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Chapter 1

Introduction
Chapter 1

The study of skin aging can occur on roughly three levels: in cell culture (in vitro), in animal models and in human subjects (in vivo). Most in vitro research on skin aging is performed on monolayer populations of skin cells. These monolayer cultures lack the structural complexity of the skin and can not represent the interactions between different cell types and molecules that the skin consists of. Traditionally, animal models could be used to bridge the gap between the relative simplicity of the culture dish and the in vivo human skin. However, more and more it becomes clear that the skin of most laboratory-animals is not very similar to human skin. In addition, the use of animal experimentation is becoming more restricted for obvious ethical reasons. For example, animal experiments are banned for testing of cosmetic ingredients in the EU (1). This has led to a need for different models in order to translate basic skin aging research to the in vivo human skin. Complex in vitro tissue models, such as human skin equivalents (HSEs), could be such a model. HSEs are in vitro tissue cultures of skin cells that have a high similarity to in vivo skin morphology and function. However, currently there is no HSE model available that is tailored to represent skin aging in vitro. Therefore, the main goal of this thesis was to contribute to the development of an in vitro HSE model that mimics characteristics of skin aging.

In the following introduction, the skin aging process and its properties will be discussed first. Thereafter, the HSEs will be introduced and their application to skin aging research will be described.

Skin aging

Skin aging is typically divided in two types: intrinsic- and extrinsic skin aging. The most common cosmetic changes caused by skin aging are caused by extrinsic skin aging (2). These include wrinkle formation, pigmenary irregularities and elastosis. Ultraviolet (UV) light is the most important external cause of extrinsic aging. Other external factors are for example electromagnetic radiation (such as infrared), ozone and tobacco smoke (3).

Intrinsic aging can not be related to any external factors, but is related to the general organismal deterioration with the passage of time. Intrinsic skin aging occurs throughout the entire skin, but due to the dominant effect of external factors, the effects of intrinsic aging on skin are only observed in parts of the body not often exposed to external factors. These parts are often called unexposed skin, which is based on our habit to cover them with textiles. Characteristics of intrinsically aged skin are for example thin, translucent skin, sagginess, dryness, hair greying and hair loss (4). The focus of this thesis, and therefore also of the rest of this introduction, is on intrinsic skin aging.

Skin aging not only causes cosmetic, but also functional changes. There is a general decline in all skin functions, such as thermo- and water regulation, immune response and
regenerative capacity (5). This leads to an increase in skin conditions that, although rarely fatal, often impact the quality of life in people affected by these conditions and present a burden on healthcare. The spectrum of disorders of aged skin contains a whole range of morbidities, ranging from idiopathic conditions such as general (or senile) dry skin and itching, impaired wound healing and hypothermia to an increased incidence of specific skin diseases such as dermatitis and eczema (6).

**Histology of intrinsic skin aging**

The skin consists of two compartments: the epidermis and the dermis. The epidermis is a layer of cells, mainly consisting of keratinocytes, which constantly divide and differentiate, eventually dying and getting sloughed off. It forms a barrier against the outside environment that offers protection from harmful substances, pathogens, UV radiation and dehydration. The dermis consists mostly of extracellular matrix (ECM). It gives structure and elasticity to the skin. The most common cell in the dermis is the fibroblast, which produces and maintains the ECM. In addition, one can find blood vessels and several skin appendages, such as hairs, (smooth) muscles, sebaceous glands and sweat glands in the dermis (7).

Sections of (intrinsically) aged skin are characterized by general atrophy, especially in the dermis. Therefore, the histological picture of aged skin can be summarized quite concisely: the number/volume of most skin components is decreased and the ones that remain look abnormal compared with young skin (3). Since the goal of this thesis is to mimic some changes of aged skin in HSEs, some specific characteristics of aged skin need to be defined. The characteristics of importance to this thesis are discussed below.

The most characteristic property of aged skin is the disappearance of epidermal ridges; a flattening of the dermal-epidermal junction (8). In contrast to photoaged skin, the interfollicular epidermal thickness is hardly affected in intrinsic aging. The number of proliferating keratinocytes in aged skin is not decreased, but the proliferation speed decreases, which leads to an overall reduction in proliferation (9). A consequence of this is slower epidermal turnover, which in turn could lead to the loss of the rete ridges.

The differentiation process of keratinocytes appears not to be affected by age directly. However, the increased prevalence of dry skin in elderly people hints at an increased predisposition to aberrations in terminal differentiation, cornification and desquamation of keratinocytes (10, 11). Other cell types in the epidermis, mainly the Langerhans cells and melanocytes, decrease in number during skin aging (12).

