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Organotypic in vitro models of human cutaneous squamous cell carcinoma

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General introduction

Cutaneous squamous cell carcinoma (SCC)

Cutaneous SCC in society

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Incidence of cutaneous SCC

Skin cancer is the most common type of cancer in fair-skinned Caucasian populations. Each year, there are two to three million new cases worldwide and skin cancer incidence rates outnumber those of all other major cancers combined in the United States (US).¹⁻⁵ In The Netherlands, over 20 000 new cases of skin cancer were diagnosed in 2000.⁶ In 2005, this number was projected to increase by 80% to more than 37 000 per year in 2015.⁶ However, current estimations of the Dutch Cancer Society have already reached 36 000 new cases of skin cancer per year for 2012, pointing out that the incidence of skin cancer among the Dutch population grows even more rapidly than foreseen.⁷ The majority of skin cancers are carcinomas, also referred to as keratinocyte skin cancers or non-melanoma skin cancers (NMSC), comprising basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). In general, the absolute incidence of BCC is about five times the incidence of SCC, with an annual 2011 estimate of 27 000 cases of BCC and 5 400 cases of SCC in The Netherlands, comprising about 75% and 15% of all skin cancer diagnoses in the Netherlands, respectively.⁷ Despite the lower incidence of SCC compared to BCC, SCCs have a greater propensity for invasion, local recurrence and metastasis.⁸ This problem is particularly dramatic in organ transplant recipients (OTRs) receiving immune suppressive medication to prevent graft rejection.⁹ Based on standardized incidence ratios for the Northern regions of Europe and the US, the risk for cutaneous SCC in these patients is approximately 70 times increased compared to the general population in those regions.¹⁰ Cutaneous SCC are often claimed to be more aggressive in OTRs than in immune competent individuals, but this statement is currently insufficiently supported by literature.¹¹⁻¹³ Increased development of successive lesions is particularly pronounced in renal transplant recipients, having a 75% risk of developing another cutaneous SCC within 5 years after diagnosis of the first lesion, compared to a risk of only 20% of developing another SCC within 5 years in the general population.¹⁴

Risk factors for cutaneous SCC

In addition to immune suppression in OTRs, cutaneous SCC is also increased in patients whose immune system is otherwise compromised, e.g. by immune deficiency diseases and chemotherapy.¹⁵ Other exposure-related risk factors for development of cutaneous SCC include ultra-violet radiation (UVR) exposure, radiotherapy and smoking.¹⁶ Although the absolute risk of UVR exposure is relatively low, massive and prolonged sun exposure of the

population make this the most well-known and most relevant risk factor for cutaneous SCC.¹⁷ An indirect viral risk factor for cutaneous SCC, in specific genus β human papilloma virus (β -HPV), is suspected.¹⁸⁻²⁰ However, recent findings suggest that it is unlikely that any currently known virus is commonly responsible for induction or maintenance of cutaneous SCC.²¹ Host-related risk factors for cutaneous SCC include increased age, male gender and light skin color.¹⁷ Furthermore, chronic ulcers, chronic inflammatory lichen sclerosis, burn wounds and various specific genetic traits also increase the risk for cutaneous SCC.¹⁷

Management of cutaneous SCC

Management of cutaneous SCC in OTRs exists in preventive patient education minimizing excessive sun exposure, intensive skin surveillance, active early-stage treatment of all premalignant lesions and surgical removal of established carcinomas.^{22, 23} Premalignant lesions are generally cleared by topical lesion-directed cryotherapy and/or field-directed topical treatment.²⁴ Established SCCs are mostly surgically excised and histologically examined.^{25, 26} Although cutaneous SCCs can often be successfully removed and are rarely lethal, their UVR exposure-related high incidence on the face, their rapid growth and their removal in itself may be cosmetically disfiguring, negatively impacting the quality of life of cutaneous SCC patients.²⁷ The cost associated with treatment of SCC is substantial. In 2004, the total direct cost associated with the treatment of NMSC in the US was \$1.5 billion.²⁸ Taken together, although cutaneous SCC does not present a large mortality risk, its high and rising prevalence and expense of treatment render this skin cancer a major public health problem.

Pathology of cutaneous SCC

Cutaneous SCC originates from a malignant population of proliferating keratinocytes in the suprabasal layers of the epidermis. These skin tumors can develop *de novo* or from the precursor lesion actinic keratosis (AK). AKs, or solar keratoses, are premalignant, dysplastic, intra-epidermal lesions (Figure 1). Approximately 8% of AKs ultimately progress to SCC.²⁴ During this process, the tumor expands through the basement membrane (BM) and invades the underlying dermis (Figure 1). Cutaneous SCC and its precursor lesions are predominantly located on sun-exposed areas of the skin, including the face, scalp, lip, forearms and back of the hands. It is therefore not surprising that UVR exposure is the predominant driver in the pathogenesis of cutaneous SCC. UVR exerts its oncogenic effects directly through DNA damage, and indirectly through local and systemic immune suppression. Direct UVR induced DNA damage involves dimerization of successive pyrimidine bases resulting in cyclobutane-pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PP). During DNA replication, these dimers may lead to specific point mutations (mostly cytosine to thymine transitions) and double strand breaks in the DNA,

