Aus dem Institut für Veterinär-Anatomie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

From porcine skin samples in situ to three-dimensional human skin constructs in vitro. Studying skin with a focus on the 3R principles.

Inaugural-Dissertation
zur Erlangung des Grades eines
Philosophiae doctor (PhD)
in 'Biomedical Sciences'
an der Freien Universität Berlin

vorgelegt von
Maneenooch Khiao-in
Tierärztin
aus Phetchaburi, Thailand

Berlin 2022
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List of abbreviations

2D	two dimensions
3D	three dimensions
ANOVA	oneway analysis of variance
BSA	bovine serum albumin
DAB	diaminobenzidine
EC	endothelial cell
ECs-ECs	endothelial cells-endothelial cells
EGF/TGF-alpha	epidermal growth factor/transforming growth factor alpha
EGM	endothelial growth medium
ELISA	enzyme-linked immunosorbent assay
EU	European Union
FBS	fetal bovine serum
FBs-FBs	fibroblasts-fibroblasts
FBs-ECs	fibroblasts-endothelial cells
FGF	fibroblast growth factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
hEGF	human epidermal growth factor
hFGF-B	human fibroblast growth factor B
HUVEC	human umbilical vein endothelial cells
IFN	interferon
IGF	insulin like growth factor
IGFBP7	insulin growth factor binding protein 7
IL	interleukin
KCFB	Keratinocytes and Fibroblasts
KCFB-EC	Keratinocytes, Fibroblasts and Endothelial cells.
KGM	keratinocyte growth medium
LM	light microscope
MCP	monocyte chemoattractant protein
MMP	matrix metalloproteinase
OECD	Organisation for Economic Cooperation and Development
P_{app}	apparent permeability
PK	pharmacokinetics
SPARC	secreted protein acidic and rich in cysteine
TEM	transmission electron microscopy
TGF	transforming growth factor

TNF	tumor necrosis factor
UV	ultraviolet radiation
VEGF	vascular endothelial growth factor

1. Introduction

Interest in anatomy and histology of skin has increased in recent years both in pharmaceutical and cosmetic development and research. The use of human skin models has become widely accepted in the evaluation of dermato-cosmetic products (Abd et al., 2016; Andrade et al., 2015; Robinson et al., 2002). Human skin, on the other hand, is only available in small quantities from cosmetic surgery clinics or cadavers (Abd et al., 2016). A further problem is that human skin histology is very varied due to gender, age, and anatomical donor location variances (Kakasheva-Mazhenkovska et al., 2011; Lee & Hwang, 2002; Mohammed et al., 2012; Otberg et al., 2004; Robertson & Rees, 2010; Sandby-Møller et al., 2003; Taylor & Machado-Moreira, 2013).

A variety of models have been developed to replace or mimic human skin in vitro, including ex vivo porcine skin models and artificial or reconstructed skin models. Ex vivo porcine skin, for example, was used to determine the impact of fire size and fire service water application on the relative risk of skin burns (Traina et al., 2019) or to investigate the safety and skin barrier-improving effects of hydroxy acids (Hwang et al., 2021). In the case of toxicity, pharmacology, effectiveness, and drug transport studies, a reconstructed skin model is useful (Wever et al., 2015).

Fresh animal skin is readily available in abundant amounts compared to human skin. Hence, numerous mammalian species, including primate, porcine, mouse, rat, and guinea pig have been utilized for skin research (Abd et al., 2016; Abdullahi et al., 2014; Oesch et al., 2014). Porcine skin ex vivo has been frequently used for skin research (Ganderup et al., 2012; Glerup et al., 2013; Helke et al., 2016; Jung et al., 2013; Monteiro-Riviere & Riviere, 1996) because remarkable anatomical and physiological similarities have been reported between human and porcine skin (Helke et al., 2016; Liu et al., 2010a; Qvist et al., 2000; Simon & Maibach, 2000). Earlier studies comparing human and pig skin reported variations in; the epidermal and dermal thickness, the skin architecture, the wound healing mechanism, the hair coat, the blood vessels in the dermis, the epidermal enzyme patterns, the cellular turnover of the epidermis, the keratinous proteins, and the composition of the lipid film of the skin surface (Dahiya, 2009; Meyer et al., 1978; Sheu et al., 2014; Sullivan et al., 2001; Swindle et al., 2011). While regional variations in porcine skin have been noticed, there is a paucity of data in porcine skin structure and composition associated with different body sites (Turner et al., 2015). The first part of the present study focuses on gaining better understanding of the morphology of porcine skin associated with different anatomic locations in comparison to human abdominal skin. This knowledge is essential to replace human abdominal skin by porcine skin.

Recently, worldwide laws and regulations have become more restrictive regarding the use of animals in research, including skin research. Consequently, there is a demand for novel materials and in vitro methods to simulate human skin for future research. Human skin in vitro reconstructs, consisting of human keratinocytes and fibroblasts, have been especially developed (Abaci et al., 2017; Carlson et al., 2008; Küchler et al., 2011; Van Gele et al., 2011). Developing a blood supply is crucial for accomplishing the functionality of skin constructs. In previous work, skin constructs have been studied by co-culturing endothelial cells within the dermal compartment and stimulating capillary formation extrinsically by adding growth factors such as VEGF and FGF (Gibot et al., 2010; Groeber et al., 2016; Marino et al., 2014; Supp et al., 2002; Tonello et al., 2003). In an endeavor to optimize in vitro constructs of human skin, the influence of endothelial cells in human three-dimensional skin constructs should be investigated in the second part of this study.

2. Literature review

2.1. The pig as an animal model in skin research

The domestic pig (Sus scrofa domestica) has been adopted as suitable model for many research undertakings because it shares similar characteristics with human anatomy. The anatomy of the cardiovascular system, major parts of the gastrointestinal tract, the nasal cavity, and the urogenital system are reported to be similar in pigs and humans (Glerup et al., 2013; Swindle et al., 2011; Vodicka et al., 2005). Large breeds such as the Landrace, Yorkshire, Duroc, or Hampshire have weights and sizes similar to humans as well as similar organ dimensions and structures (Swindle et al., 2011). However, the size of the larger domestic pig breeds requires large housing areas and costly feeding regimes. In addition, they can be difficult to handle. Consequently, several minipig breeds have been bred specifically for biomedical research because of their small size even at maturity, economical husbandry, and ease of handling. Whilst there are at least 45 breeds of minipigs available worldwide (Smith & Swindle, 2006), one of the most common breeds used is the Göttingen pig (Gutierrez et al., 2015).

Because humans and pigs share many similarities in the anatomy and physiology of their skin, pigs have been used as models for human skin in studies of preclinical dermal toxicology, dermal pharmacokinetics, dermal phototoxicity, dermal wound healing and in a broad array of other biomedical research applications (Stricker-Krongrad et al., 2016). The epidermis and dermis of the pig have similar thicknesses. The porcine epidermis ranges from 30 to 140 μm, whilst that of human ranges from 50 to 120 μm (Khiao In et al., 2019; Sullivan et al., 2001). The dermo-epidermal junction of the pig is similarly and more firmly attached to the dermis as seen in humans (Summerfield et al., 2015). Humans and pigs are similar in the number and morphology of their hair follicles (Mangelsdorf et al., 2014). Additional similarities of human and porcine skin include the size, orientation and distribution of blood vessels of the skin (Sullivan et al., 2001), the epidermal enzyme patterns of skin (Meyer & Neurand, 1976; Oesch et al., 2018), the epidermal turnover time which is 30 days in pig and 27-28 days in human (Liu et al., 2010a; Weinstein & Van Scott, 1965), the presence of keratin proteins type 1, 5, 6 and 10 (Debeer et al., 2013) and the composition of the skin surface lipid film (Dahiya, 2009; Gray & Yardley, 1975). All these similarities appear to make pig skin an excellent animal model for human skin (Abdullahi et al., 2014).

Skin permeation assays, which examine percutaneous absorption of molecules, are important tests in the evaluation of any topical drug-delivery system or formulation. Porcine skin has been used widely in skin permeation studies. For example, Qvist et al. (Qvist et al., 2000) found that the permeation of salicylic acid, nicotine, and testosterone of both the

Göttingen minipig and other domestic pigs correspond to the permeation through human skin. The stratum corneum is the first barrier to substances that permeate through the skin. The stratum corneum structure of the pig resembles that of human skin with a comparable thickness (approximately 10 µm) and several layers (approximately 9-18 in humans and 15 in pigs) (Gerstel et al., 2016; Khiao In et al., 2019; Ya-Xian et al., 1999).

In addition, the cutaneous lipids are known to be important regulators of skin permeability. These lipids: ceramide, cholesterol and fatty acid are similar in intercorneocyte space in humans and pigs. However, the arrangement of these lipids differs. In humans, they are arranged in an orthorhombic packing pattern while in pig lipids are arranged predominantly in the hexagonal lattice (Caussin et al., 2008; Todo, 2017). The difference in the lipid architectural composition may explain the differing skin permeability results (Takeuchi et al., 2012; Todo, 2017). Moreover, the transfollicular pathway of percutaneous transport seems to be particularly important (Mohd et al., 2016). Pigs have a similar structure and distribution of hair follicles to that of humans (Jacobi et al., 2007; Mangelsdorf et al., 2014; Otberg et al., 2004). However, there are variations in the number of hair follicles between different body locations. The average hair follicle density in human skin, except for the head, typically ranges between 14 and 32 hairs/cm^2. In contrast, in porcine skin it ranges from 4 hairs/cm^2 in the caudal belly to 42 hairs/cm^2 in the outer ears (Otberg et al., 2004; Turner et al., 2015). Earlier studies indicate that the follicular contributions in drug absorption of porcine skin correlates well with follicular contributions in human (Frum et al., 2008; Takeuchi et al., 2011).

2.2 Human skin: Essentials of histology and use in research

Skin, the largest organ of the human body, consists of three distinct layers. A thin outer is epidermis overlying a supporting dermis that lies above a hypodermis of variable thickness. Most skin regions also are characterized by adnexa, particularly skin glands and hair (Herkenne et al., 2008; McGrath & Uitto, 2010). The stratum corneum, the epidermis' outermost layer, comprises 15-20 layers of flattened cells. The stratum granulosum, stratum spinosum, and stratum basale compose the deeper epidermis. A stratum lucidum exists between the stratum corneum and the stratum granulosum in palmoplantar skin (McGrath & Uitto, 2010). The dermis consists of connective tissues and a complex circulatory network that lies underneath the epidermis (Moteriro-Riviere, 2013). The use of human skin in biomedical research is varied. Hence the locations from which skin is harvested depends on the research requirements (Varani, 2012). Transdermal absorption studies, for example, require vast amounts of skin. Hence the human abdomen, breast, and back skin are the best places to get the skin for these procedures (Abd et al., 2016; Barbero & Frasch, 2016). UV exposure study was performed on the forearm and upper back skin because both are naturally exposed to

sunshine. (Downs et al., 2009; Lopes & McMahon, 2016). Likewise, skin from the hip region which is normally not exposed to sunlight is used as the control site for pre-UV treatment experiment (Kim et al., 2016; Rhie et al., 2001; Tian et al., 2014). More specifically, scalp skin is used in research focused on hair follicles (Adley et al., 2005; Gherardini et al., 2019). In studies of specific disease processes, skin lesions can be of value. Psoriatic plaque skin, for example, is used in ex vivo protocols for testing antipsoriatic agents (Varani, 2012).

Because in vivo tests on human skin are undertaken under near-natural conditions and a normal physiological status of the body, they are considered the gold standard experimental model for skin research (Abd et al., 2016). However, in vivo experiments on humans have many limitations including high costs, ethical considerations, and difficulties to measure and interpret results (Abd et al., 2016; Trauer et al., 2009). An alternate option is to use ex vivo human skin that has similar physical and anatomical structures to in vivo skin (Abd et al., 2016; Liu et al., 2014; Schaudinn et al., 2017). Explanted human skin retains its biological functions for three days (Ng et al., 2009). For example, inflammatory cytokines can still be detected and quantified by the enzyme-linked immunosorbent assay (ELISA) technique after 20 hours (Schaudinn et al., 2017). Cryopreserved skin seems to be viable for five years for use in transdermal absorption testing (Franz et al., 2009). Ex vivo human skin for research is commonly sourced from plastic surgery off-cuts, neonatal foreskin as well as from cadavers. The type of skin used experimentally varies greatly. Full thickness skin is commonly used in transdermal absorption tests (Berthet et al., 2017; Franz et al., 2009; Trauer et al., 2009) and in skin irritation tests (Varani, 2012). Skin without its stratum corneum is used in skin barrier function studies (Danso et al., 2015) and in studies of wound infections (Schaudinn et al., 2017).

Whilst ex vivo skin retains some of its biological characteristics, it loses others. The dislocation of the skin's blood supply, for example, stops any inflammatory responses because immunological reactors such as white blood cells are no longer present (Corzo-León et al., 2019; Varani et al., 2007). Sterne et al. (Sterne et al., 2000) reported impairments of intracellular systems including membrane integrity, aerobic respiration, protein, and DNA repair and synthesis over time culminating in tissue necrosis.

