microenvironment.

Global histology of SCC models revealed extensive lateral migration and proliferation of biopsy-associated keratinocytes, constituting an epidermal sheet of several cell layers. In addition, a subpopulation of expanded SCC-associated keratinocytes formed squamous cell nests in the dermal equivalent of some SCC models, which was never observed in
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Figure 4 | Immunohistochemical staining of putative squamous cell carcinoma (SCC) marker Axl in normal skin (a), normal skin explant model (b), primary SCC (c) and SCC explant model (d). Explant models were cultured for 4 weeks under air-exposed and serum-free conditions prior to processing for immunohistochemical analysis. Counterstained with haematoxylin. Scale bars indicate 100 µm.

healthy control material. These squamous cell nests resembled those of invasive well-differentiated primary SCC, with well ordered islands and keratin pearls.\textsuperscript{18} Furthermore, immunohistochemical analyses showed that keratinocytes in the SCC explant model were in an activated state, indicated by abundant expression of intermediate filament keratins K6, K16 and K17. These keratins are known to occur in the process of keratinocyte activation, which is characterized by hyperproliferation, migration, extensive cytoskeleton adaptation and BM component formation.\textsuperscript{19} These keratins are known to be absent in healthy epidermis, but present in hyperproliferative epithelial cells in pathologic conditions, including chronic wounds, psoriasis and cutaneous squamous SCC.\textsuperscript{19-22} Interestingly, explant models of normal buttock skin of immunocompromised renal transplant patients also stained positive for both K16 and K17, indicating that normal skin of these individuals might be in an activated state. This may be due to the immunosuppressive therapy of the patients concerned, consisting of the purine synthesis inhibitor azathioprine in combination with prednisone. Future research on higher numbers of immunocompromised normal skin explant cultures is needed to confirm this
observation. Detection of cell-cycling specific marker Ki67 of SCC explant models resembled that of normal skin and was not comparable to that of primary SCC. This implicates that the proliferation index of SCC models was normalized in culture. SCC explant models exhibited distorted expression of differentiation-specific keratins K1 and K10, indicating an aberrant differentiation pattern that was also seen in primary SCC. In addition, in primary SCC and in vitro skin cancer models differentiation-specific keratins K4 and K13 were present. Both keratins have been shown to be absent in normal skin. Aberrant staining of K1, K4, K10 or K13 was not observed in normal skin explants derived from immunocompetent or immunocompromised donors. SCC explant models showed a terminal differentiation pattern that is similar to primary SCC, characterized by patchy cytoplasmic staining but with increased intensity in invasive squamous nests. As a soluble protein precursor of the cross-linked envelope found in normal stratum corneum, involucrin was detected in the suprabasal layers of normal skin and explant cultures thereof, derived from both immunocompetent and immunocompromised donors. The current SCC explant model presented with a fully functional and continuous BM, displaying all of its main components including collagen types IV and VII, laminin 332 and integrin α6β4. The squamous cells nests observed in these in vitro SCC models showed a similar pattern, implicating the functionality of these keratinocytes to form a normal BM surrounding these invasive areas.