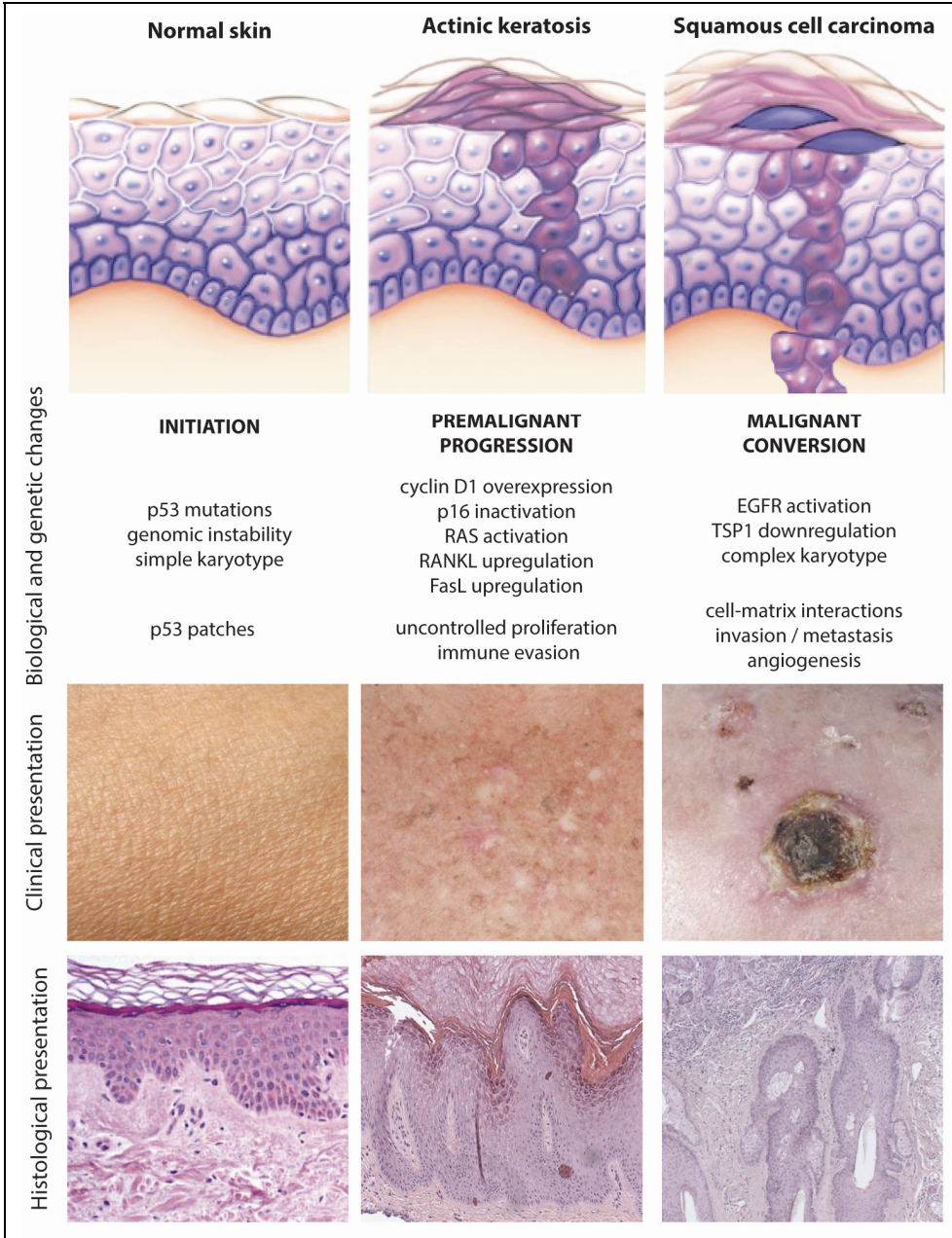


Figure 1 | Overview of genetic, biological, clinical and histological changes in initiation, premalignant progression and malignant conversion of normal skin to actinic keratosis and cutaneous squamous cell carcinoma. Images adapted and reused with permission (Nancy Heim of the Columbia University Medical Center (schematics), iStockphoto/Eugene Llacuna (normal skin), Department of Dermatology of the Leiden University Medical Center).

the latter of which may also induce chromosomal aberrations.²⁹ When left unrepaired, UVR-induced DNA damage accumulates over many years and leads to actual mutations in cellular DNA, altering genes regulating normal cellular growth. Importantly, inactivating UVR-induced mutations in the p53 tumor suppressor gene are very common early events in SCC carcinogenesis, both in immune competent individuals and in immune suppressed OTRs.³⁰⁻³² These p53 mutations result in unchecked mitosis (cell cycle deregulation) and loss of apoptosis in affected epidermal keratinocytes, allowing for clonal expansion and formation of premalignant lesions (Figure 1). Upon further UVR exposure, genomic instability increases and additional genetic lesions may accumulate in these cells, including inactivation of the CDKN2A (p16 INK4A) tumor suppressor gene, overexpression of the cell cycle regulator cyclin D1 gene and activation of the proliferative RAS pathway – events all associated with progression of AK to SCC (Figure 1).³³⁻³⁶ As mutations in the RAS family of proto-oncogenes are rare events in cutaneous SCCs, increased RAS signaling may result from amplification of its gene locus and increased expression and activation of upstream factors including the transmembrane tyrosine kinase epidermal growth factor receptor (EGFR).³⁷ EGFR overexpression has been inconsistently found in 0% to 78% of cutaneous SCCs and may result from highly variable numerical aberrations or genetic amplifications. However, increased EGFR activation and upregulated EGFR signaling have been consistently found in 25% to 100% of cutaneous SCCs.^{36, 38-40} EGFR is directly activated by its ligands as well as through decreased dephosphorylation by UVR.⁴¹ Indeed, EGFR ligands have been found upregulated in cutaneous SCCs when compared to adjacent normal skin.⁴⁰ Of all genetic aberrations characteristic of cutaneous SCC, many have been found at the chromosomal level, as supported by complex karyotypes with large numbers of allelic imbalances often displayed by immune competent AKs and SCCs.⁴²⁻⁴⁵ Genetic and phenotypic differences between SCCs may be explained by functional equivalence of various genetic changes, allowing a large number of genotypes to be tumorigenic. Furthering on this concept, intratumoral (genetic) diversity may result from multiple genetic clones existing in different regions and at different times within the same tumor.