2.3. In vitro models of skin research

In vitro skin models have been developed to mimic human skin because of; the increase in regulatory restrictions governing the use of animals, ethical issues, and economics. In vitro skin has been grown to mimic both healthy and diseased skin. They have been developed as alternative methods to test the efficacy and safety of pharmaceutical products and medications, specifically dermatological medications, to study

wound healing and inflammatory skin conditions such as psoriasis (Küchler et al., 2013).

Two-dimensional (2D) and three-dimensional (3D) skin cell cultures are examples of in vitro methods used as alternatives to animal testing (Teimouri et al., 2018). For skin research purposes 2D cell cultures of keratinocytes are grown as a monolayer on a solid flat surface such as a petri dish. The morphology of the individual cell in 2D cultures is influenced by cell proliferation, differentiation, apoptosis, as well as gene and protein expression (Tibbitt & Anseth, 2009). However, cells grown in 2D culture are usually flatter and more elongate than they are in vivo, and cells appear not to behave as they do in an in vivo microenvironment (Edmondson et al., 2014). Therefore, the results have not translated to in vivo studies (Teimouri et al., 2018). Consequently, in vitro 3D models have become increasingly in demand as they are considered to resemble the in vivo skin more closely.

As 2D cultured cells poorly mimic the situation found in normal live skin, 3D skin models have been developed. These models use keratinocytes as epidermal equivalents and fibroblasts as dermal equivalents. The primary function of the fibroblasts is to produce extracellular matrix constituents such as ground substance, collagen, and elastin. Cells then form aggregates or spheroids within the matrix. The interactions between cells and the extracellular matrix of 3D models more closely mimic that found in an in vivo environment (Edmondson et al., 2014). The in vitro skin models are designed to mimic the epidermis (reconstructed human epidermis models) or the full human skin (living skin equivalents) (Küchler et al., 2013). There is commercially available reconstructed human epidermis e.g., EpiSkin®, SkinEthic®, and EpiDerm® and living skin equivalents e.g., GraftSkin®, EpiDermFT®, and Pheninon®. Some reconstructed skin models are produced in-house for individual research purposes such as drug evaluations and toxicological screenings (van Drongelen et al., 2014).

Nevertheless, these models are still poor matches to normal live human skin. The most significant limitation is their relatively weak barrier function that limits their applicability for dermal penetration studies and may cause confusing results in other tests (Küchler et al., 2013; Netzlaff et al., 2005). Morphologically, these models lack cell types found in normal human skin such as melanocytes, Langerhans cells, Merkel cells, dermal dendritic cells, resident T cells, and endothelial cells (Wever et al., 2015). In order to, closely mimic natural skin, it will be necessary to add these cells into future constructs (Abaci et al., 2017). Particularly, representative cells from the vascular systems are important for sending fluid and protein balance, cell nutrition and immunologic functions in the tissue. One of the reasons why skin graft fail is the absence of vascularization (Marino et al., 2014). The attempt to create a vascular supply to in vitro 3D models include the addition of endothelial progenitor cells or mesenchymal stem cells or endothelial cells into the tissue construct (Dai

et al., 2018; Supp et al., 2002; Wang et al., 2017). However, there are still many limitations and the strong need to improve the vascular elements of 3D skin models (Randall et al., 2018).

2.4. The 3Rs principles

The use of animals for scientific purposes has been and continues to be vital in the development of modern knowledge and advanced technology. Many countries have adopted strict regulatory regimens in their endeavor to protect these animals. For example, the European Union's Directive 2010/63/EU protects animals used under its jurisdiction (Olsson et al., 2017). In this context, the “Principles of Humane Experimental Technique” (Russell & Burch, 1959) are imperative. The 3Rs are Reduction, Refinement and Replacement of experimental animals. Russell and Burch (Russell & Burch, 1959) developed the concept of the 3Rs, meaning that if animals were to be used in experiments, every effort should be made to Replace them with non-sentient alternatives, to Reduce to a minimum the number of animals used and to Refine the experiments that used animals so that they caused minimal pain and distress.

2.4.1. Replacement of experimental animals

Replacement is either the use of methodologies that avoid using animals or the use of less sentient species. It includes using in vitro cultures, mathematical and computer modelling as well as the replacements of vertebrates with animals that are lower on the phylogenetic scale. To date, in vitro methods consist of cell culture, organoids, organs-on-a-chip, and 3D printed tissues or organs (Kendall et al., 2019).

This dissertation considers cell cultures using skin epithelium and dermal fibroblasts. Initially, 2D models were produced in which a cellular monolayer culture was grown, but these did not match the natural environment of cells. Following that, 3D cultures in which cells were grown in a scaffold or matrix were produced. Here cellular behavior is more reflective of in vivo cellular responses. 3D cultures play a vital role in understanding the pathogenesis of infectious diseases (Andersson et al., 2017), development of therapeutic drugs (Edmondson et al., 2014; Reichl et al., 2011), cytotoxicity of chemical agents (Riss et al., 2011; Srivastava et al., 2018) and skin barrier functions (Niehues et al., 2018).

2.4.2. Reduction of experimental animals

Reduction refers to reducing the numbers of animals used without meaningfully diminishing the amount and quality of information collected from experiments. Reducing the number of animals used in an experiment can be achieved by changes in experimental design

and analysis (Kendall et al., 2019; Parker & Browne, 2014). In some instances, different treatments can be compared to the same control group. This has the added advantage that the treatments can be directly compared with each other, which cannot be done in separate experiments (de Boo & Hendriksen, 2005). Using appropriate minimal sample-sizes is crucial to reliably and validly answering the research question of interest, whilst using no more animals than necessary (Festing & Wilkinson, 2007).

In addition, the re-use of animals for research reduces the overall number of animals required. The sequential use of an animal for unrelated animal experiments as well as using control animals from a previous study, in which they were not exposed to factors that might have lasting effects, can be considered for a subsequent experiment (de Boo & Hendriksen, 2005; Törnqvist et al., 2014).

2.4.3. Refinement of experimental animals

Refinement is the development of methods or technologies that minimize an animal's pain or distress. This includes the processes that deliver enhanced animal care, housing, and handling (Kendall et al., 2019). Even non-invasive experiments in which animals are subjected to repeated procedures, may cause stress to them. In these cases, stress can sometimes be lessened by training animals to cooperate in routine repeated procedures. In invasive experimental protocols anesthesia and analgesia to minimize pain or distress experienced by animals can be used. In addition to reducing numbers of animals used, micro-sampling provides a refinement because it is quicker and less stressful (Auer et al., 2007; Lilley et al., 2015; Taylor & Emerson, 2018).

3. Objectives and hypotheses

The overall objective of this dissertation was to support the 3Rs principles in skin research by morphological and in vitro studies. The first part focuses on gaining better understanding of the morphology of porcine skin associated with different anatomic locations in comparison to human abdominal skin. This knowledge is essential to replace human abdominal skin by ex vivo porcine skin. In the second part of the dissertation the influence of endothelial cells in human three-dimensional skin constructs is investigated.

Study 1:

The objective of this study was to identify histologically and ultra-structurally the various regions of German Landrace pig skin that could be used as the best possible substitute for human abdominal skin. For this, porcine skin collected from the ear, flank, back, and caudal abdomen was compared to human abdominal skin excised during plastic surgery. Epidermis and dermis were investigated in situ by light and transmission electron microscopy, with focus on the dermo-epidermal thickness ratio, dermo-epidermal interface length, density of hair follicles, arrector pili muscles, blood vessels and sweat glands. Additionally, in porcine skin, the barrier function of the four regions was analyzed.

Hypothesis: Amongst the different locations of pig skin, one location is suited optimally for replacing human abdominal skin.

Study 2:

The objective of this study was to assess morphologically the effect of endothelialization on the epidermal differentiation in two different human three-dimensional skin constructs. The two variations of human full-thickness skin constructs consisted either of keratinocytes and fibroblasts only (type 1, KCFB) or of keratinocytes as well as fibroblasts and vascular endothelial cells (type 2, KCFB_EC). Samples were examined using light and transmission electron microscopical techniques.

Hypothesis: The differentiation of keratinocytes is influenced by the presence of endothelial cells.

4. Histological and functional comparisons of four anatomical regions of porcine skin with human abdominal skin

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Histological and functional comparisons of four anatomical regions of porcine skin with human abdominal skin

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Abstract

Because of the shortage of human skin for research purposes, porcine skin has been used as a model of human skin. The aim of this study was to identify the region of German Landrace pig skin that could be used as the best possible substitute for human abdominal skin. Porcine samples were collected from the ear, flank, back and caudal abdomen; human abdominal skin samples were excised during plastic surgery. Histological and ultrastructural assessments were carried out on the epidermis and dermis, with emphasis on the dermo-epidermal interface length, dermo-epidermal thickness ratio as well as densities of; hair follicles, arrector pili muscles, blood vessels and sweat glands. In the pig, the barrier function of the four anatomical regions was assessed. Results showed that both histologically and ultrastructurally, all four regions of porcine skin were similar to human skin. These include the shapes of keratinocytes, structure of cell contacts and presence of Weibel Palade bodies in endothelial cells. Other parameters such as the thickness of epidermis, the thickness of stratum basale, spinosum and granulosum and the number of cell layers in the stratum corneum were similar in human abdominal and in all four regions of porcine skin. However, there were also significant differences especially in the thickness of the stratum corneum, the dermo-epidermal interface length and the blood vessel density.

KEYWORDS
anatomical region, comparison, German Landrace, human, pig, skin

1 | INTRODUCTION

Skin is used extensively in many areas of research such as analysing the effects of irradiation (Kim et al., 2013), studying the absorption and effects of transdermal drugs (Mendoza-Garcia, Sebastian, Alonso-Rasgado, & Bayat, 2015), screening toxicity of topically applied compounds (Nakamura et al., 2017) and studying wound repair (Leal, Cordery, Delgado-Charro, Bunge, & Guy, 2017). Ideally, skin testing addressing questions involving humans should be conducted on human skin. However, while the demand for human skin for research purposes is very high, the supply is severely limited. Human skin is obtained during plastic surgery procedures mostly from abdominal, breast and back regions and is stored in skin banks for scientific purposes. Similarly, this is the case with skin obtained from deceased donors (Calota, Nitescu, Florescu, & Lascar, 2012; Mahdavi-Mazdeh et al., 2013; Pirnay et al., 2010).

As an alternative to human skin, in vitro cultured skin constructs and biosynthetic skin substitutes have been developed for many biomedical applications (Abdullahi, Amini-Nik, & Jeschke, 2014; Jung & Maibach, 2015; MacNeil, 2007). Over the past decade, skin reconstructs having all epidermal strata made from keratinocytes, some even including melanocytes and Langerhans cells, have been

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developed (Bechetoille et al., 2007; Gledhill et al., 2015; Swope, Supp, & Boyce, 2002; Wojtowicz et al., 2014). Likewise, there are epidermal-dermal skin reconstructs that include keratinocytes, fibroblasts and endothelial cells (Khiao-In et al., 2015; Liu et al., 2013). Khiao-In et al. (2015) reported that some features typical of human skin such as a basement membrane and hemidesmosomes in the stratum basale were absent in the reconstructs. Similarly, in artificial skin, adnexal structures such as sweat and sebaceous glands as well as hair follicles are absent. On top of these structural inadequacies, the costs associated with the development of the skin substitutes are extremely high (Abdullahi et al., 2014; Netzlaff, Lehr, Wertz, & Schaefer, 2005; Varkey, Ding, & Tredget, 2015).

Therefore, the search for alternative skin from non-human sources such as primates, pigs, dogs, cats, rodents, rabbits and fish, for use in medical research including studies on skin xenotransplantation, has been a major focus over the past decade (Abdullahi et al., 2014; Avci et al., 2013; Badyal & Desai, 2014; Boneva, Folks, & Chapman, 2001; Ge, Zheng, & Wei, 2009).

Concerning the skin, the most obvious similarity—with the exception of the woolly pig breeds—is that pigs and humans share the characteristic of having sparse body hair. This is particularly important, as hair follicles together with their sebaceous glands have been recognised as pathways for percutaneous penetration of topically applied drugs (Stricker-Krongrad, Shoemake, & Bouchard, 2016a). Because of the similarity of their integument to that of humans, pigs have been used extensively in dermal research resulting in many significant publications (Fujii et al., 1997; Gad, 2006; Jung Y., Son, Kwon, Kim, & Han, 2013; Monteiro-Riviere & Riviere, 1996).

However, in both humans and pigs, regional variations in skin architecture occur in different body regions. These include parameters such as the thickness of the stratum corneum, as well as the number and distribution of sweat glands in the human (Bouslimani et al., 2015; Holbrook & Odland, 1974; Kakasheva-Mazhenkovska, Milenkova, Gjokik, & Janevska, 2011; Otberg et al., 2004; Robertson & Rees, 2010). Turner, Pezzone, and Badylak (2015) examining 11 different skin locations of American Yorkshire crossbreed pigs reported significant differences in their histology particularly in the thickness of epidermis and dermis as well as the amount of collagen and elastin in each region. Similarly, regional differences in the distribution of sweat glands over the body of Large White Yorkshire pigs were described by Sumena, Lucy, Chungath, Ashok, and Harshan (2010).

