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Design and analysis of a squamous cell carcinoma *in vitro* model system☆

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ABSTRACT

Tissue-engineered skin equivalents based on primary isolated fibroblasts and keratinocytes have been shown to be useful tools for functional *in vitro* tests, including toxicological screenings and drug development. In this study, a commercially available squamous cell carcinoma (SCC) cell line SCC-25 was introduced into epidermal and full-thickness skin equivalents to generate human-based *disease-in-a-dish* model systems. Interestingly, when cultured either in the epidermis or dermis of full-thickness skin equivalents, SCC-25 cells formed hyper-keratinized tumor cell nests, a phenomenon that is frequently seen in the skin of patients afflicted with SCC. Raman spectroscopy was employed for the label-free cell phenotype characterization within the engineered skin equivalents and revealed the presence of differential protein patterns in keratinocytes and SCC-25 cells. To conclude, the here presented SSC *disease-in-a-dish* approaches offer the unique opportunity to model SSC in human skin *in vitro*, which will allow further insight into SSC disease progression, and the development of therapeutic strategies.

1. Introduction

Among basal cell carcinoma, squamous cell carcinoma (SCC) is the most common type of skin cancer. Every year, an estimated 400,000–600,000 new cases of cutaneous SCC are diagnosed worldwide, predominantly occurring in sun-exposed skin areas of elderly patients [1]. Although SCC is curable in most cases, treatment at early disease stages is advisable since SCC has the potential to spread and form metastases [1,2]. The development and expansion of SCC is considered to be an accumulative process of cellular malignant alterations within the epidermal skin layer [3]. SCC can arise from so-called precancerous lesions, the most frequent being actinic keratosis, which is induced by UV-radiation. Although actinic keratosis may stay inconspicuous for several years, it is estimated that 8–20% of untreated actinic keratosis turn into SCC [1]. Histopathologically, actinic keratosis is associated with atypical keratinocytes that are present within a thickened epidermal layer due to abnormal keratin expression [1]. An advanced, pre-invasive stage of cutaneous SCC is known as Bowen's disease, which occurs as an asymptotic, but well-defined scaly plaque that affects all layers of the epidermis [1]. Morphologically, the spatial organization of the epidermis is destructed in Bowen's disease; parakeratosis and sometimes hyperkeratosis can be observed [1]. Pathological keratinocytes proliferate, laterally expand and can appear as multinucleated, dyskeratotic cells [1]. An invasive, aggressive SCC is characterized by cancerous keratinocytes passing the basal lamina and infiltrating the dermis, in which case a high risk for reoccurrence and the formation of metastases was reported [3].

Various risk factors have been identified to be involved in tumor formation and progression, including exposure to UV-radiation, immunosuppression, individual skin type or previous therapies [4,5]. Less is known about the alterations that occur in the skin due to risk factor exposure, which can finally lead to a malignant high-risk SCC. *In vitro* toxicological screening on malignant cancer cells

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have shown that a three-dimensional (3D) environment impacts the response of malignant cells significantly [6]. Furthermore, previous work from our group has demonstrated the critical importance of 3D environments for *in vitro* models of the intestine, trachea, and myocardium [7–9]. The establishment of a three-dimensional *in vitro* test system for SCC would be of great benefit for the evaluation of therapies and a general better understanding of the underlying mechanisms of the disease. *In vitro* skin equivalents have been shown to be suitable tools to mimic functional and structural key characteristics of human native skin [10]. Accordingly, various tissue-engineered products are already commercially available for topical substance testing or toxicity screening [10]. This *skin-in-a-dish* approach has the potential to be modified to meet different application requirements. *In vitro* SCC skin models were previously generated by the incorporation of different SCC cell lines into the epidermis. However, these SCC *in vitro* models could only be weakly discriminated from standard skin equivalents using morphological and tumor marker expression analyses [11,12]. Here, we generated a simplified epidermal skin equivalent that omits the dermis as well as a full-thickness skin equivalent in order to mimic the different aspects of SCC *in vivo*. An SCC cell line (SCC-25), derived from tongue tissue of a 70-year-old patient afflicted with SCC [13], was introduced in either epidermal or full-thickness skin equivalents (Fig. 1). Although the epidermal *disease-in-a-dish* models enabled the study of 3D culture effects on SCC-25 cells, they did not allow for the monitoring of the impact of fibroblast signaling, which is crucial for the development of an invasive SCC state *in vivo*. In previous studies, spontaneous invasion of SCC cell lines could not be accomplished within an *in vitro* skin equivalent [11,12]. Here, SCC cells were directly diffused within the dermal collagen gel to establish an SSC model system that also mimics the invasive SCC morphology. The co-culture of both healthy human skin cells and malignant cells is important for the reproduction of *in vivo* cell–cell interactions and the *in vivo* microenvironment that influences and directs cell behavior [14]. Since cellular markers were not sufficient to discriminate SCC *in vitro* models from standard skin equivalents [11], we employed Raman spectroscopy on full-thickness standard *skin-in-a-dish* and late-stage *disease-in-a-dish* models. Raman spectroscopy is an emerging technology in the field of biomedical research that possesses great potential for the non-invasive discrimination between healthy and pathological cells *in vitro* [15]. In skin cancer diagnosis, Raman spectroscopy was explored *in vivo* showing its suitability to differentiate between cancerous skin tissue and healthy skin regions [16]. Raman spectroscopy generates fingerprints of the molecular constitution of a biological sample; monochromatic laser light is thereby used to excite vibration modes in molecules. Dependent on the excitation mode, the photons from the incident beam can experience a frequency-shift when scattered [17]. The frequency-shifted scattered light is detected as a spectrum, where the resulting peaks are specific for molecular bonds and their secondary structure. Moreover, Raman spectroscopy permits non-destructive measurements on living cells and native tissues without the need for staining or processing steps prior to the measurements [15]. In cancer research, achievements using Raman spectroscopy as a diagnostic tool were presented in various *in vitro* and *in vivo* studies [18,19]. In a previous study, Raman spectroscopy enabled an accuracy of more than 90% for the differentiation between non-tumorigenic and tumorigenic skin cell lines [20]. Krishna et al. investigated Raman spectroscopy on biopsy sections of oral squamous carcinoma and non-malignant tissues. Here, spectral differences revealed increased protein and DNA signals in the epidermal cell layer of malignant tissue [21]. Our previous studies indicated that Raman spectroscopy is a promising technology to analyze skin cells within their 3D microenvironment [22], as well as key extracellular matrix proteins such as collagen and elastin [15].