In addition to DNA damage, UVR exposure induces both systemic and local immune suppression in the skin. UVR-induced soluble mediators tempering the systemic immune response include cis-urogenetic acid and interleukin 10.⁴⁶ UVR exposure results in local immune suppression in epidermal keratinocytes by upregulation of the receptor activator of NF- κ B ligand (RANKL), thereby inducing dendritic cell-mediated proliferation of regulatory T cells (Tregs), which in turn locally reduce the peritumoral immunological response by effector T cells in the skin.⁴⁷ In addition, epidermal Langerhans cells are reduced in number and function upon UVR exposure, hampering their response to transformed cells.⁴⁶ Furthermore, cutaneous AK and SCC cells display simultaneous

downregulation of cell death receptor Fas (CD95) and upregulation of Fas ligand (FasL), providing them with an active defense from systemic Fas-expressing immune effector cells.^{48, 49} Adding to this, UVR and viral infections (e.g. HPV) are suggested to synergistically support oncogenesis in the skin.⁵⁰⁻⁵²

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Tumor-stroma interactions in cutaneous SCC

Microenvironmental barriers to proliferation

The process of cutaneous SCC initiation, progression, invasion and eventually metastasis does not occur in isolation, as the (pre)malignant epidermal cells are surrounded by their microenvironment. Moreover, epidermal cells need to overcome various microenvironmental barriers in order to expand and eventually invade the dermis. These barriers select for specific adaptive cellular properties during the different stages of carcinogenesis. Ultimately, such microenvironmental barriers define the sequence with which hallmarking phenotypical and genotypical changes occur during SCC carcinogenesis.⁵³ In normal epidermis, detachment-induced apoptosis (anoikis) and contact inhibition are dominant proliferation barriers. Resistance to these barriers confers early properties of premalignant epidermal cells that are hallmarked by genetic aberrations in cell cycle regulators, resulting in insensitivity to anti-growth signals and uncontrolled proliferation. Then, independent growth signaling caused by upregulated expression of growth factors and/or receptors enable premalignant cells to overcome normal proliferation limits. In cutaneous SCC, this is mostly reflected by upregulation of the proliferative RAS pathway, including increased activation of EGFR and increased transcription of EGFR ligands.^{39, 40}

Invasion through the basement membrane

The development of invasive SCC is not simply accomplished by unrestricted proliferation of the cancer cell population, but rather requires an active collaboration of malignant epidermal SCC cells and various dermal components. Epidermal cells are anchored to and separated from the underlying dermis by an intact basement membrane (BM), in which the basal layer (basal lamina) facing the epidermis and the reticular layer (lamina reticularis) facing the dermis are fused (Figure 2). The basal lamina typically harbors laminins, integrins, entactins, dystroglycans and an underlying network of collagen type IV fibrils, whereas the reticular layer mainly contains collagen type I and collagen type III fibers. The two layers are connected by collagen type VII anchoring fibrils and microfibrils. Through the BM, substrates, metabolites and signaling molecules are exchanged between the epidermis and the dermis. The dermis consists of extracellular matrix (ECM),

containing large amounts of collagen type I, collagen type IV and fibronectin, in which fibroblasts, immune cells and vasculature reside. The BM acts as a mechanical barrier preventing premalignant cells from invading the dermis and deeper tissues.

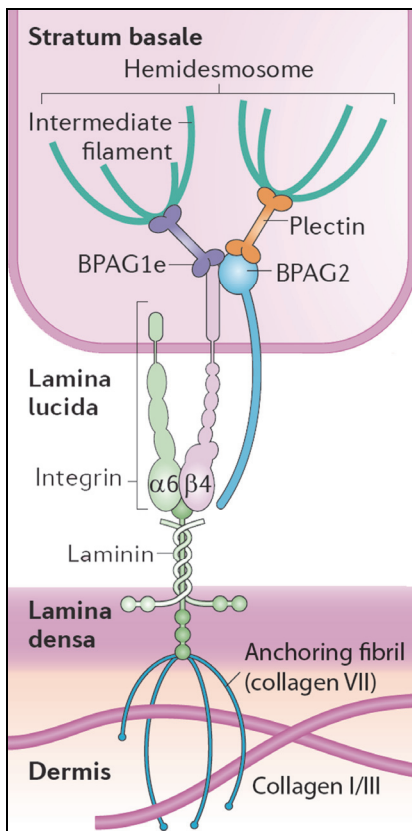


Figure 2 | Schematic representation of the basement membrane (BM) separating the epidermis from the dermis in human skin. The BM is composed of the lamina lucida and lamina densa. Hemidesmosomes anchor keratinocytes from the basal layer to the BM, mainly through the interaction of laminin 332 with the $\alpha_6\beta_4$ integrin receptor and the transmembrane protein bullous pemphigoid antigen 2 (BPAG2, also known as collagen XVII). This interaction also relays signals from the epidermal and extraepidermal environment to a network of keratin intermediate filaments. Hemidesmosomes are tethered to intermediate filaments by the plakin family members plectin and BPAG1e. Anchoring of the epidermis to the dermis depends on the interaction of laminin 332 with collagen VII, the major component of anchoring fibrils that extend from the BM into the dermis. Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Molecular Cell Biology, Simpson et al, copyright (2011).

Cancer-associated fibroblasts

Cutaneous tumor invasion is a manifestation of disrupted epidermal homeostasis resulting from interactions between the epidermal cancer cells and their stromal counterparts in the dermis, mainly represented by cancer-associated fibroblasts (CAFs).⁵⁴ In literature, these cells are also termed tumor-associated fibroblasts, reactive stromal fibroblasts, activated fibroblasts, myofibroblasts, peritumoral fibroblasts, pericytes or fibrocytes.⁵⁵⁻⁵⁸

CAFs have been found responsible for breakdown of BM structures and dermal ECM remodeling in concert with epithelial cancer cells, thereby facilitating tumor invasion.^{53, 59, 60}