These examples indicate that general statements on the similarity of human and pig skin have to be considered with caution and that a single harvest site of skin for all individual specific research questions may not be appropriate.

As a measure to overcome the persistent shortage of human skin, pig skin should be considered for use as an alternative in research. To identify the region of pig skin most similar to that of human abdominal skin, the aim of this study was to compare the morphology and ultrastructure of pig skin from the ear, flank, back and caudal abdomen to that of human abdominal skin using light microscopy, morphometry, immunohistochemistry and transmission electron microscopy. In addition, the skin barrier function of the different regions of the pig skin has been compared via a skin permeation assay using Franz diffusion cells.

2 | ANIMALS, MATERIALS AND METHODS

2.1 | Animals and samples

Human abdominal skin samples from three healthy females (age 47–63 years old) were obtained after plastic surgery from the Division of Plastic Surgery and Hand Surgery, Helios Clinic Emil von Behring, Berlin, Germany, with the informed consent of the donors. The time of excision was between mid-morning and mid-afternoon.

Eight weaned German Landrace pigs (25–30 kg, 10–11 weeks old) supplied by the Institute of Animal Nutrition, Department of Veterinary Medicine, Freie Universität Berlin and the Institute for Animal Nutrition, Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig/Germany were used in this study. The animals had been killed for other experiments approved by German authorities not involving skin treatment. Skin samples were taken mid-morning, immediately following each pig's death.

Skin samples were collected from four locations of the pig's body; the ear (dorsum auriculae), the flank (regio abdominis media/fossa paralumbalis), the back (Regio dorsi/regio lumbalis) and the caudal abdomen (regio abdominis caudalis/regio pubica). All skin samples were dissected through to the underlying subcutaneous fat layer. The size of skin samples for light microscopy and immunohistochemistry was 2 x 2 cm, and for transmission electron microscopy (TEM), it was 0.5 x 0.5 cm. Immediately after removal, the samples were placed in 4% neutral buffered formalin for light microscopy and in Karnovsky's fixative (7.5% glutaraldehyde and 3% paraformaldehyde in phosphate buffered saline) for TEM.

2.2 | Light and transmission electron microscopy

For light microscopy, skin samples were embedded in paraffin blocks and sectioned at a thickness of 5 µm with a microtome (Leica, Histoslide 200R, Germany) for both light microscopy and for immunohistochemistry. Slides for light microscopy were deparaffinised stepwise using a descending series of ethyl alcohol concentrations (100% to 70%) and then stained with haematoxylin and eosin according to standard procedures (Böck, 1989). To visualise collagen and elastic fibres, sections were stained according to Volkmann-Strauss (Böck, 1989). Semi-thin sections (0.5 µm) of specimens processed for electron microscopy (see next paragraph) were stained with modified Richardson's solution and then analysed by light microscopy. For transmission electron microscopy, samples were fixed overnight in Karnovsky's solution and processed according to standard methodologies (see Khiao-in et al., 2015).

2.3 | Immunohistochemistry

Paraffin sections were heated in citrate buffer (0.01 M, at pH 6.0) for antigen retrieval. The slides were pre-incubated with 3% (w/v) bovine serum albumin (BSA) in incubation buffer (0.05 M Tris-HCl pH 7.4 + 0.9% v/w NaCl +0.66 mM $MgCl_2$ + 2% w/v DNS +0.1% w/v gelatine +0.01% v/v Tween20) before incubation with rabbit anti-pig polyclonal CD31 antibody (1:80 in antibody dilution buffer; Abcam Cambridge, UK). Blocking of endogenous peroxidase activity was achieved by immersion in TBS with 3% (v/v) H_2O_2. The slides were incubated with donkey anti-rabbit secondary antibody (1:300 in antibody dilution buffer; Biotium, Fremont, USA), and the enzyme–substrate reaction was visualised using diaminobenzidine (DAB, Roth, Dautphetal-Buchenau, Germany). Nuclei were counterstained with Mayer's haemalum (Fluka-Chemie, Buchs, Switzerland).

2.4 | Semiquantitative and quantitative analysis

Pictures of paraffin and semi-thin sections from each specimen were taken and recorded using an Axioskop light microscope (Carl Zeiss, Oberkochen, Germany), DS-Ri1 digital camera (Nikon, Düsseldorf, Germany) and a NIS-Elements version 3.0 imaging program (Nikon, Düsseldorf, Germany). Pictures were taken at magnifications of 1.25×, 5×, 10×, 20×, 40× and 100×. Five pictures from different areas were made at each magnification. Qualitative interpretation of cell morphology and tissue architecture was undertaken at all magnifications. Quantitative analysis/morphometry was done using the 40× magnification images.

Quantitative analysis included: Measurement of the thickness of the epidermis as well as of each stratum of the epidermis (stratum basale, spinosum, granulosum, corneum); counting the numbers of layers of each epidermal stratum; measurement of the thickness of the dermis; calculation of the dermal : epidermal thickness ratio; measurement of the length of the dermo-epidermal interface by drawing a line along this junction using computer software; counting the number of sweat gland clusters, hair follicles and arrector pili muscles in the dermis; counting the number of blood vessels in the dermal superficial vascular plexus from paraffin sections stained with anti-CD31. In each slide, the number of sweat glands, hair follicles, arrector pili muscles and blood vessels was counted and the area of dermis was measured. The data are presented as average number per average cm^2 or mm^2, respectively. Measurements of the strata of the epidermis were made in the semi-thin sections stained with modified Richardson's solution; all other measurements were

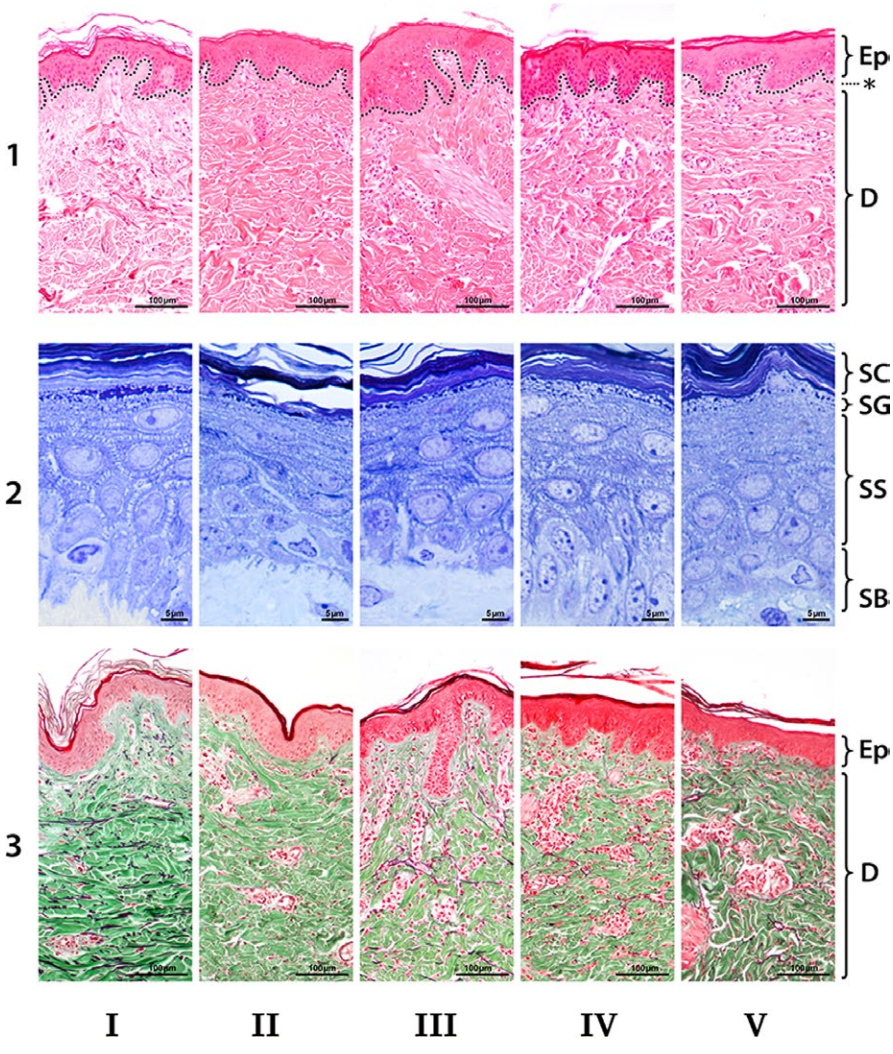

FIGURE 1 Light microscopy, human abdominal skin (I) and pig skin from the ear (II); flank (III); back (IV) and caudal abdomen (V). Sections in row (1) H&E stain, scale bar 100 µm; Ep, epidermis; D, dermis; asterix (*), dermo-epidermal interface line. Sections in row (2) stained according to Richardson, scale bar 5 µm; SC, stratum corneum; SG, stratum granulosum; SS, stratum spinosum; SB, stratum basale. Sections in row (3) Volkmann-Strauss stain, scale bar 100 µm; Ep, epidermis (red); D, dermis with collagenous fibres (green) and elastic fibres (dark purple)

made in haematoxylin and eosin-stained sections. The thickness of the different strata was defined as follows and measured accordingly (Figure 1): Stratum basale: one cell layer above the dermo-epidermal junction; Stratum spinosum: the rows of cells from the stratum basale out to the layer of granular cells; Stratum granulosum: cells which contain keratohyalin granules; Stratum corneum: flattened enucleated cells without granules.

2.5 | Skin permeability studies

Skin permeability studies were performed according to standardised and validated test procedures from the Organisation for Economic Cooperation and Development (OECD) for predictive testing of percutaneous absorption (Schäfer-Korting et al., 2008). The standard model lipophilic compound testosterone was used. Initially, the pig skin was adjusted to an even thickness of 1 mm using a Dermatome™ (Aesculap, Tuttlingen, Germany). A testosterone stock solution (40 µg/ml, 2% [v/v] Igepal® CA-630; Sigma-Aldrich, Munich, Germany) was spiked with an appropriate amount of 2,4,6,7-^{3}H-testosterone (100 Ci/mmol, Amersham, Freiburg, Germany) to achieve a total radioactivity of 2 µCi/ml. Permeation experiments were performed for 24 hr using Franz diffusion cells (PermeGear, Hellertown, PA, USA). The total amount of permeated testosterone was quantified using radiochemical detection (HIDEX 300 SL, HIDEX, Turku, Finland). The permeation rate for testosterone was calculated as the apparent permeability coefficient (P_{app}).

2.6 | Statistics

Statistical analysis was performed to compare quantitative parameters of each skin region to human abdominal skin. Mixed linear regression models were used to investigate the influence of the skin samples on continuous variables such as thickness of the epidermis and its layers (stratum basale, spinosum, corneum), dermo-epidermal ratio, length of dermo-epidermal interface, number of mitotic cells/high power field, number of hair follicles/cm^2, number of arrector pili/cm^2 and number of blood vessels/mm^2. As some investigations took place several times in the same sample, the sample was included as random effects in the model, while the skin regions were included as fixed effects.

When data were not normally distributed, the non-parametric Kruskal–Wallis-test was used to investigate the influence of the skin regions thickness of stratum granulosum, papillary dermis, reticular dermis and number of sweat gland clusters/cm^2.

The number of cell layers (stratum spinosum and stratum corneum) was investigated by analyses of variance tests. No model could be built for the stratum granulosum due to lack of variation. A p-value of less than 0.05 was considered statistically significant. Analyses were carried out using SPSS version 24 (IBM Deutschland GmbH, Ehningen). All data are presented as Mean±SD.

3 | RESULTS

3.1 | Epidermis

The epidermis of both, human and pig skin, consisted of four layers; stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Measurements (Table 1) revealed that there was no significant difference in the thickness of the epidermis of human abdominal skin and pig skin from all four locations, that is, ear, flank, back and caudal abdomen.