Since the dermis is a more static tissue than the epidermis, alterations and aberrations can have longer effects. For example, the half-life of collagen, an important constituent of ECM, is 15 years in the skin (13). Therefore, changes in ECM components and homeostasis
Figure 1: Histological characteristics of intrinsic skin aging. The most common and obvious characteristic of aged skin is the loss of the rete ridges (arrows); a flattening of the dermal-epidermal junction. The amount of collagen is reduced and the matrix is more disorganized. In addition, skin appendages and blood vessels decrease in number and become more fragile. SC: stratum corneum, Epi: epidermis, PD: papillary dermis, RD: reticular dermis. Scale bars: 50 µm.

Fibroblasts and skin aging

As experimentally confirmed in HSEs, the dermis populated and maintained by fibroblasts is crucial to skin appearance and mechanical properties, and clearly affects epidermal development and homeostasis (17). Hence, the studies in this thesis are based on the
premise that the fibroblasts are pivotal in skin aging. We therefore studied two processes involving dermal fibroblasts that may be important drivers of skin aging: fibroblast senescence and loss of papillary fibroblasts in the dermis. Both processes are described below and depicted in figure 2.

**Senescence**

The term cellular senescence was originally coined to describe the state that normal (i.e. untransformed), primary cells enter when they lose their replicative capacity *in vitro*. After a certain number of population doublings most cells will stop dividing permanently (18). Later it was discovered that cell senescence is part of the cellular stress response (19). Basically, after a significant amount of genotoxic stress cells will block their cell cycle progression. Based on the severity of damage and the cell's ability to repair damage, three options are possible: the cell can repair the damage and re-enter the cell cycle, go into senescence or remove itself by apoptosis. Due to its role in genotoxic stress response, senescence is seen as a tumor suppressive mechanism (20, 21).

It appears as if senescence is an intermediate between complete recovery and apoptosis, but this may be an oversimplification. Besides losing their replicative ability, senescent fibroblasts undergo a number of changes. These include alterations in morphology, secretory phenotype and ECM production. These changes are suspected to contribute to aging (22, 23).

Based on the *in vitro* alterations that occur in senescent cells, it is inferred that senescence plays a role in *in vivo* aging (24, 25). The number of senescent cells is increased in aged skin (and other tissues as well) (26-28). Senescent cells appear to contribute to aging in the skin by creating a more pro-inflammatory environment and by promoting matrix degradation. However, the exact role and the extent of the contribution of senescent cells in aging are not completely clear.

**Papillary and reticular fibroblasts**

The dermis can be divided in two morphologically distinct parts. The upper part, called papillary dermis, is characterized by a high cell density and loose matrix. The lower part, called reticular dermis, is characterized by a low cell density and dense matrix (7). The fibroblasts from these layers look and behave differently in culture (29). For example, compared with reticular fibroblasts, papillary fibroblasts show a leaner and more spindle-shaped morphology and show increased proliferation (30-32).

Since papillary fibroblasts are closer to the epidermis, it is assumed that papillary fibroblasts are better able to support the epidermis. For example, they differ in the secretion of a number of growth factors and papillary fibroblasts are better at stimulating
Figure 2: Proposed model of the role of fibroblasts in skin aging. Skin aging is characterized by a general atrophy of the skin and its cellular components. The most common characteristic of skin aging is flattening of the dermal-epidermal junction. This is correlated to a reduction of the papillary dermis. In this thesis two mechanisms involved in skin aging were investigated in HSEs: (I.): the decrease in the papillary dermis and papillary fibroblasts and (II.): the appearance of senescent fibroblasts in aged skin.
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Epidermal (terminal) differentiation in HSEs (33, 34).

A study by Mine et al. showed that papillary fibroblasts are more affected by aging than reticular fibroblasts (35). During skin aging, the rete ridges and part of the papillary dermis are lost, which leads to a relative increase of the reticular dermis and reticular fibroblasts. The main hypothesis of this thesis is that these changes in the dermis have significant effects on the entire skin, because reticular and papillary fibroblasts have different effects on skin homeostasis; the (relative) increase of reticular fibroblasts and the decrease of papillary fibroblasts will alter skin homeostasis.

Myofibroblasts and Transforming growth factor-β

Transforming growth factor-β (TGF-β) is an important regulator in normal skin homeostasis. Its two main effects are stimulation of matrix formation and inhibition of epidermal proliferation (36, 37). If the skin is injured, TGF-β recruits immune cells, stimulates the production of new ECM and stimulates the closure of the wound (38). An important step in this process is the differentiation of fibroblasts to myofibroblasts, which is also induced by TGF-β.

Myofibroblasts are known for their role in tissue remodelling; they play an essential role during wound healing (39). In the wound, the first action of the recruited myofibroblasts is to attach to the damaged dermis and contract, thus closing the wound as much as possible. Then the damaged tissue needs to be regenerated, to which the myofibroblast contributes considerably by generating ECM components. After regeneration is completed, myofibroblasts should disappear from the healed tissue. If the healing process is not properly terminated, myofibroblasts can remain in healed tissue. Improperly stopped healing processes can lead to fibrotic diseases, such as liver and lung fibrosis and hypertrophic scar formation. Myofibroblasts are strongly implicated in these disorders (40).