The functional contribution of CAFs to carcinogenesis is supported by their structural properties, as their phenotype is similar to fibroblasts associated with wound healing (Figure 3). To synthesize large amounts of ECM constituents, CAFs typically contain a large euchromatic nucleus with one or two nucleoli and a prominent rough endoplasmic reticulum and Golgi apparatus.⁵³ These changes in nuclear volume and cellular composition are partly driven by pro-fibrotic growth factors released by the cancer cells and include transforming growth factor- β (TGF- β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), all of which are key mediators of fibroblast activation and tissue fibrosis. In turn, cutaneous SCC-associated CAFs themselves have been shown to produce increased amounts of growth factors including hepatocyte growth factor (HGF) and ECM-degrading proteases such as matrix metalloproteinase 2 (MMP2), MMP3, MMP9 and MMP10, facilitating increased ECM turnover and remodeling.⁶¹⁻⁶⁴ Many growth factors bind to dermal ECM components like proteoglycans, fibronectin and vitronectin.⁶⁵ This renders the dermal ECM a reservoir of growth factors that can be released upon ECM degradation, thereby establishing stable gradients of growth factors supporting tumorigenesis. The extensive crosstalk between cancer cells and their activated stroma ultimately leads to increased tumor cell motility, for which BM and dermal ECM degradation is a substrate. Beyond invasion, cancer cells may acquire metastatic properties, thereby successfully overcoming other microenvironmental barriers including hypoxia and ischaemia, often marked by increased angiogenesis. Late-stage invasion of cutaneous SCC cells into subcutaneous tissues is indeed characterized by increased stromal angiogenic responses and increased density of both blood and lymphatic vessels, which have not been found in the dermis of normal skin, AKs and less aggressive, early-stage SCCs.⁶⁶⁻⁶⁸ Although metastasis is rare in cutaneous SCC, it is hallmarked by increased angiogenesis at the invasive tumor front in immune suppressed OTRs.^{12, 69}

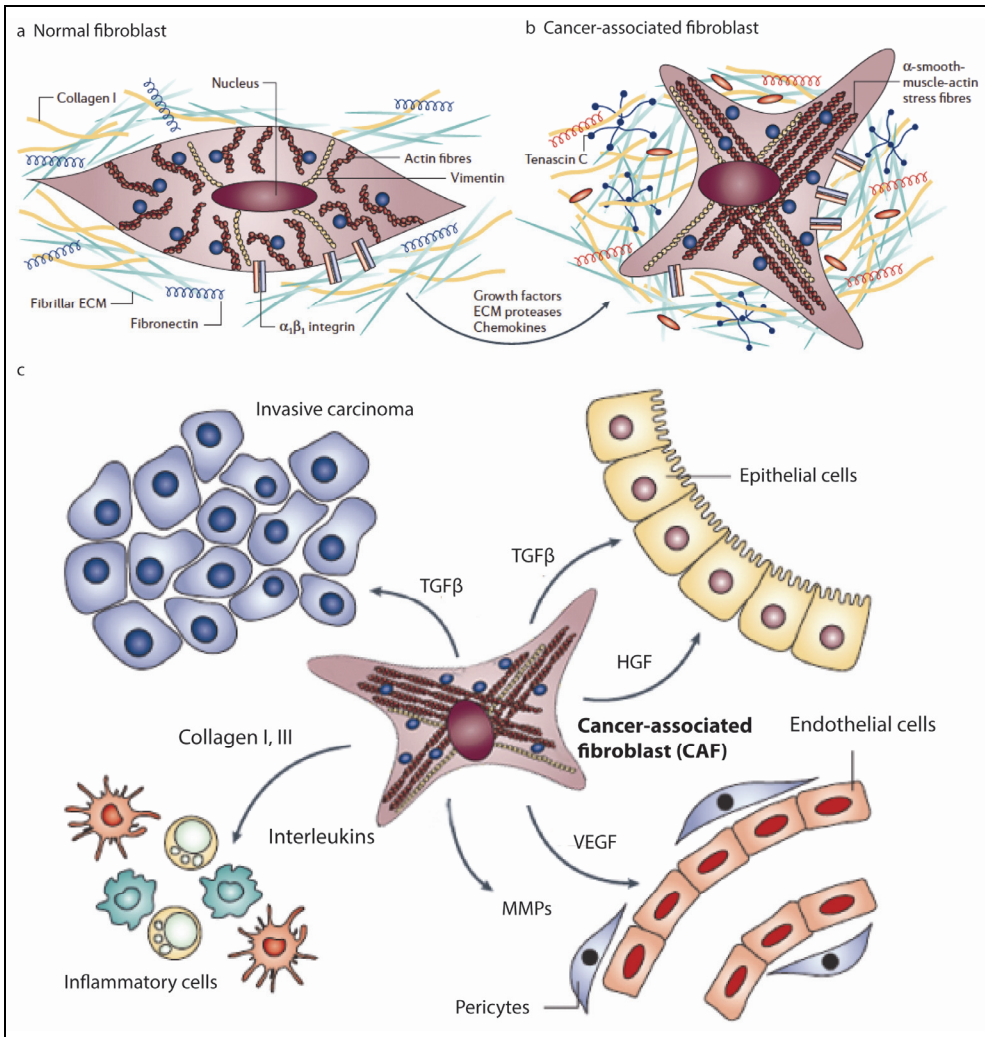


Figure 3 | In human skin, normal fibroblasts are embedded in the fibrillar extracellular matrix (ECM) of the dermis, which largely consists of type I collagen and fibronectin (a). Upon interaction with epithelial tumor cells, cancer-associated fibroblasts (CAFs) acquire an activated phenotype, which is associated with α -smooth-muscle actin expression and enhanced secretion of ECM proteins (b). In the tumor stroma, CAFs communicate with cancer cells, resident epithelial cells, endothelial cells, pericytes and inflammatory cells through the secretion of growth factors and chemokines (c). Through the increased deposition of collagens and modulation of the inflammatory response, CAFs induce and maintain a potentially oncogenic microenvironment. Adapted with permission from Macmillan Publishers Ltd, copyright 2006.⁵³