The thickness of the stratum basale in human skin was not different from all four regions of pig skin ($p = 0.123$) (Table 1). Basal keratinocytes of human and porcine epidermis were low columnar or cuboidal in shape with either round or ovoid nuclei. In both human and pig skin, the basal aspect of the keratinocytes connected with

TABLE 1 Measurements of thicknesses (mean ± *SD*) of human and pig epidermis and counts of cell layers

Sample site	Human abdomen ($n = 3$)	Pig caudal abdomen ($n = 8$)	Pig back ($n = 8$)	Pig ear ($n = 8$)	Pig flank ($n = 8$)
Thickness of epidermis (µm)	59.9 ± 8.7	53.4 ± 2.2	62.7 ± 2.3	51.6 ± 3.5	59.7 ± 2.4
Thickness of stratum basale (µm)	9.8 ± 0.8	10.1 ± 0.4	10.1 ± 0.4	8.9 ± 0.4	10.6 ± 0.4
Thickness of stratum spinosum (µm)	32.9 ± 6.3	32.4 ± 2.5	37.8 ± 2.1	34.0 ± 1.8	34.5 ± 1.8
Thickness of stratum granulosum (µm)	3.2 ± 0.7	3.2 ± 0.1	2.6 ± 0.1	2.7 ± 0.3	2.9 ± 0.2
Thickness of stratum corneum (µm)	13.2 ± 2.3	7.8 ± 0.3	9.8 ± 0.9	6.6 ± 0.5	9.4 ± 0.5
Number of layers of stratum spinosum	4.0 ± 0.7	7.0 ± 0.4	7.4 ± 0.4	7.6 ± 0.4	6.9 ± 0.4
Number of layers of stratum granulosum	1 ± 0.0	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2	1.5 ± 0.2
Number of layers of stratum corneum	13.3 ± 1.4	10.2 ± 0.9	15.6 ± 0.9	9.4 ± 0.9	13.4 ± 0.9

finger-like projections (0.3–1.4 μm in length) to the adjacent dermis. In both species, a few cells with clear cytoplasm and lobulated nuclei, possibly melanocytes and Merkel cells, were observed within the stratum basale (Figure 2).

In both pig and human skin, rete ridges (length 0.2–0.8 μm) were present at the dermo-epidermal junction (Figure 1). Transmission electron microscopy showed that the epidermis and dermis were separated by a basement membrane. The lamina rara and lamina densa of the basement membrane were clearly visible, together with the anchoring fibrils of the dermis and those of the hemidesmosomes in the plasma membrane of the basal cells. Ultrastructurally, the cell membranes of the basal cells had digit-like protrusions, 0.2–0.8-μm long, extending into the dermis. Tonofilament bundles, arranged as dense fibrils, were present in the cytoplasm of basal cells of all skin samples. Lateral cell borders interdigitated with neighbouring cells and desmosomes were visible at the interdigitations.

While the thickness of the stratum spinosum of the human skin was similar to that of pig skin, the number of cells layers was significantly lower in human skin (average 4 ± 0.7) than in all regions of the pig skin (average 7 ± 1.2; $p = 0.001$ ear, $p = 0.008$ flank, $p = 0.002$ back and $p = 0.006$ caudal abdomen; see Table 1). Light microscopically, the stratum spinosum was characterised by a lower zone and an upper zone. The lower zone consisted of three layers of polygonal shaped keratinocytes in both humans and pigs. In the upper zone, elongate flattened cells were oriented parallel to the epidermal surface. It consisted of only one layer in humans but 3–4 layers in pigs. Occasionally, cells with a clear cytoplasm, possibly Langerhans cells, were observed in both species (Figure 2).

Keratinocytes of the stratum spinosum of both species possessed round nuclei. Ultrastructurally, the keratinocytes´ outer cell membranes had typical spiny protrusions (0.5–3 μm) that attached to neighbouring cells by desmosomes. Large numbers of tonofilaments formed bundles in the cytoplasm except in the perinuclear zone. Bundles of tonofilaments extended from the inner cytoplasm towards the desmosomes.

The thickness of the stratum granulosum of the human abdominal skin was similar to that measured in all pig skin samples (Table 1). The stratum granulosum in all pig skin samples consisted of 1–2 cell layers whereas human skin usually had a single layer.

Cells of both species were elongated and arranged parallel to the skin surface. Long slender nuclei were present, and cell membranes were poorly defined. Basophilic keratohyalin granules were scattered throughout the cytoplasm. Measurements from transmission electron microscope images of the keratohyalin granules ranged from lengths of 0.7–3 μm and widths of 0.1–0.4 μm. In both species, cells were found to be connected with the corneocytes of the stratum corneum and with the cells of the stratum spinosum by distinct desmosomes.

The stratum corneum of human skin was significantly thicker than that of pig skin ($p < 0.001$ from the ear region, $p = 0.009$ of the flank, $p = 0.019$ of the back and $p < 0.001$ of the caudal abdomen). However, there was no significant difference in the numbers of cell layers (9–15) in this stratum between the two species (Table 1). Stacks of electron-dense, flattened corneocytes devoid of nuclei and organelles were observed in both species. They were attached closely to each other by corneodesmosomes. In the intercellular spaces between them, less electron-dense layers were observed, possibly lipid layers.

Mitotic cells in the stratum basale and stratum spinosum in all sections examined were rare. Only one or two mitotic figures were detected per section; however, these were found in fewer than 50% of the samples. Therefore, statistics could not be performed for this parameter.

3.2 | Dermo-epidermal junction

The dermo-epidermal junction in human abdominal skin and in all porcine skin samples had an undulating appearance with distinct pillars of dermis rising up between epidermal rete ridges (Figures 1 and 3a). The length of the human dermo-epidermal interface was similar to that of the pig's abdomen, flank and back ($p = 0.054$, 0.463 and 0.073, respectively (Table 2). The length of the dermo-epidermal

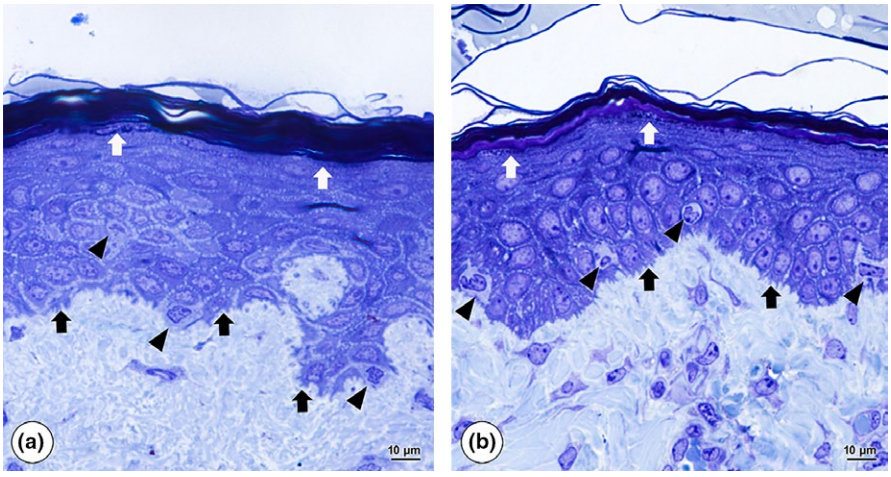

FIGURE 2 Light microscopy of (a) human abdominal skin and (b) porcine skin from the ear, stained according to Richardson. Black arrowheads, cells with clear cytoplasm in the stratum basale and stratum spinosum; black arrows, dermo-epidermal junction with finger-like projections of basal cells; white arrows, keratinohyalin granules in the stratum granulosum

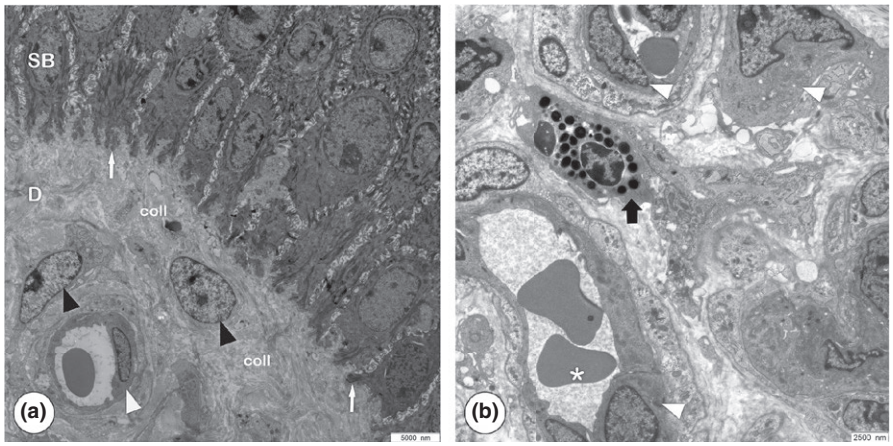

FIGURE 3 Transmission electron microscopy of pig skin. Image (a) presents the interface between stratum basale (SB) and the dermis (D). Image (b) shows a mast cell close to a capillary. White arrows, dermo-epidermal junction, white arrowhead, endothelial cells of capillaries; asterix (*), erythrocyte; black arrow, mast cell; black arrowheads, fibroblasts; coll, collagen

TABLE 2 Measurements of different parameters (mean ± *SD*) of human and pig dermis

Parameter	Human abdomen (*n* = 3)	Pig caudal abdomen (*n* = 8)	Pig back (*n* = 8)	Pig ear (*n* = 8)	Pig flank (*n* = 8)
Length of dermo-epidermal interface (μm)	1419.5 ± 46.7	1213.3 ± 61.0	1228.7 ± 44.3	1131.2 ± 39.4	1343.1 ± 70.1
Thickness of dermis (μm)	1831.5 ± 29.6	1167 ± 78.8	1620 ± 122.2	952.4 ± 127.0	1877.0 ± 68.5
Epidermal-dermal thickness ratio	1:19	1:16	1:21	1:14	1:24
Number of blood vessels/mm^2	104.2 ± 13.4	223.3 ± 15.7	211.5 ± 22.4	208.9 ± 14.9	217.1 ± 10.8
Number of hair follicles/cm^2	7.0 ± 3.3	12.9 ± 1.9	49.4 ± 8.8	30.7 ± 4.3	35.4 ± 8.0
Number of sweat gland clusters/cm^2	15.7 ± 1.1	16.2 ± 3.4	22.8 ± 5.0	30.8 ± 8.1	19.0 ± 3.1
Number of arrector pili muscles/cm^2	13.1 ± 3.4	11.6 ± 6.9	161.0 ± 48.0	13.1 ± 3.4	91.9 ± 27.0

interface in skin of the pig's ear was significantly longer ($p = 0.009$) (Table 2).

3.3 | Dermis

The two layers of the dermis, that is the outer narrow stratum papillare and the inner wider stratum reticulare, were visible in skin of both species. No significant difference was found between dermal thickness of human abdominal dermis and those from the pig's flank and back regions ($p = 0.999$ and 0.789, respectively). In contrast to this, human abdominal dermis was significantly thicker than those from the pig's ear and abdominal regions ($p = 0.001$ and 0.012, respectively).

The epidermal-dermal thickness ratio of human abdominal skin differed significantly to that of the pig from the ear ($p = 0.048$), but not to that from the flank ($p = 0.519$), back ($p = 0.897$) and caudal abdomen ($p = 0.379$) (Table 2).

Volkmann-Strauss staining allowed clear identification of a network of collagenous and elastic fibres in the dermis. Whereas collagenous fibres were of comparable sizes and distributions in all samples, this was not true for elastic fibres. In the human skin, more and thicker elastic fibres were seen than in any of the porcine samples. Compared to human skin, elastic fibres were particularly thin and sparse in porcine skin from the ear; however, no quantitation was done.

The subpapillary vascular plexus was well developed in the dermal papillary dermis in both species. Small non-myelinated nerves were scattered throughout the dermis of both species.

Ultrastructural examination of human and pig dermis showed fibroblast-like cells with irregularly shaped nuclei and with an abundance of rough endoplasmic reticulum, mitochondria and Golgi apparatus. Blood vessels were lined with endothelial cells in which Weibel Palade bodies were observed occasionally. In pigs only, occasionally, cells with an abundance of electron-dense granules in their cytoplasm, probably mast cells, were found near blood vessels (Figure 3b).

Endothelial cells of blood vessels were identified by CD31 immunolabelling in both species allowing counting of the number of blood vessels (Figure 4). The number in human dermis was significantly lower than found in the pig (ear $p = 0.002$; flank $p = 0.001$; back $p = 0.002$; caudal abdomen $p = 0.001$; Table 2).

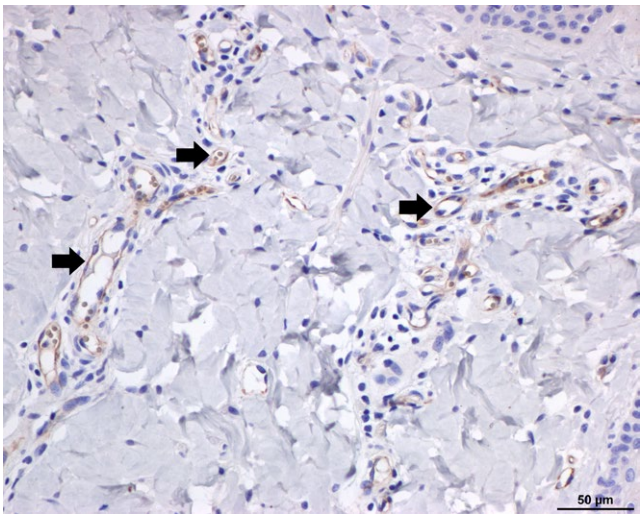

FIGURE 4 Light microscopy of the dermis of porcine abdominal skin labelled with the endothelial marker anti-CD31 (brown colour), arrows indicate representative blood vessels

Hair follicles were found in both human and pig dermis. Light microscopically, the morphology and layering of pig hair follicles were similar to that of human hair follicles. The number of hair follicles in the human abdominal skin was significantly lower than that counted in the flank ($p = 0.023$) and the back ($p = 0.001$) regions of the pig (Table 2). The number of hair follicles in human and porcine abdominal and ear skin was similar. Hair follicles in both humans and pigs were accompanied by sacculated sebaceous glands.