2. Materials and methods

2.1. Human tissue samples

All research was carried out in compliance with the rules for investigation of human subjects as defined in the Declaration of Helsinki. Written informed consent of the patients or parents of the patients was obtained according to the approval of the Landesärztekammer Baden-Württemberg (F-2012-078; for normal foreskin from elective surgeries) or the clinical ethics committee of the University Hospital Tübingen (2012B02; for squamous cell carcinoma biopsies).

2.2. Cell isolation and culture

Primary human fibroblasts and keratinocytes were isolated from foreskin samples (age: 2 months–9 years). The isolation of primary cells was performed as previously described [22]. SCC-25 cells were purchased from LGC Standards (Wesel, Germany (=ATCC; CRL1628)). SCC-25 cells were cultured in DMEM/F-12 medium (Invitrogen, Karslruhe, Germany), supplemented with 1 mM sodium pyruvate (Life Technologies GmbH, Darmstadt, Germany), 1.1 mM hydrocortisone (Sigma Aldrich, Steinheim, Germany), 10% fetal calf serum (Life Technologies) and 1% gentamycin (Invitrogen). Media exchange was performed every 2–3 days and cells were passaged after reaching 70–80% confluence.

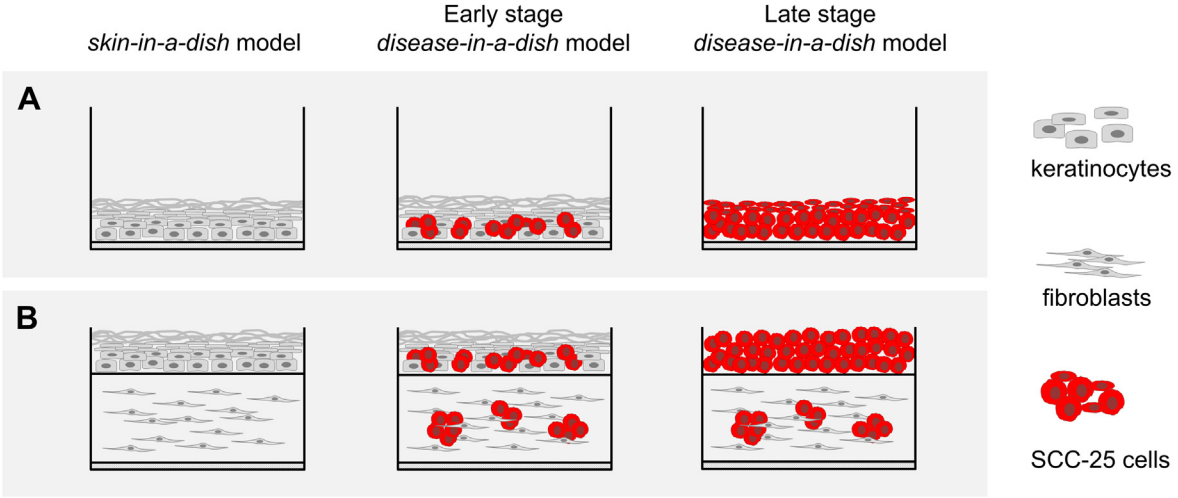

Fig. 1. Schematic of (A) epidermal and (B) full-thickness skin equivalents depicting the standard *skin-in-a-dish* model and the modified *disease-in-a-dish* models with SCC-25 cells.