Modeling cutaneous squamous cell carcinoma

In vivo SCC models

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To develop rational preventive and therapeutic strategies and to study functional consequences of genetic aberrations found in clinical research, basic research models are needed. Experimental models for cutaneous SCC include *in vivo* and *in vitro* models. *In vivo* models are living, intact animals in which human disease is studied without the risk of harming human beings. For cutaneous SCC, traditional *in vivo* models are based on chemically, radiation- or virally induced carcinogenesis in mice. An old but still widely used approach is the two-stage model of chemical skin carcinogenesis, in which genotoxic agents are applied to murine skin for tumor initiation, followed by repeated exposure to a mitogenic tumor promoter to induce hyperplasia.⁷⁰ The affected skin forms mainly benign papillomas, some of which eventually progress to cutaneous SCC.⁷¹ In radiation-induced skin carcinogenesis models, mice develop cutaneous SCCs upon repeated UVR exposure without prior chemical tumor initiation. The immune competent outbred hairless albino mouse strain SKH1-*hr* is the most widely used model organism to study UVR-induced cutaneous SCC.⁷² Upon chronic UVR exposure, these mice develop cutaneous SCC resembling human cutaneous SCC both at the morphological and molecular level.⁷³ Virally induced *in vivo* skin carcinogenesis models include transgenic mice with expression of specific regions of the HPV8 genes, leading to spontaneous development of papillomas, dysplasia and SCC of the skin.⁷⁴ Expression of specific HPV38 genes greatly increases their susceptibility to chemical and UVR-induced skin carcinogenesis.⁷⁵ Another approach to study skin carcinogenesis *in vivo* is intradermal or subcutaneous injection or surface transplantation of human cancer cell line xenografts into immunodeficient mice.⁷⁶⁻⁷⁸ Severe combined immunodeficient (*scid*) mice, immune deficient Rag1 mice and athymic nude mice are often used for such xenograft models.⁷⁹ Genetically engineered mice with germline alterations further add to the spectrum of model organisms available for studying cutaneous SCC.⁸⁰ Refined genetically engineered models include mosaic or conditional models, in which a latent allele is phenotypically inactive until stimulated in a tissue- and time-specific manner with exogenous chemicals or viruses, often utilizing the Cre-lox recombination system.^{81, 82} Examples of successfully combined approaches include surface transplantation with genetically engineered human SCC cell lines and UVR-induction of SCC in a xenograft mouse model.^{78, 83} All *in vivo* models available have certain features that render them suitable for studying a specific research question (Table 1).

Table 1 | Key features of *in vivo* models of human cutaneous SCC.

<i>In vivo</i> model	Transformed cell origin ¹	Genetically defined ²	Intact immunity ³	Relevant microenvironment ⁴	Genomic instability ⁵
Chemical carcinogenesis	Mouse or human*	No	Possible*	Yes	Possible*
UVR-induced carcinogenesis	Mouse or human*	No	Possible*	Yes	Yes
Viral carcinogenesis (human papillomavirus, HPV)	Mouse	Yes	Yes	No	Unknown
Xenograft (injected or transplanted)	Human	Possible	No	No	Yes
Genetically engineered	Mouse	Yes	Yes	Yes	No
Genetically engineered (non-germline)	Mouse	Yes	Yes	Yes	No

¹ **Transformed cell origin** defines whether the model carcinomas consist of either human or animal cells.
² **Genetically defined** states whether the genetic background of the host animal is fully known.
³ **Intact immunity** defines whether the host animal has an intact immune system.
⁴ **Relevant microenvironment** defines the similarity of the surrounding tumor stroma to that in human SCC.
⁵ **Genomic instability** defines the similarity of the genomic instability of the tumors to that found in human SCC.
 * When combined with xenograft approaches, human cells may be used at the expense of intact immunity.

***In vitro* SCC models**

Primary cells and cell lines

The natural complexity of living organisms present in *in vivo* models is a great barrier to the identification and functional study of individual biological components. Therefore, *in vitro* models are used in which components of an organism that have been isolated from their native biological context permit simplified and more accessible analyses than can be done with whole organisms. To study human cutaneous SCC *in vitro*, both two- and three-dimensional tissue culture approaches are available. Two-dimensional monolayer cell cultures are either based on primary cells or cell lines. Primary cutaneous SCC cell cultures are notoriously difficult to establish, and can be obtained either by direct primary SCC explant culture or enzymatic isolation of primary SCC cells.⁸⁴ The resulting primary monolayer SCC cell cultures are often very heterogenic, have a limited lifespan and mostly require feeder layers of immortalized dermal fibroblasts.⁸⁵ However, upon successful *in vitro* propagation, primary cutaneous SCC cell cultures may retain the ability to proliferate indefinitely, thereby establishing immortal SCC cell lines. These cultures provide useful subsets of precisely defined stages of tumorigenic differentiation. To date, only a limited number of cutaneous SCC cell lines are available (Table 2). Among these, the MET1 to MET4 cell lines represent a clonally related progression series of cutaneous SCC, as these cell lines are derived from a primary cutaneous SCC, two recurrences and a metastasis from a single patient. Furthermore, genetic engineering of the HaCaT cell line, originating from spontaneously immortalized healthy human keratinocytes, resulted in a series of malignant tumorigenic and even metastatic variants.^{86, 87} Such cell line series representing different stages of skin carcinogenesis enable the *in vitro* study of sequential acquisition of transformation-related traits. In addition, using such SCC cell lines, both phenotypic and genetic characteristics can be correlated to their direct clinical origin.

Three-dimensional *in vitro* SCC models

Isolated cultures of primary SCC cells and established SCC cell lines lack any microenvironmental context. Therefore, co-cultures of either primary SCC cells or SCC cell lines and dermal human fibroblasts are performed to study the interaction between both cell types.⁸⁸ Also, either primary SCC cells or SCC cell lines can be cultured in ECM-like matrices (e.g. Matrigel[®], Cultrex[®]) to study the interaction between SCC cells and ECM components *in vitro*.⁸⁹ However, such SCC models still lack a fully organized representation of the relevant human SCC microenvironment. More advanced *in vitro* models of cutaneous SCC are therefore represented by three-dimensional cell culture systems, integrating SCC cells in a relevant microenvironment. In literature, these models

Table 2 | Characteristics of human cutaneous SCC cell lines currently available.