Arrector pili muscles were found in significantly smaller numbers in human skin than in the porcine skin of the back ($p = 0.008$), and to a lesser extent, but not statistically significant, of the flank. The number of arrector muscles from the ear and the caudal abdomen did not differ significantly from the number found in human abdominal skin.

In humans eccrine and in pigs, apocrine sweat glands were found. Both types of sweat glands were arranged in clusters in the reticular dermis; in pigs, they extended to the subcutis. Glands were surrounded by myoepithelial cells in both species. Their number in human abdominal skin was lower than that found in pig skin, but the differences were not statistically significant (Table 2).

3.4 | Skin permeability studies

The permeation for back skin after 24 hr was so low to the effect that no valid apparent permeability (P_{app}) values could be determined. For ear, flank and abdominal skin, no significant differences were observed. The P_{app} value was $0.27 \pm 0.3 \times 10^{-6}$ [cm/s] for ear skin, $0.23 \pm 0.17 \times 10^{-6}$ [cm/s] for skin from the flank and $0.1 \pm 0.22 \times 10^{-6}$ [cm/s] for abdominal skin ($n = 4$).

4 | DISCUSSION

A domestic species and experimental animal that in many respects is considered to be similar to the human is the pig (Debeer et al., 2013; Stricker-Krongrad et al., 2016a; Sullivan, Eaglstein, Davis, & Mertz, 2001). In the present study, four different regions of German Landrace pig skin were examined by light microscopy, morphometry, immunohistochemistry and transmission electron microscopy to identify a region that is most similar to human abdominal skin. Our findings corroborate the similarity in qualitative cellular and ultrastructural characteristics between human abdominal and porcine skin. These include the shapes of keratinocytes as well as their nuclei in the different epidermal layers, the structure of cell contacts between the keratinocytes and between the corneocytes, the architecture of the stratum corneum and the presence of Weibel Palade bodies in dermal capillary endothelial cells.

In our study, the thickness of the human abdominal epidermis was similar to that reported by Qvist, Hoeck, Kreilgaard, Madsen, and Frokjaer (2000). The reason for discrete differences in epithelial thickness measurements may be due to different sources of skin samples and the utilisation of different fixation procedures. It is known that histological methods including fixation and even staining may alter proportions of skin specimens and thereby influence measurements. Fixation alone followed by paraffin wax embedding may result in up to 33% shrinkage (Chatterjee, 2014). Interestingly, Therkildsen et al. (1998) comparing epidermal thickness in paraffin and cryostat sections found that "it has been stated that thickness values from conventional histological sections are likely to be too low due to the shrinkage of the samples during the process of dehydration but this was not confirmed in the present study."

In our study, the most striking similarities between human abdominal skin and porcine skin from the ear, flank, back and caudal abdomen were the thicknesses of the epidermal stratum basale, stratum spinosum and stratum granulosum. The stratum basale consisted of a single cell layer and had similar thicknesses in the human, and all four porcine locations examined. Mitotic cells were uncommon in the stratum basale and in the adjacent stratum spinosum of all samples examined. Indeed, the number was so low that a statistical calculation could not be performed. This can probably be attributed to the fact that keratinocytes undergo mitosis and cytokinesis at night (Janich et al., 2013; Scheving, 1959).

Although the stratum spinosum and the stratum granulosum were of similar thickness in both species, there were fewer cell layers in human skin. The latter result is corroborated by the study of Debeer et al. (2013) who also found 1–2 cell layers in the stratum granulosum in skin samples of young Seghers Hybrid pigs.

We found that the stratum corneum thickness of the human was significantly greater than in all porcine samples. Qvist et al. (2000) found similar values in human abdominal and breast skin. Likewise, Liu et al. (2010) reported similar thicknesses of the stratum corneum in human back skin and young Bama minipigs, although there was no significant difference in the number of cell layers. As corneocytes have a constant thickness throughout the stratum corneum (Jacobi et al., 2005), it can be concluded that human corneocytes are thicker than porcine ones. This is an important result as the stratum corneum is the main barrier to the penetration of exogenous substances into the skin because the

rate of diffusion and thus penetration of molecules as well as reservoir properties are dependent upon the length of the diffusion pathway (Abd et al., 2016; Jacobi et al., 2005). The length of the diffusion pathway is equated to the thickness of the stratum corneum (Holbrook & Odland, 1974); however, the number of layers of the stratum corneum may influence solute permeation possibly due to lateral bilayer diffusion (Johnson, Blankschtein, & Langer, 1997). These histological differences could be an explanation of the results of the in vitro permeation assay using the OECD reference substance testosterone. Permeation rates of testosterone ranked: ear skin > flank >abdominal skin. No permeation of the lipophilic substance testosterone was observed for back skin. Because of the high affinity of testosterone to the stratum corneum and viable epidermis, the lacking permeation of porcine back skin is probably due to the comparably high number of stratum corneum layers and high thickness of its epidermis (Table 1). In conclusion, with respect to its barrier properties, ear and flank skin from the pig is most comparable to human skin, which has a P_{app} value according to the literature of 0.5 ± 0.467 x 10^{-6} [cm/s] (*n* = 4) (Schäfer-Korting et al., 2008).

The dermis accounts for most of the skin thickness. In the present study, it was found that dermal thicknesses of human abdominal and porcine flank and back regions were similar, whereas that of the porcine abdomen and ear were thinner. This is corroborated by Jacobi et al. (2007), who found dermal thickness to be approximately the same in the porcine and human back. According to Forbes (1969), Montagna and Yun (1964), Sullivan et al. (2001) and Vardaxis, Brans, Boon, Kreis, and Marres (1997), the dermal blood supply of pigs is similar to that of humans. However, Debeer et al. (2013) considered the porcine dermal vascular plexus to be more complex than that seen in the human abdomen. They found one capillary vessel per dermal papilla in humans compared to several capillaries or small clusters of vessels in each porcine dermal papilla. Our study also found a significantly higher number of blood vessels in the porcine samples.

The length of the dermo-epidermal interface of the human abdominal skin is similar to that of the porcine abdomen, flank and back. This is a parameter that is consistent in human and three out of the four porcine skin locations tested. It is important because the dermo-epidermal interface aids in barrier function, both from and into the epidermis, allows for firm attachment of epidermis to dermis and serves as a base for re-epithelialisation in wound healing (Haschek, Rousseaux, Wallig, Bolon, & Ochoa, 2013).

The hair follicle is an important storage and penetration organ as it provides diminished barrier properties allowing fast access for smaller substances into skin layers and acts as a potential reservoir for topically applied substances (Patzelt & Lademann, 2015). Otberg et al. (2004) found massive body region-dependent hair follicle characteristics in humans and concluded that testing of topically applied drugs and cosmetics on different skin areas is mandatory. Our study identified no significant difference between the number of hair follicles in human and porcine abdominal and ear skin. Sumena et al. (2010) who examined abdominal skin in Large White Yorkshire pigs reported similar numbers. In accordance with our results for human and porcine abdominal skin, Stricker-Krongrad, Shoemake, Liu, Brocksmith, and Bouchard (2016b) found that humans and minipigs have approximately 11 follicles per cm^2. They reviewed the importance of minipigs in dermal safety assessment and underlined the significance of hair follicles and sebaceous glands as pathways for percutaneous penetration of topically applied lipophilic drugs via the pilosebaceous route.

Our results show that the number of arrector muscles from porcine abdomen and ear skin did not differ significantly from the number found in human abdominal skin. Debeer et al. (2013) found a strikingly similar antigen pattern in arrectores pili of both species as several antibodies used to detect normal human skin structures (including desmin, caldesmon, actin and alpha smooth-muscle actin) showed equivalent immunoreactivity on porcine skin. These immunoreactivity observations may be of interest for future studies by stem cell researchers because very recently, arrector pili muscles have been found to bridge the follicular stem cell niche and the interfollicular epidermis (Torkamani, Rufaut, Jones, & Sinclair, 2017).

A considerable dissimilarity between human and porcine skin is that humans have mostly eccrine sweat glands, whereas pigs have mostly apocrine glands. The latter extend into the porcine subcutis and do not significantly contribute to thermoregulation (Debeer et al., 2013). Porcine eccrine sweat glands are limited to the planum nasolabiale and carpus, whereas human apocrine glands are only found in the axillary and rectogenital areas (Rittié, Sachs, Orringer, Voorhees, & Fisher, 2013). Sweat glands play a role in transdermal drug delivery, and researchers hope to exploit sweat glands to enhance drug penetration (Palmer & DeLouise, 2016). In our study, the number of eccrine sweat glands in human abdominal skin was lower than the number of apocrine ones found in pig skin, but the differences were not statistically significant. Despite the functional differences between the two glandular types, there is a similarity in that both, human eccrine and porcine apocrine sweat glands, have a key function in re-epithelialisation of skin wounds (Rittié et al., 2013).

In our study, in pigs, occasional cells with an abundance of electron-dense granules in their cytoplasm, probably mast cells, were found near blood vessels. Liu et al. (2010), who compared skin from the back of minipigs and humans, emphasised that densities of mast cells that act as regulators in inflammatory processes are similar in both species. Janssens et al. (2005) found differences in the numbers of mast cells between proximal and distal body sites.

The pigs examined by us were between 10 and 11 weeks old. While a pig's age does not correlate readily to human age, morphologically, the skin of young pigs appears to be more similar to human adult skin than does adult pig skin (Liu et al., 2010).

Currently, there are no standardised methodologies for the comparative examination of human and porcine skin. Existing studies have used a variety of sampling sites, ages and pig breeds. To date, many similarities and differences have been found between human and porcine skin. The following parameters were found to be similar in human abdominal skin and porcine skin from at least one or more locations: thickness of the epidermis of all four regions, thickness of

stratum basale, spinosum and granulosum of all four regions, rare mitotic figures in the stratum basale of all four regions, numbers of cell layers in the stratum corneum of all four regions, thickness of the dermis of the porcine back and flank, length of the dermo-epidermal interface of the porcine abdomen, back and flank, epidermal-dermal thickness ratio of porcine abdomen, back and flank, number of hair follicles of porcine abdomen and ear, number of arrector pili muscles of porcine abdomen and ear, number of sweat glands in porcine skin of all four regions. The most striking differences were the significantly thicker stratum corneum and the lower number of blood vessels in human abdominal skin. The stratum corneum is particularly important in the penetration of substances. It can be concluded that for each individual question and study, it has to be carefully checked which part of the porcine skin is most similar to the human one and differences must be considered when selecting an alternative area of skin for use in penetration and absorption studies using porcine skin.

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5. The effect of endothelialization on the epidermal differentiation in human three-dimensional skin constructs – A morphological study

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The effect of endothelialization on the epidermal differentiation in human three-dimensional skin constructs – A morphological study

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Abstract.
INTRODUCTION: Inducing vascularization in three-dimensional skin constructs continues to be difficult. In this study, two variations of human full-thickness skin constructs were examined. Type KCFB consists of keratinocytes (epidermal equivalent) and fibroblasts that were embedded in a collagen matrix (dermal equivalent). Type KCFB-EC consists of keratinocytes as well as fibroblasts and vascular endothelial cells. The epidermal equivalent of KCFB-EC constructs underwent cellular alterations in their differentiation possibly induced by the presence of endothelial cells. The objective of the study was to assess the effect of endothelial cells, i.e., endothelialization of the dermal equivalent on the differentiation of keratinocytes by comparing the morphology and ultrastructure of the two types of skin constructs, as well as to excised normal human skin. Hypothesis: The differentiation of keratinocytes is influenced by the presence of endothelial cells.
METHODS, PATIENTS, MATERIAL: KCFB constructs (keratinocytes, fibroblasts) and KCFB-EC skin constructs (kera-tinocytes, fibroblasts, endothelial cells) were prepared according to Küchler et al. [25]. After two weeks, the skin constructs were processed for analysis by light microscopy (LM) and electron microscopy (TEM), followed by quantitative, semi-quantitative as well as qualitative assessment. For comparison, analysis by LM and TEM of excised normal human skin was also performed.
RESULTS: Both KCFB and KCFB-EC skin constructs and the human skin had all strata of stratified soft-cornified epidermis present. The comparison of the respective layers of the skin constructs brought the following characteristics to light: The KCFB-EC constructs had significantly more mitotic cells in the stratum spinosum, more cell layers in the stratum granulosum and more keratohyalin granules compared to KCFB skin constructs. Additionally, the epidermal architecture was unorganized in the endothelialized constructs and features of excessive epidermal differentiation appeared in KCFB-EC skin constructs.
CONCLUSION: The endothelialization of the dermal equivalent caused changes in the differentiation of the epidermis of KCFB-EC skin constructs that may be interpreted as an unbalanced, i.e., uncontrolled or enhanced maturation process.