Reticular fibroblasts share some characteristics with myofibroblasts, such as increased contractility and formation of dense matrix. However, it is not known whether reticular fibroblasts represent a precursor state to myofibroblasts, have a “mild” myofibroblast-like phenotype or are not related to myofibroblasts at all.

The number of alpha smooth muscle actin (α-SMA, a myofibroblast marker) positive fibroblasts is higher in reticular populations than in papillary populations in vitro. However, the percentage of positive fibroblasts, even in reticular populations, is minimal (<5%). Expression of α-SMA is not found in dermal fibroblasts in vivo (41).
**Human Skin equivalents**

There are several ways to perform experiments with skin. The experimental systems differ in complexity, ranging from monolayer cultures of skin cells to *in vivo* studies (both in humans and in animal models). Human skin equivalents (HSEs) fit in between both ends of this spectrum. HSEs are more complex than monolayer cultures and more representative of the tissue morphology of *in vivo* skin. While HSEs lack the sheer complexity of *in vivo* skin, which contains a multitude of different cell types and is influenced by many hormonal, metabolic, nervous and immune processes, HSEs are not hampered by the ethical and practical constraints of *in vivo* skin. HSEs allow the study of processes in a dedicated, specially designed experiment.

HSEs can be generated in several ways. They can consist of only an epidermis or both a dermal and epidermal part: a full thickness equivalent. Most HSEs only contain fibroblasts and keratinocytes, but other cell types, such as melanocytes, vascular cells and several types of immune cells can be included as well (42). HSEs can be used to model several skin diseases, for example skin cancer, skin infections and skin irritation (43-50).

The main type of HSE used in this thesis is the Fibroblast Derived Matrix (FDM) HSE (17). In this type of HSE fibroblasts are seeded in a transwell and stimulated to produce ECM. This combination of ECM and fibroblasts is then used as a dermal matrix onto which the keratinocytes are seeded. In most full-thickness HSEs the dermal part contains or consists of artificial components, for example a bovine or rattail collagen type I gel seeded with fibroblasts. The FDM HSE more clearly represents the dermis of human skin than other types of full-thickness equivalents, since the ECM is generated by the fibroblasts themselves.

**Aims**

The main goal of this thesis was to contribute to the development of an *in vitro* HSE model that mimics characteristics of skin aging. In **chapter 2**, we started by examining if FDM HSEs can be representative of *in vivo* skin and used for studies on skin aging. For this purpose, FDM HSEs were treated with a known anti-aging compound: Bio Marine Complex (51). The effects of this compound on *in vivo* skin collagen production and keratinocyte proliferation are well described and thus allowed us to show the similarities between the response of HSEs and *in vivo* skin to this compound.

After showing the validity of HSEs as a model for skin aging *in vitro*, several alterations were made to the composition and culture process of FDM HSE, with a focus on the fibroblasts. The alterations were based on the two processes involved in skin aging discussed above: the appearance of senescent fibroblasts and the loss of papillary fibroblasts.
in aged skin. We hypothesized that alterations to the HSE culture process that mimic these processes can introduce features of skin aging in HSEs.

Serial passage is commonly used to induce replicative senescence *in vitro* and is often used as a model for aging in *in vitro* monolayer cultures (52, 53). In chapter 3, HSEs were generated with long-cultured (and often passaged) fibroblasts and compared with skin equivalents generated with short-cultured fibroblasts of a few passages to determine whether the inclusion of senescent fibroblasts introduces features of aged skin to the HSE.

The remainder of the chapters focusses on papillary and reticular fibroblasts. Unlike senescent fibroblasts, there is no clear definition for these populations in monolayer culture yet. Therefore, we first tried to identify markers for both fibroblasts populations in chapter 4. In chapter 5 and 6, we investigated if papillary fibroblasts can differentiate into reticular fibroblasts. In chapter 5 the fibroblasts were serially passaged and in chapter 6 TGF-β1 was used to induce differentiation. In both chapters 5 and 6 HSEs were generated with papillary and reticular fibroblasts. Based on the results obtained in these HSEs a final study was performed to elucidate how the different fibroblast populations interact with keratinocytes *in vitro*. This is described in chapter 7. The results from these four chapters led to an extension of the hypothesis of Mine et al. regarding the role of the fibroblast populations in skin aging (35). The future perspectives of the presented research and discussion concerning our hypotheses are presented in chapter 8.

**References**

1. EU: final ban on animal experiments for cosmetic ingredients implemented. ALTEX 2013: 30:268-269.