Cell line	Tumor stage	Origin	Characteristics	Reference
SCC-12B2	Primary, well-differentiated	Male OTR (60), immune suppression, face	- p53 mutated - poorly differentiated - cystic in mice	Rheinwald et al (1981) ⁸⁵
SCC-12F2			- p53 mutated - well-differentiated - not tumorigenic in mice	
SCC-13	Primary, well-differentiated	Female OTR (56), radiotherapy, face	- p53 mutated - well-differentiated - feeder layer dependent - cystic in mice	
HSC-1	Primary, poorly differentiated	Male (75), untreated, dorsal hand (HSC-1), ear (HSC-5)	- p53 status unknown - not tumorigenic in mice	Kondo et al (1981) ⁹⁰
HSC-5	Primary, highly differentiated			Hozumi et al (1990) ⁹¹
SCL-I	Primary, moderately differentiated	Female (74), untreated, face	- p53 mutated - moderately tumorigenic in mice	Boukamp et al (1982) ⁹²
SCL-II	Primary, poorly differentiated	Male (91), radiotherapy, face	- p53 mutated - not tumorigenic in mice	Tilgen et al (1983) ⁹³
HaCaT	None (healthy skin)	Male (62), upper back (distant melanoma periphery)	- spontaneously immortalized - UVR-specific p53 mutations - chromosomal aberrations characteristic of human SCC	Boukamp et al (1988) ⁹⁴
A388.6TG.c2	Primary	Male (86), location unknown	generated and selected by exposing a primary SCC to carcinogenic chemicals	Gioeli et al (1997) ⁹⁵
MET1	Primary, invasive, moderately differentiated	Male OTR (36), immune suppression, dorsal hand (MET1-3), axillary lymph node (MET4)	- no p53 mutations - poorly differentiated - tumorigenic in mice	Proby et al (2000) ⁹⁶
MET2	Recurrence, deeply invasive, poorly differentiated			
MET3				Popp et al (2000) ⁹⁷
MET4	Metastasis, poorly differentiated			

are designated three-dimensional, full-thickness, organotypic, (bio)engineered or reconstructed human skin cancer models, equivalents, constructs or rafts.

Three-dimensional *in vitro* human SCC models contain an epidermal compartment and a dermal compartment (Figure 4). The epidermal compartment may harbor any epidermal representation of human SCC, including genetically or chemically transformed primary keratinocytes, established SCC cell lines or complete primary SCC explants. The dermal compartment of three-dimensional SCC models may be composed of any biological or artificial matrix representing human dermis, including rat-tail type I collagen, human fibroblast-derived matrix (FDM), de-epidermized human dermis (DED) or artificial matrix. The dermal matrix may be either acellular or seeded with any type of human stromal cell, including healthy or disease-associated human fibroblasts, endothelial cells or immune cells. The epidermal and dermal compartments of three-dimensional SCC models are ideally separated by a functional BM, allowing for the study of SCC invasion. In general, the dermal compartment is prepared and seeded with stromal cells before adding the epidermal compartment. Depending on the type of matrix, this may take one to four weeks. In this phase, the culture medium is optimized towards creating a dermal microenvironment capable of fully supporting the prospective epidermal compartment. For example, human fibroblast-derived matrix is prepared during four weeks in standard fibroblast medium supplemented with high concentrations of ascorbic acid (vitamin C) to stimulate ECM formation.⁹⁸ Next, the epidermal compartment is added to the culture system. The cultures are then kept submerged in culture medium to promote attachment of epidermal cells to the dermal compartment and to stimulate proliferation of the epidermal cells to form a three-dimensional multilayered epidermis. This submerged culture period typically covers four to seven days allowing a functional BM to form. Culture medium in this phase is typically supplemented with fetal bovine serum (FBS) or equivalent non-animal additives to promote proliferation. Subsequently, the three-dimensional models are cultured at the air-liquid interface, leaving the epidermal compartment air-exposed to stimulate cellular differentiation.^{99, 100} The air-exposed culture period typically spans two weeks, but may range from one to twenty weeks depending on the type of model and the research question. The culture medium of three-dimensional SCC models in the air-exposed culture phase is supplemented with various agents specifically stimulating proliferation, differentiation and maintenance of the epidermal compartment, including serum, hormones (e.g. insulin, hydrocortisone), vitamins (e.g. ascorbic acid, retinoids), fatty acids (e.g. palmitic acid, arachidonic acid) and growth factors (e.g. EGF, keratinocyte growth factor (KGF)). Typical examples of three-dimensional *in vitro* cutaneous SCC models include those generated by Borchers et al (1997), Gioeli et al (1997), Ponc et al (2000), Martins et al (2009) and Obrigkeit et al