Keywords: Full-thickness skin constructs, human skin, epidermis, keratinocyte, fibroblast, endothelial cell

1. Introduction

The skin covering of our bodies is an excellent barrier against environmental influences and prevents water loss from the human body. It is a multi-layered organ that consists of an outermost epidermis and

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a subjacent dermis. Due to its complex composition and organization the epidermis has an important protective role against e.g. toxic substances, microbe invasion and dehydration [34, 43]. The epidermis is in a state of continuous renewal and the mechanisms responsible for triggering epidermal morphogenesis are the particular focus of interest for several lines of research both in industry and academia, e.g. medical and pharmaceutical research as well as in the cosmetic industry [53].

In human skin research over recent years a growing emphasis has been put on developing alternatives to animal experimentation. Specific reasons for this are; the complete ban of animal testing in cosmetic research, which came into effect just recently, as well as the fact that the 3R concept (refinement, reduction, replacement of animal experiments) has become of increasing relevance. Reconstructed epidermis constructs and full-thickness skin constructs of healthy skin have become well accepted as potential alternatives to animal use for toxicological and basic preclinical studies [14, 26]. Recently, the development of constructs of diseased skin states have come into focus [10, 32, 52]. Several organotypic *in vitro* systems are now commercially available or have been developed as in-house skin constructs [10, 25, 44].

Most skin constructs consist of co-cultured skin-derived cells, particularly keratinocytes which constitute the epidermal equivalent and fibroblasts, that are embedded in a three-dimensional biopolymer scaffold presenting the dermal equivalent. However, in order to more closely mimic the architecture of human skin, it is essential to include additional cellular elements that are residents of the skin such as endothelial cells (EC). As many functions of the skin rely upon the multifaceted interplay of the different cell types within this organ, it is a demanding task to create skin constructs that are closely representative of the complex composition of human skin [48]. These ideas conform to ontogenic investigations, which prove that the morphogenesis and function of the cells of an organ are entirely dependent on cell-cell as well as cell-matrix interactions, including the effects of specific extruded extracellular matrix components and soluble factors [3, 41]. Without their typical environment, cells lose their organotypic characteristics. During their differentiation, the keratinocytes undergo metamorphosis and develop a complex epidermal specific barrier system [11]. In the case of skin research, the architecture and permeability of that barrier system is the focus of interest, therefore the maturation and differentiation of the epidermal keratinocytes into corneocytes plays a pivotal role. Only when grown in an adequate inductive milieu that includes the elevation of the epidermal layer to the air-liquid interface, will the keratinocytes exhibit the desired maturation features *in vitro* [50, 53].

Furthermore the inclusion of blood vessels for tissue engineering is an area of active investigation. However, it is still decidedly difficult to form capillary-like networks within these constructs [14, 24, 31, 42, 49]. While the molecular aspects of angiogenesis in the skin constructs are being investigated in depth, little to no attention is paid to the physical interaction, communication and exchange between the cells themselves [28]. Pilot studies in our laboratory using "live cell imaging" revealed the exchange of corpuscular material between endothelial cells during the critical phases of angiogenesis [8]. Moreover, in a recent study [22], we found strong evidence that lung carcinoma cells when co-cultured with lung endothelial cells modify their cellular and molecular features that encourages alternative ways of providing their blood supply.

In order to optimize a 3D in-house skin construct, endothelial cells were added to well-established skin constructs [25]. Results revealed changes in the epidermal layer of the endothelialized skin constructs. In order to gain a deeper insight, the aim of the current study was to identify and quantify these changes in the epidermal layer, which are obviously due to the influences of the endothelial cells. Consequently, in the present project, the morphology and ultrastructure of two types of skin constructs were analyzed, namely skin constructs without endothelial cells (KCFB) and skin constructs with endothelialized dermal equivalent (KCFB-EC). Results were compared to findings of normal human skin.

2. Material and methods

Qualitative and quantitative analyses of the epidermal morphology and architecture of KCFB skin constructs and KCFB-EC skin constructs as well as of samples from excised human skin were performed.

2.1. Preparation of skin constructs without endothelial cell layer

The *in vitro* skin constructs were generated according to previously published procedures [25, 52]. Briefly, primary keratinocytes and fibroblasts were isolated from juvenile foreskin, the remnant tissue of circumcision surgery (with permission). Fibroblasts, fetal calf serum (Biochrom, Berlin, Germany) and bovine collagen I (PureCol, Advanced BioMatrix, San Diego, USA) were brought to neutral pH and poured into 3D cell culture inserts with a growth area of 4.2cm^2 (BD Biosciences, Heidelberg, Germany) and cultivated for 2 hours at 37°C. Keratinocyte growth medium (KGM, Lonza, Walkersville, USA) was added on top of the collagen-matrices and after 2 hours the primary human keratinocytes were added on top. After 24 hours, the skin constructs were lifted to the air-liquid interface and a specific differentiation medium was added. The skin constructs were cultured for 14 days with media changes every other day.

2.2. Preparation of skin constructs with endothelial cell layer

For the implementation of an endothelial cell layer in the *in vitro* skin construct, the preparation was adjusted slightly. After solidification of the collagen-fibroblast-matrix, the cell culture inserts were turned upside-down and human umbilical vein cord endothelial cells (HUVEC) were seeded onto the basal side of the insert membranes. After 1 hour, the inserts were placed into a 6-well companion plate (BD Biosciences, Heidelberg, Germany), containing endothelial growth medium (EGM-2, Lonza, Walkersville, USA). KGM was added on top of the collagen-matrices and after 2 hours the primary human keratinocytes were added. After 24 hours, the skin constructs were lifted to the air-liquid interface and a specific differentiation medium was added. Therefore, the proprietary differentiation medium was mixed (1:1) with EGM-2 and supplemented with 1.36 mM 2-phospho-L-ascorbic acid (Sigma-Aldrich, Munich, Germany) [46]. The skin constructs were cultured for 14 days with media changes every other day.

2.3. Preparation of human skin

To compare the skin constructs including endothelial cells with the *in vivo* situation, excised human abdominal skin from healthy donors undergoing plastic surgery was used (all patients signed informed consent). Following removal of subcutaneous fat tissue the skin was stored at –20°C for up to 6 months until usage. For preparation, the skin was thawed, a disc of 2 cm diameter was punched and afterwards fixed with Karnovsky solution (7.5% glutaraldehyde and 3% paraformaldehyde in phosphate buffered saline).

2.4. Sample preparation for light and electron microscopic examination

Four batches of each skin construct and samples of human skin from three different donors were processed using the following methodologies. Samples were fixed in Karnovsky solution for 4 hours, washed in 0.1M cacodylate buffer (cacodylic acid sodium salt trihydrate, Roth, Karlsruhe, Germany), incubated in 1% osmium tetroxide (Chempur, Karlsruhe, Germany) for 120 min, dehydrated in a descending series of

ethanol and washed in intermedium propylene oxide (1,2 Epoxypropan; VWR, Germany). Subsequently, samples were embedded in a mixture of agar 100 (epoxy resin), DDSA (softener), MNA (hardener) and DMP 30 (catalyst) (Agar Scientific, Stansted, GBR). Polymerization was done at 45°C and 55°C, each for 24 hours. Semi- and ultrathin sections were cut by an ultra-microtome Reichert Ultracut S (Leica, Wetzlar, Germany). Samples were divided into four equal parts and one level cut from each. Semi-thin sections (0.5 μm) were stained with modified Richardson solution [39] for 45 seconds on an electric hotplate adjusted to 80°C. Sections were examined under light microscopy (Olympus CX21, Olympus, Stuttgart, Germany). Ultrathin (80 nm) sections were mounted on nickel-grids (Agar Scientific, Stansted, GBR) and contrasted with lead citrate and uranyl acetate.

2.4.1. Light microscope

Pictures from the four sections of each specimen were taken and recorded using an Axioskop light microscope (Carl Zeiss, Oberkochen, Germany), DS-Ri1 digital camera (Nikon, Düsseldorf, Germany) and NIS-Elements Version 3.0 imaging program (Nikon, Düsseldorf, Germany). For quantitative and morphological evaluation, pictures were taken from semi-thin sections at magnifications 10×, 20×, 40× and 100×. Five pictures from different areas were taken per magnification. Quantitative analysis was undertaken using the 20× magnification images. These included counting the number of cellular layers, of mitotic cells in the stratum basale and spinosum, of keratohyalin granules in the stratum granulosum. The number of keratohyalin granules in the stratum granulosum was counted in ten cells of each sample (100× magnification) by a NIS-Elements Version 3.0 imaging program (Nikon, Düsseldorf, Germany). In addition measurements of the thickness of each stratum of the epidermis and epidermal equivalents were taken. Qualitative assessment of the morphology of cells and the tissue architecture was undertaken at all magnifications.

2.4.2. Electron microscope

Semi-quantitative analysis of ultra-structural morphology was done by electron microscope (Zeiss EM 903, Oberkochen, Germany).

Five cells were analyzed for each morphological feature. Photos were taken and edited using an Adobe Photoshop Program (Adobe System, Unterschleissheim, Germany).

2.4.2. Statistics

The independent-samples *T*-Test and one way analysis of variance (ANOVA test) of the software package IBM SPSS 22 (IBM Deutschland GmbH, Ehningen, Germany) were used for data analyses. $P \leq 0.05$ was defined as the level of significance.

3. Results

Both KCFB and KCFB-EC skin constructs and the human skin had all strata of stratified soft-cornified epidermis present: a) stratum basale, in the constructs defined as the layer of cells with direct contact to the dermal equivalent and mitoses; b) stratum spinosum, defined as the cells with spinous processes and with mitosis besides the stratum basale; c) stratum granulosum defined as the layer with intracellular granules and d) stratum corneum, defined as layer with signs of cellular degeneration and loss of nuclei using transmission electron microscopy a basement membrane at the epidermal-dermal interface was detectable only in the skin, but not in the skin constructs.

3.1. Statistic evaluations

The KCFB-EC constructs had significantly greater numbers of mitotic cells in the stratum spinosum, more cell layers in the stratum granulosum and greater numbers of keratohyalin granules compared to KCFB skin constructs.

In the stratum spinosum mitotic cell numbers, with a mean of 11 (2–22) mitoses for the KCFB-EC skin constructs, were significantly ($p = 0.013$) greater than in the KCFB constructs (5.5; 2.5–15). In the stratum basale the number of mitoses of both skin construct types revealed no significant difference ($p = 0.966$): KCFB skin construct had a mean of 6 (3–9) mitotic cells and the KCFB-EC skin constructs a mean of 6.5 (2–13).

Significantly more cell layers (p-value of 0.025) were present in the stratum granulosum of KCFB-EC constructs, i.e., 2.5 (1–4). In KCFB, 2 (1-2) layers were counted. In contrast, the number of the cell layers in the stratum spinosum ($p = 0.970$; KCFB 9 (6–13), KCFB-EC 10 (5–17)) and in the stratum corneum ($p = 0.993$; KCFB 4 (3–8), KCFB-EC 5 (2–5)), showed no significant differences (Fig. 1a–c).

The cell layers of the stratum spinosum and stratum corneum of the skin constructs were readily distinguishable. The number of layers of the stratum granulosum in the skin samples could not be counted, as it was not possible to distinguish the cell borders using light microscopy. Significant differences in the number of layers in the skin constructs and the normal skin ($p < 0.001$) were observed in the stratum spinosum: human skin 4.5 (3–5) (KCFB 9 (6–13), KCFB-EC 10 (5–17)) and also in the stratum corneum: human skin 13 (12–15) with a $p < 0.001$ ((KCFB 4 (3–8), KCFB-EC 5 (2–5)).

The epidermal thicknesses of the two types of skin constructs were not significantly different. The average thickness of all epidermal strata was 105.23 μm (65.10–159.84) in KCFB samples and 119.51 μm (44.18–206.92) in KCFB-EC ($p = 0.752$). The stratum basale had a mean thickness of 19.37 μm (10.15–32.81) in KCFB, and 16.43 μm (11.03–20.37) in KCFB-EC ($p = 0.199$). The stratum spinosum had a mean thickness of 70.66 μm (43.91–140.07) in KCFB and 82.04 μm (25.26–162.25) in KCFB-EC ($p = 0.782$). The thickness of the stratum granulosum in KCFB had a mean of 7.77 μm (3.05–11.19) and in KCFB-EC a mean of 12.83 μm (4.11–24.91; $p = 0.169$). The average thickness of the stratum corneum was 10.32 μm (4.34–22.06) in KCFB and 11.10 μm (4.71–25.41) in KCFB-EC ($p = 0.933$).