Although our in vitro SCC explant model displayed invasive behavior by the formation of squamous nests, extensive invasion as seen in primary SCC is not present. This may be due to the facts that our culture conditions were not specifically selective for tumor cells and that the dermal equivalents in these models were seeded with fibroblasts of healthy origin, providing a healthy microenvironment. Recent advances in cell biology indicate the vital importance of cancer-associated fibroblasts in tumor-stroma interactions and invasion of SCC. Thus, the squamous cell nests observed in our model may reflect the invasive capacity of lesional keratinocytes alone, or perhaps supported by few biopsy-associated fibroblasts, or the limited effects of SCC-associated keratinocytes on healthy fibroblasts seeded in the dermal equivalent. Of interest, skin models seeded with SCC cell line SCC-12B2 showed extensive invasion of the dermal compartment, which was also seeded with fibroblasts of healthy origin. This may support a mechanism in which epidermal keratinocytes provide molecular cues to associated dermal fibroblasts, which in turn enable tumor invasion. Therefore, incorporation of SCC-associated fibroblasts in the current model may improve the invasive characteristics of primary SCC-derived keratinocytes.
Furthermore, the current in vitro SCC explant model deviated from primary SCC in the lack of aberrations in composition and function of their BM. The most important components of the human cutaneous BM include collagen types IV and VII in anchoring fibrils, and laminin 332 and integrin $\alpha_6\beta_4$ in anchoring filaments, which are all confined to a continuous EDJ in normal skin.\textsuperscript{28, 29} SCC however, showed striking aberrations in localization and intensity of BM components and presented with great variability within and between tumors. These differences have been demonstrated to be correlated with the differentiation status and the degree of keratinization of primary SCC. In well-differentiated and highly keratinized SCC, laminin 332 and collagen type IV were strongly expressed in the tumor nest near the BM without loss of continuity.\textsuperscript{30} In poorly differentiated and poorly keratinized SCC, these proteins were largely lost, suggesting that these SCC cause greater enzymatic degradation of BM components than well-differentiated SCC. This hypothesis is supported by a study in which a negative correlation was demonstrated between protease activity and the level of cellular differentiation in SCC.\textsuperscript{31} Positive correlation between BM degrading activity of tumor cell lines and their metastatic potential in vivo has been established as well, demonstrating that poorly differentiated tumors have a higher potential to metastasize than well-differentiated ones.\textsuperscript{32} In addition to this concept of tumor metastasis facilitation by protease degradation of the BM and other components of the host extracellular matrix, an important role is indicated for other components of the extracellular matrix, including fibronectin and collagen type VII.\textsuperscript{33, 34} Moreover, extracellular cues of the tumor microenvironment are required for the complex processes of tumor growth and cellular or tissue invasion.\textsuperscript{34, 35} Permeation of the BM is a central determinant of malignancy in a wide array of epithelial cancers, including breast, prostate, lung, kidney and skin.\textsuperscript{36} The importance of laminin 332 in this process has been widely demonstrated in SCC in vivo, showing that the collagen type VII non collagenous domain is required for SCC tumorigenesis and that the expression of both laminin 332 and integrin $\beta_4$ correlate with tumor invasion, progression and prognosis of RAS-driven epithelial tumors.\textsuperscript{6, 37-39}

Recently, studies aimed at the identification of genes that are differentially expressed in SCC revealed the overexpression of the transmembrane receptor tyrosine kinase Axl in these tumors.\textsuperscript{40} In normal skin of both immunocompetent and immunocompromised donors and explants thereof, Axl was virtually absent in the epidermis. In primary SCC however, Axl was highly present both membranous and cytoplasmic, and this staining pattern was also found in skin cancer explant models. In earlier studies, Axl was shown to have transforming potential and was found to be upregulated in metastatic SCC cell lines MET1 and MET4 when compared to premalignant SCC cell line PM1 and frequently overexpressed in primary SCC when compared to normal skin and BCC.\textsuperscript{11, 40} These findings
were supported by previous studies in murine SCC where Axl overexpression was also noted.\textsuperscript{41} Axl has been shown to modulate a number of cellular processes including adhesion, migration and proliferation. Also, Axl was shown to confer resistance of SCC cells to apoptosis.\textsuperscript{42} Further studies are suggested to elucidate the potential role of Axl in SCC, as it may represent a novel therapeutic target for intervention in skin cancer development.

In conclusion, the \textit{in vitro} expanded human SCC models presented here exhibited a normal proliferative character based on the Ki67 proliferation index, but highly increased expression of activation-associated keratins K6, K16 and K17, and presented with invasive behavior through the \textit{in vitro} formation of invasive nests of keratinocytes. In addition, differentiation of the \textit{in vitro} SCC models was disturbed, based on altered expression of differentiation-specific keratins 1, 4, 10 and 13, and of the putative SCC-specific Axl protein. Concerning BM composition and related invasion, \textit{in vitro} SCC representation was still limited, leaving room for improvement of the tumor-stroma interaction in the current \textit{in vitro} SCC explant model. Further validation of these and future \textit{in vitro} SCC models will be focused on the role of SCC fibroblasts in SCC formation and genomic analysis to assess whether SCC specific genetic aberrations are maintained \textit{in vitro}. Ultimately, development of the present model may help to extend the basic understanding of the pathogenic mechanisms of SCC in the clinically relevant group of immunocompromised organ-transplant recipients. Subsequently, it may lead to identification of novel therapeutic targets by exploration of reversal or prevention of tumor development in the organotypic \textit{in vitro} human skin carcinoma models. Finally, this work may add significantly to advances in the field of alternatives to animal experimentation in skin cancer research, thereby meeting moral dilemmas and increasing opposition.

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References


Chapter 2


An in vitro model of primary cutaneous squamous cell carcinoma