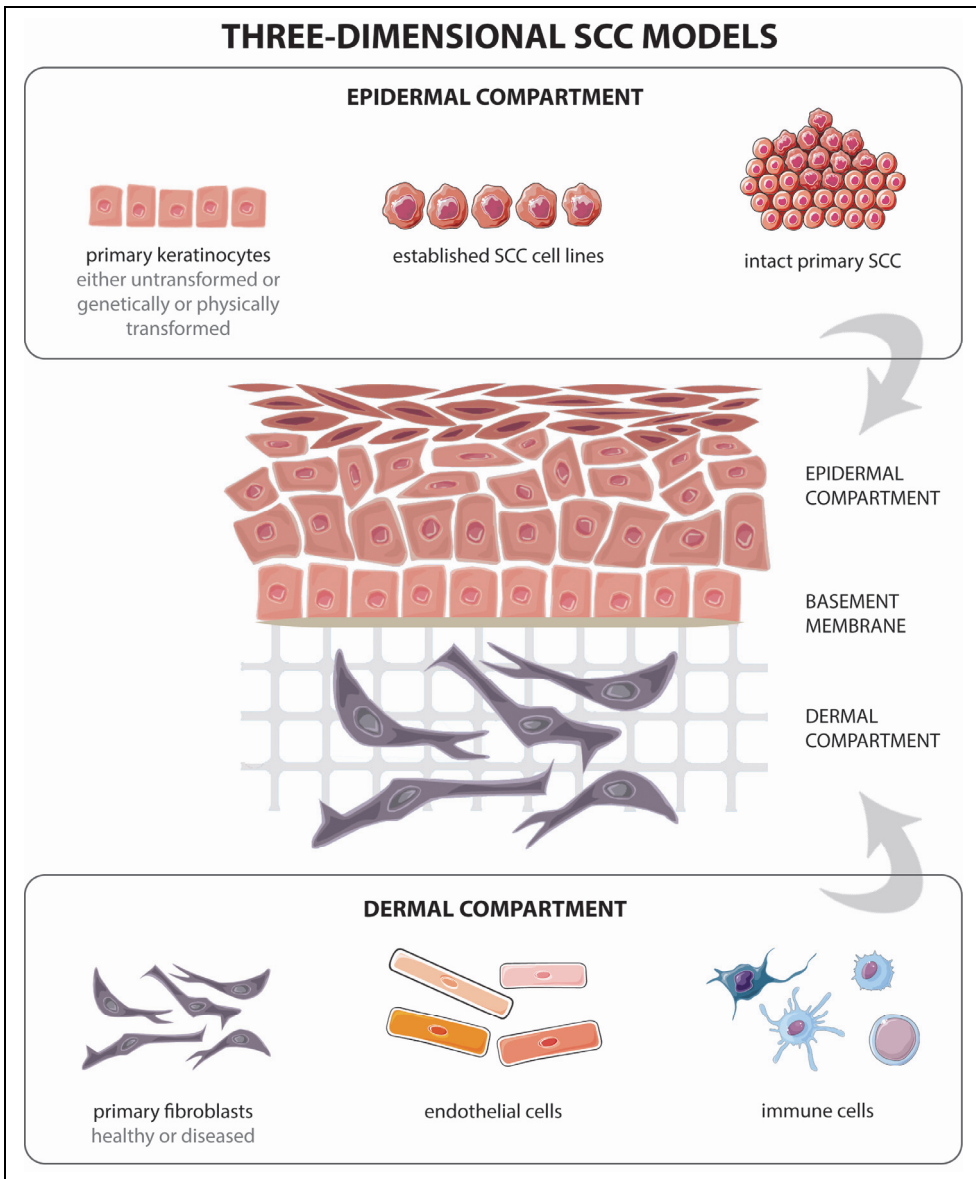


Figure 4 | Three-dimensional *in vitro* cutaneous SCC models offer great versatility. The epidermal compartment can be generated with any epidermal representation of human SCC, including (transformed) primary keratinocytes, SCC cell lines or primary SCC explants. The dermal compartment can be any biological or artificial matrix representing human dermis, acellular or seeded with any type of human stromal cell, including healthy or disease-associated human fibroblasts, endothelial cells or immune cells.

(2009) (Table 3).^{95, 101-104} Without exception, these models have been generated with SCC cell lines in their epidermal compartment and all but one were cultured with relatively high amounts (10%) of FBS. Altogether, various cell-based models are currently available to study human cutaneous SCC *in vitro*. These models all have their own characteristics regarding the number of cell types involved, representation of a relevant microenvironment, reproducibility, experimental time span and availability (Table 4). As with *in vivo* models for cutaneous SCC, the *in vitro* model of choice can be matched to each specific research question.

Table 3 | Historical overview of three-dimensional *in vitro* cutaneous SCC models.


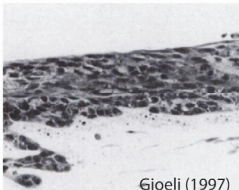

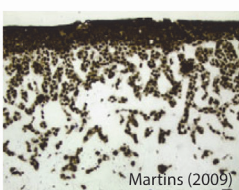
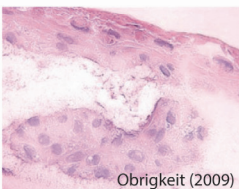
	EPIDERMAL COMPARTMENT	DERMAL COMPARTMENT	CULTURE SPECS
 Borchers (1997)	Ha-ras transformed HaCaT II-4 cell line	rat tail collagen seeded with normal human dermal fibroblasts	Regimen 1 day submerged 14 days air-exposed Medium 10% FCS
 Gioeli (1997)	SCC cell line A388.6TG.c2	rat tail collagen seeded with normal human fibroblasts	Regimen 1 day submerged 14 days air-exposed Medium 10% FCS
 Rhee (2000)	SCC cell line SCC-12F2	rat tail collagen seeded with normal human dermal fibroblasts	Regimen 1 day submerged 21 days air-exposed Medium 5% > 1% > 0% FCS
 Martins (2009)	SCC cell line MET1 with siRNA-mediated collagen VII knockdown	rat tail collagen mixed with Matrigel (1:1) seeded with normal human dermal foreskin fibroblasts	Regimen 1 day submerged 10 days air-exposed Medium 10% FCS 10 ng/ml EGF
 Obrigkeit (2009)	normal human epidermal foreskin keratinocytes + SCC cell line SCC-12	rat tail (?) collagen seeded with normal human dermal foreskin fibroblasts	Regimen 1 week submerged 5 days air-exposed Medium 10% FCS 10 ng/ml EGF

Table 4 | Key features of *in vitro* models of human cutaneous SCC.

<i>In vitro</i> model	Microenvironment ¹	Reproducibility ²	Timespan ³	Availability ⁴
Two-dimensional (2D) models				
Primary SCC cells	-	low	weeks	low
SCC cell lines	-	high	infinite	high
Co-culture of SCC cells and fibroblasts	stromal fibroblasts	medium	weeks	medium*
Three-dimensional (3D) models				
ECM with SCC cells e.g. Matrigel [®] , Cultrex [®]	ECM (non-human) BM (disorganized)	medium/high*	days	medium*
3D SCC model	ECM, BM, stromal cells	medium/high*	weeks/months	medium*
Ex vivo primary SCC	-	low	days	low
¹ Microenvironment extracellular matrix (ECM), basement membrane (BM), stromal cells. ² Reproducibility determined by good laboratory practice (GLP) and heterogeneity of material. ³ Timespan experimental window, equivalent to <i>in vitro</i> viability of the model. ⁴ Availability primarily determined by dependence on primary human material. * Dependent on SCC cell type incorporated in the model: primary cells limit reproducibility and availability.				