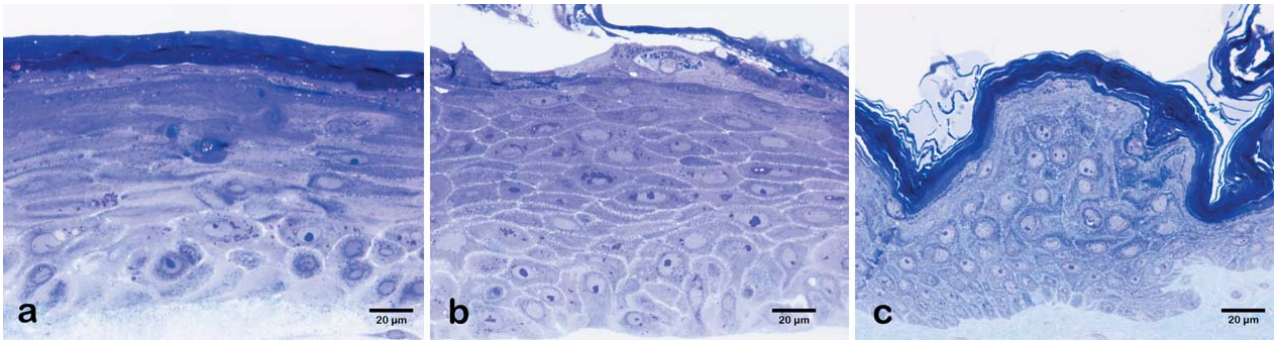

Fig. 1. Light microscopy of semi-thin sections where; (a) human full-thickness skin construct KCFB (keratinocytes/fibroblasts), (b) endothelialized human full-thickness skin construct KCFB-EC (keratinocytes/fibroblasts, endothelial cells) and (c) normal human skin. In both KCFB and KCFB-EC skin constructs and the human skin all strata of stratified soft-cornified epidermis are present. The KCFB-EC construct (b) has significantly greater numbers of mitotic cells in the stratum spinosum and more cell layers in the stratum granulosum.

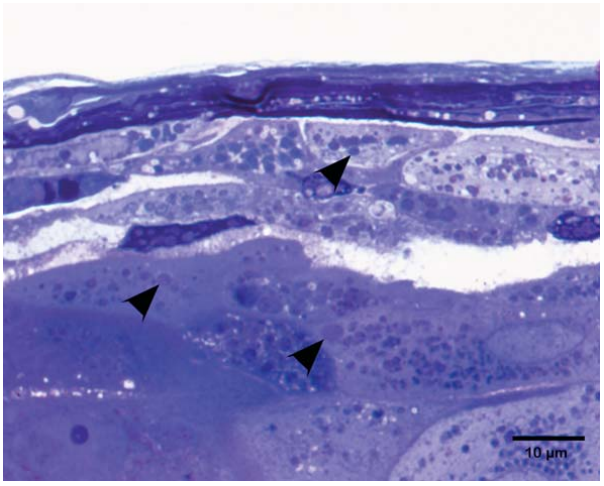

Fig. 2. Light microscopy of a semi-thin section of an endothelialized construct (KCFB-EC) demonstrating keratohyalin granules (arrowhead) present in the stratum granulosum.

In contrast, the average thickness of all layers of the human skin (58.53 µm, $p = 0.003$) and more precisely its stratum basale (9.56 µm, $p < 0.001$), spinosum (32.42 µm, $p = 0.03$) and granulosum (2.60 µm, $p = 0.01$) were all significantly thinner than those of the skin constructs, except the stratum corneum layer, which was thicker in the human skin 12.40 µm, but not significantly (Fig. 1a–c).

The keratohyalin granule counts of the KCFB-EC constructs yielded 22.88 (15.3–39.9) granules per 10 cells, which was significantly more than those found in the KCFB construct with 13.63 (7.4–18.6) granules (Fig. 2). The variation in the two skin construct types had a *p*-value of 0.01.

3.2. The epidermal architecture was disordered in the KCFB-EC constructs

Some typical features of the human skin such as the presence of hemidesmosomes on the basal side of the basal cells were not observed in the constructs. Furthermore, cellular shapes as well as strata architecture of the skin constructs were different to those of normal human skin. Additionally in both constructs the cells of the upper spinosum layers and of the granulosum cell layers were elongate and flat, while in human skin comparable cells do not flatten before reaching the upper granulosum layers. Furthermore the skin constructs displayed small intra-group variations.

Variations between the endothelialized constructs and non-endothelialized constructs were identified by light microscopy in all strata of the stratified soft-cornified epithelium. Lipid droplets of different size (between 0.417 and 15.253 µm in diameter) were present in all samples and in every single layer of the samples within the keratinocytes. There were more lipid droplets throughout all cell layers of the endothelialized constructs (Fig. 3). Seventy percent of the cells forming the basal layer of both construct types were columnar and the remainders were either cuboidal or irregular in shape. Stratum spinosum cells of both construct types formed spinous processes; however, cell shapes of the constructs differed. In the deeper layers spinosum cells of the KCFB constructs were mostly ellipsoid in shape, whereas those of the upper layers were flat (Fig. 4a). In contrast, the cells within the upper layers of stratum spinosum of the KCFB-EC constructs were flat whereas all other cells of this stratum were polygonally shaped (Fig. 4b). The stratum granulosum cells of the KCFB constructs were homogenously flat (Fig. 4c), in contrast the granulosum cells of the KCFB-EC constructs were irregularly shaped in 50 % of the samples (Fig. 4d).

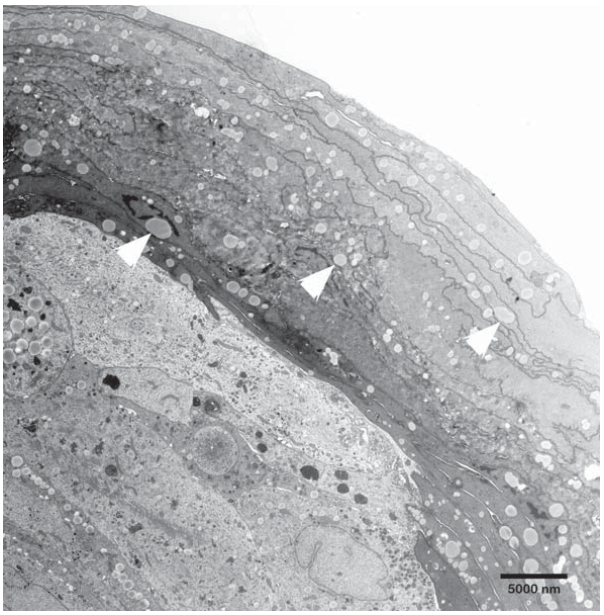

Fig. 3. Transmission electron microscopy image of a KCFB construct. Lipid droplets (arrowheads) occur in every layer of the keratinocytes.

In these KCFB-EC constructs wider intercellular spaces were obvious between the cells of the stratum granulosum (Fig. 4d). In addition, in fifty percent of the samples no cohesion between the strata granulosum and corneum was found. In the stratum corneum of the KCFB constructs the corneocytes formed a tightly packed compartment, whereas gaps were found between the corneocytes in 50% of the KCFB-EC constructs (Fig. 4c, d).

3.3. Transmission electron microscopical findings – specific features of excessive epidermal differentiation appear in KCFB-EC skin constructs

Stratum basale: In both skin constructs, desmosomes were found between neighboring cells in the basal layer and also apically on the sides of adjacent cells. The basal cells developed finger-like protrusions of around 945.74 nm (511.16–1500.59 nm) length on their basal side (Fig. 5a, b). A basement membrane or at least a part of it, i.e., a basal lamina, was not observed. Cytoskeleton fibers accumulated around the nucleus, creating the appearance of a perinuclear ring (Fig. 5c). Occasionally rounded membrane coated organelles with diameter from 900.55 to 3085.79 nm were identified in the cytoplasm. These organelles contained membrane-like structures. *Stratum spinosum:* The spinous protrusions of the stratum spinosum cells were consistently connected by numerous desmosomes. Rounded membrane coated organelles, similar to those described in the basal layer were detected in the stratum spinosum of both constructs and appeared either densely packed with diameters of 379.74–778.79 nm or loosely packed with diameters of 900.55–3085.79 nm (Fig. 6). Furthermore, lamellar bodies (mean diameter 95.57–236.65 nm) were identified in the upper layers of the stratum spinosum as membrane bound structures, containing concentric stacks of thin electron-dense lamellar lipid membranes (Fig. 7a–d). Greater numbers of membrane coated organelles and lamellar bodies were observed in the stratum spinosum of the KCFB-EC skin constructs. Intracellular filaments were seen to accumulate in the periphery of the

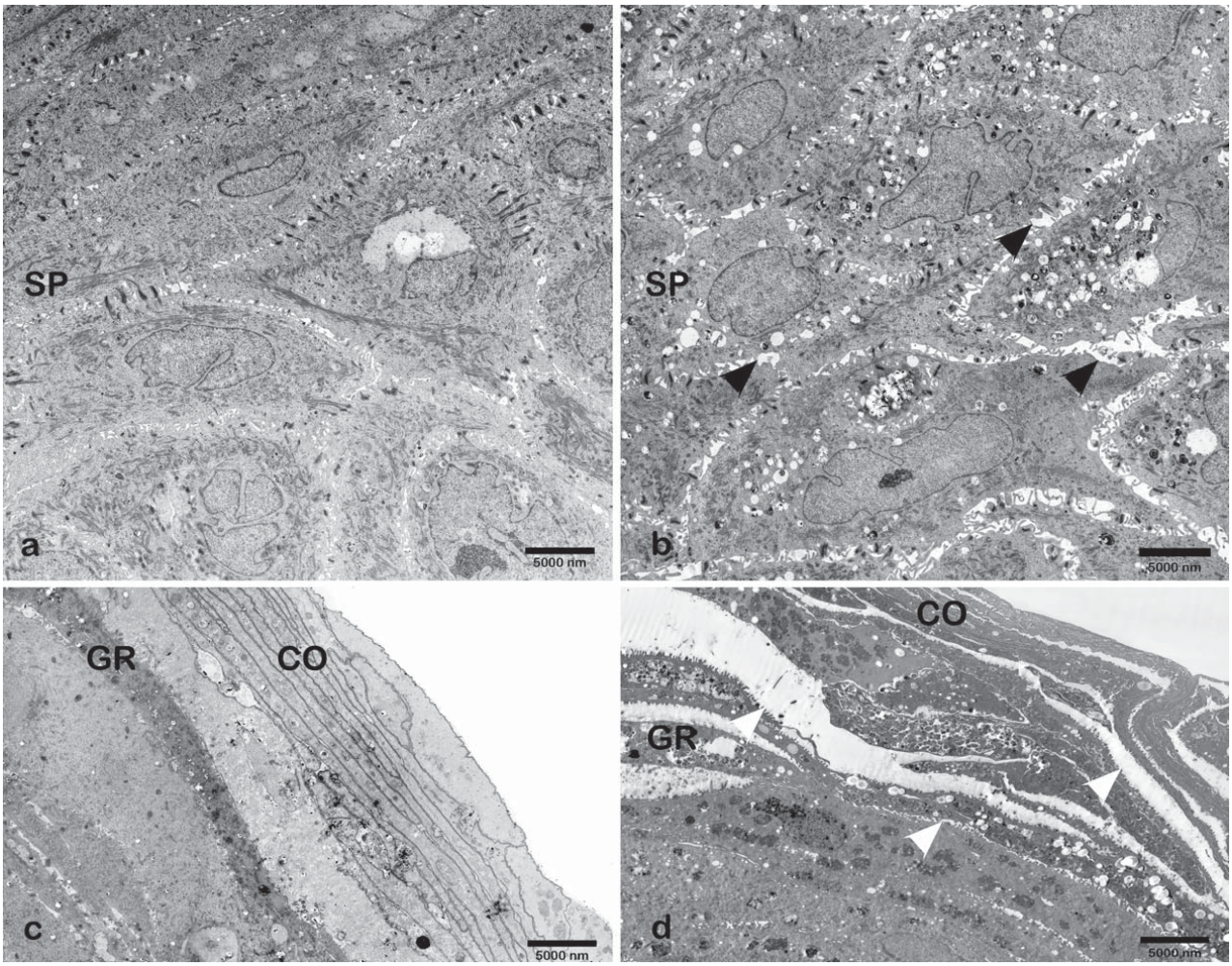

Fig. 4. Transmission electron microscopy (TEM) images of (a, c) KCFB construct and (b, d) endothelialized KCFB-EC construct. In (a) cells of the stratum spinosum (SP) and in (c) cells of stratum granulosum (GR) are of similar shape. In (b) cells within the stratum spinosum and in (d) cells within stratum granulosum are mostly polygonally shaped. Wide intercellular spaces (arrowheads) are obvious between the cells of the stratum spinosum (SP), granulosum (GR) and corneum (CO) in the KCFB-EC constructs (b, d). Note that the corneocytes form a tightly packed compartment in the KCFB constructs (c).

spinous cells. These filaments were connected to desmosomes between the cells of the stratum spinosum in both skin constructs, but to a considerably greater extent in the KCFB constructs. *Stratum granulosum:* In the stratum granulosum cells were densely packed and numerous desmosomes were seen between the cells. Many lamellar bodies were present, especially in the upper layers. At the stratum granulosum/stratum corneum interface lamellar bodies extruding their lipid content were seen frequently (Fig. 8a–c). Significantly more lamellar bodies appeared in the endothelialized constructs. Fewer membrane coated organelles containing membrane-like structures were present in the stratum granulosum. Cables of filaments accumulated beneath the cell membrane in a similar manner in both constructs. Keratohyalin granules were found in both construct types, however, noticeably more in the endothelialized ones. The keratohyalin granules varied from roundish to polygonal in shape, with a mean diameter of 856.63 nm in KCFB and 1046.74 nm in KCFB-EC (Fig. 9a–c). No stellate shaped granules were found.