***In vitro* SCC models as alternatives to animal experimentation**

Animal experimentation and the 3Rs concept

The only direct approach to study human cutaneous SCC is through clinical research. However, experimentation on human subjects is limited by ethical and technical constraints. Therefore, indirect *in vivo* and *in vitro* approaches are used for experimental research without harming human beings. The use of *in vivo* models is subject to ethical debates on inhumane animal experimentation. Researchers are obliged to weigh the distress imposed on animals during the experiment against the expected gain in knowledge. Elimination of inhumane practices from animal experimentation is generally discussed under the three headings of reduction, refinement and replacement of humane technique – the three Rs introduced by William Russell and Rex Burch in 1959.¹⁰⁵ Reduction covers any decrease in the numbers of animals used to obtain information of a given amount and precision. So, reduction methods obtain comparable levels of

information from fewer animals or more information from the same number of animals. Refinement covers any decrease in the incidence or severity of inhumane procedures. Therefore, refinement methods alleviate or minimize potential pain, suffering and distress and enhance animal well-being. Replacement covers the substitution of conscious living animals for insentient material. Replacement methods therefore achieve a given purpose without conducting any scientific procedure on animals. The 3Rs concept is currently fully implemented in standard procedures for designing animal experiments.^{106, 107}

Since the 1980's, efforts to reduce animal experimentation have been gaining momentum under the pressure of animal welfare groups and public opinion. In 1986, a European Union (EU) directive on the protection of animals used for experimental purposes officially banned animal experiments whenever a scientifically approved alternative exists.¹⁰⁸ Ever since, the number of animals used for experiments more than halved. However, recent reports show that despite this absolute decrease, the number of genetically modified animals rises drastically.¹⁰⁶ In The Netherlands, over 560.000 animals were used for scientific experiments in 2010. About 16% of these animals were genetically modified mice. In addition, almost 500.000 animals were wasted in the process of obtaining animals of only one desired sex, maintenance of specific strains or unforeseen disease. More than 70% (359.347) of these waste animals were genetically modified mice, indicating the disproportionately high pressure of genetic engineering on the total number of animals used and pointing out the persisting relevance of reducing animal experimentation.

Replacement in studying cutaneous SCC

The fidelity of mice as model organisms for human skin cancer is limited; the murine skin differs from human skin in structure and function. Architecturally, the murine epidermis with densely-distributed hair follicles and without sweat glands contrasts with the human epidermis. The epidermal thickness of normal human skin, comprising 6 to 10 cell layers together covering over 100 μm more than triples that of murine skin, which generally contains only 3 cell layers spanning less than 25 μm .¹⁰⁹ Functionally, mouse skin has greater percutaneous absorption and a decreased barrier function in comparison to human skin and mice have effective cutaneous wound regeneration without scarring, while human skin is subject to extensive hypertrophy upon wounding.^{109, 110} Furthermore, the metabolic rate of mice, including epidermal turnover, is faster than in humans.¹⁰⁹ For example, SKH1-*hr* mice have been shown to contract their SCCs approximately 250 times faster than humans at comparable daily UVR dosages, offering obvious experimental advantages, but limiting their representation of human SCC.¹¹¹ Extrapolation of experimental data obtained in mice to human cancer can be further hindered by differences in oncogenic signaling, which may cause known human tumor suppressor

genes to elicit a different spectrum of tumors in mice than in humans.^{112, 113} Besides intrinsic differences between *in vivo* and *in vitro* models, experimental efficiency, as defined by the balance between time, cost and information yield, is often in favor of *in vitro* methods. Moreover, *in vitro* cell culture models represent important replacement techniques for experimentation on human cutaneous SCC. Especially three-dimensional *in vitro* SCC models harboring multiple interacting cell types in a relevant microenvironment offer great alternatives to animal SCC models. These *in vitro* SCC models are highly discriminative, as they reproduce one or more particular features of human cutaneous SCC in detail, e.g. invasion, hyperproliferation or epidermal dysplasia. The design of every experiment to investigate human cutaneous SCC should therefore focus on the model with the highest experimental efficiency, which is the most discriminative and the least inhumane for that particular research question. Hence, a robust and reliable *in vitro* model of human cutaneous SCC is a highly desirable experimental vehicle to investigate human cutaneous SCC development and therapeutic responses.

Thesis aim and outline

The aim of the research described in this thesis was to develop an *in vitro* model of human cutaneous squamous cell carcinoma (SCC) for ultimately screening potential therapeutic compounds. To this end, we first maintained intact primary human cutaneous SCCs of immune suppressed organ transplant recipients (OTRs) on three-dimensional rat-tail collagen matrices seeded with normal human dermal fibroblasts (NHDFs). We also cultured human cutaneous SCC cell lines on NHDF-seeded rat-tail collagen matrices. The resulting invasive primary SCC models and SCC cell line models are characterized in **Chapter 2**. To study the contribution of the microenvironment to the invasive potential of our *in vitro* SCC models, we modulated the composition of the dermal compartment in two ways. Firstly, we replaced the NHDFs in the dermal matrix by primary human cancer-associated fibroblasts (CAFs) isolated from primary cutaneous SCCs of immune suppressed OTRs. *In vitro* SCC cell line models based on these SCC-associated CAFs are described in **Chapter 3**. Secondly, we replaced the rat-tail collagen matrix by normal human dermal fibroblast-derived matrix (FDM), resulting in a fully human dermal microenvironment as covered by **Chapter 4**. To validate our primary *in vitro* SCC cultures as models of human cutaneous SCC, we performed molecular profiling of primary cutaneous SCCs, allowing the comparison with their counterparts expanded *in vitro*. **Chapter 5** identifies the pathways and genomic alterations present in primary cutaneous SCCs of immune suppressed OTRs and their precursor lesions. As upregulation of the RAS pathway has been found in SCCs of both immune suppressed and immunocompetent individuals, we used our *in vitro* SCC models to investigate the effects of erlotinib, a small molecule inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase upstream of RAS. This proof of principle for the therapeutic screening potential of our *in vitro* SCC models is described in **Chapter 6**. The results presented in this thesis are summarized and discussed in **Chapter 7**.

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