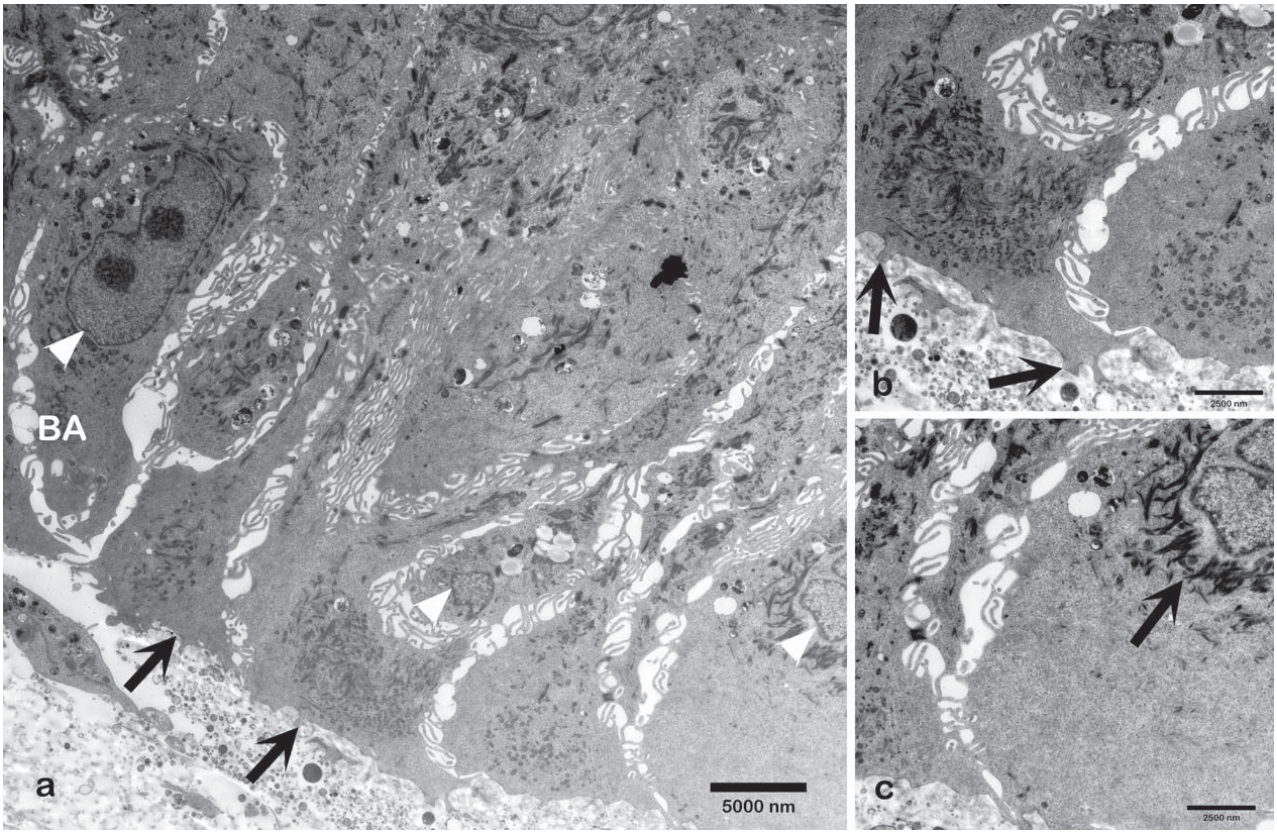

Fig. 5. Transmission electron microscopy images of a KCFB-EC construct where; (a) columnar cells of the basal layer (BA; arrow: basal pole of cells; arrowheads: nucleus). In (b) the cells have finger-like protrusions on their basal side (arrows) and in (c) cytoskeleton fibers accumulate around the nucleus (arrow).

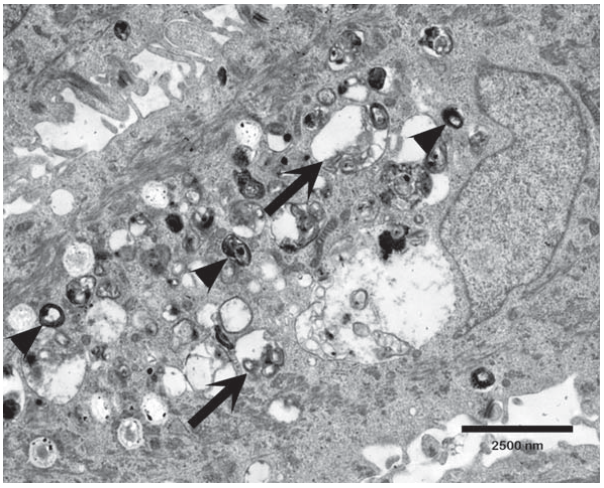

Fig. 6. Transmission electron microscopy image of a KCFB-EC construct. Rounded membrane coated organelles in the stratum spinosum are either densely packed (arrowheads) or loosely packed (arrows) with membranous content.

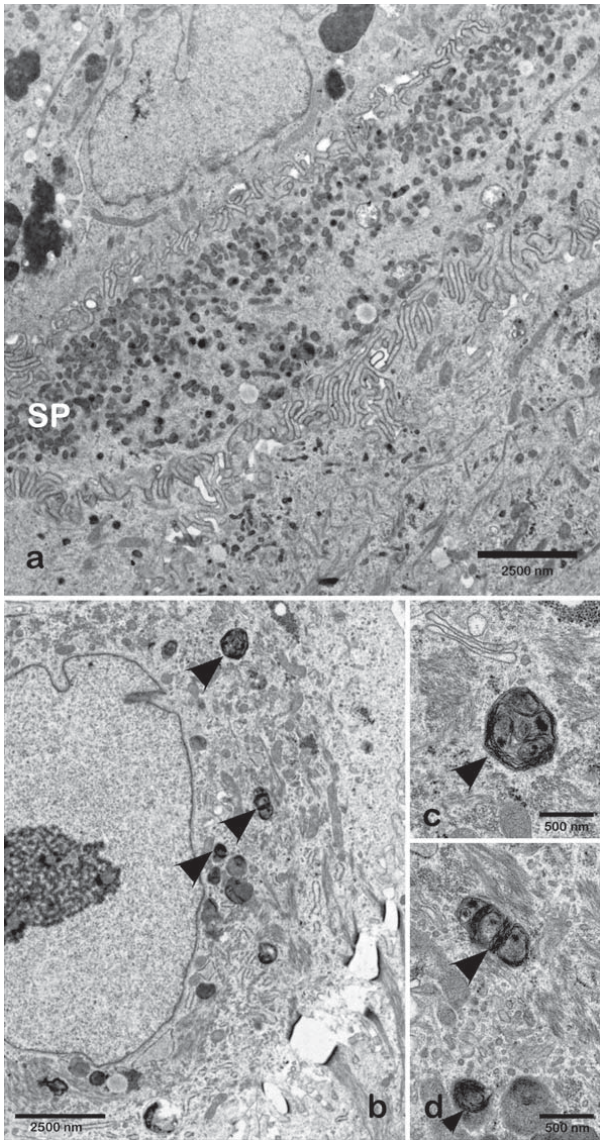

Fig. 7. Transmission electron microscopy images of a KCFB-EC construct. In (a) in the upper layers of the stratum spinosum many lamellar bodies (SP) are present. In (b, c, d) Lamellar bodies (arrowheads) containing concentric stacks of thin electron-dense lamellar lipid membranes (c, d).

Stratum corneum: Ultrastructurally; enucleated, flattened cells, mostly without cytoplasmic organelles (rare exceptions occurred in both types) characterized the cellular part of the stratum corneum. A well-developed cornified envelope characterized the corneocytes of the middle and upper stratum corneum of both construct types (Fig. 10a, b). The corneocytes adhered to each other through their abundant corneodesmosomes (Fig. 10a, b). More corneodesmosomes were found in the non-endothelialized skin constructs compared to the endothelialized ones. Throughout the KCFB construct samples and in 50% of the endothelialized construct samples the cornified cell layers were regularly and tightly packed, whereas in the other 50% of the endothelialized constructs wide intercellular spaces were seen. Intercellularly, a lipid-film organized into lamellar lipid bilayers, was seen in both construct samples (Fig. 11a, b).

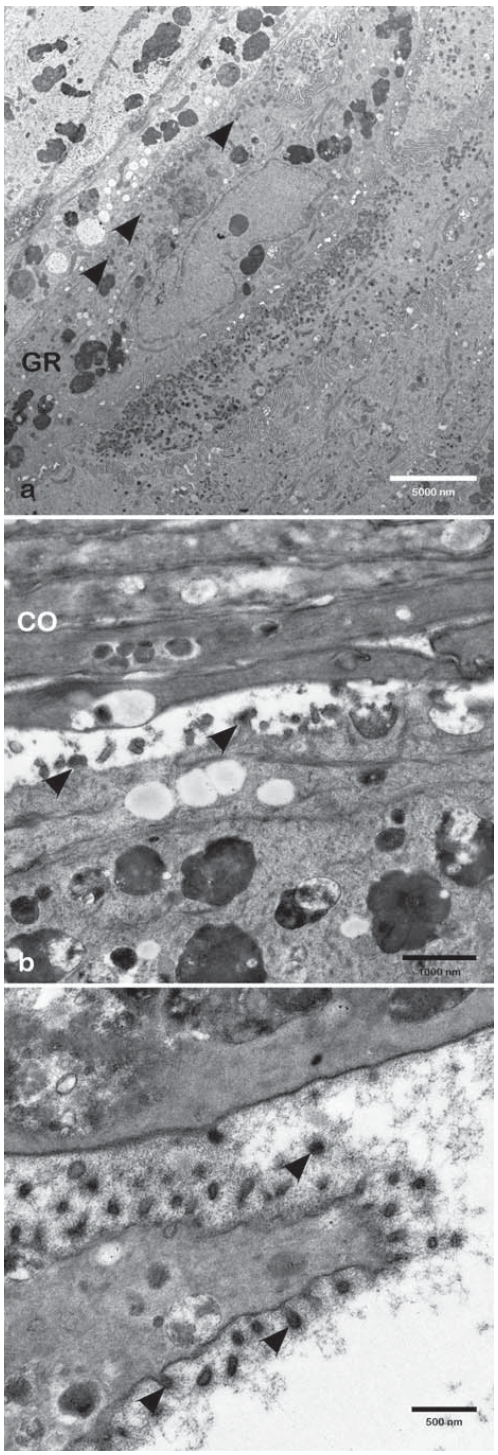

Fig. 8. Transmission electron microscopy images of a KCFB-EC construct. In (a) within the cells of the stratum granulosum (GR) lamellar bodies (arrowheads) are present. (b, c) Lamellar bodies extruding their lipid content (arrowheads) into the stratum granulosum (GR)/stratum corneum (CO) interface.

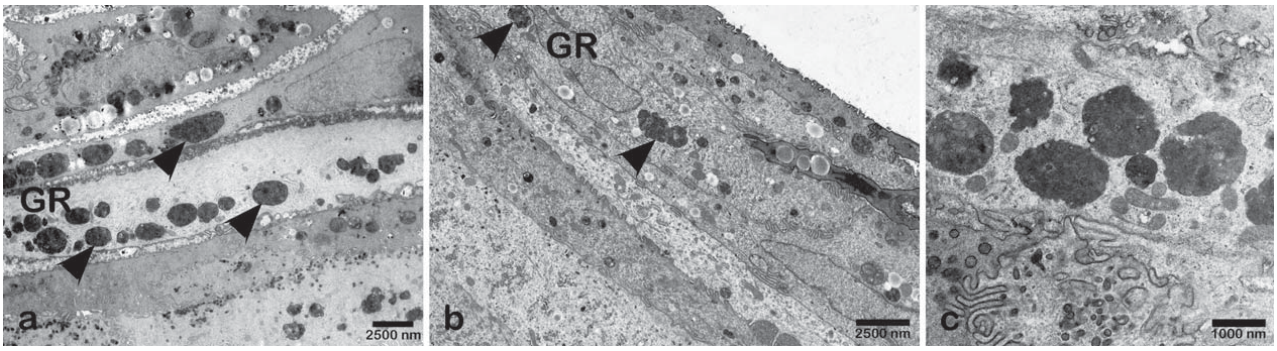

Fig. 9. Transmission electron microscopy image of KCFB and KCFB-EC constructs. More keratohyalin granules (arrowheads, GR = stratum granulosum) are found in the endothelialized constructs (a) compared to the non-endothelialized ones (b). The keratohyalin granules vary from rounded to polygonal in shape (c).

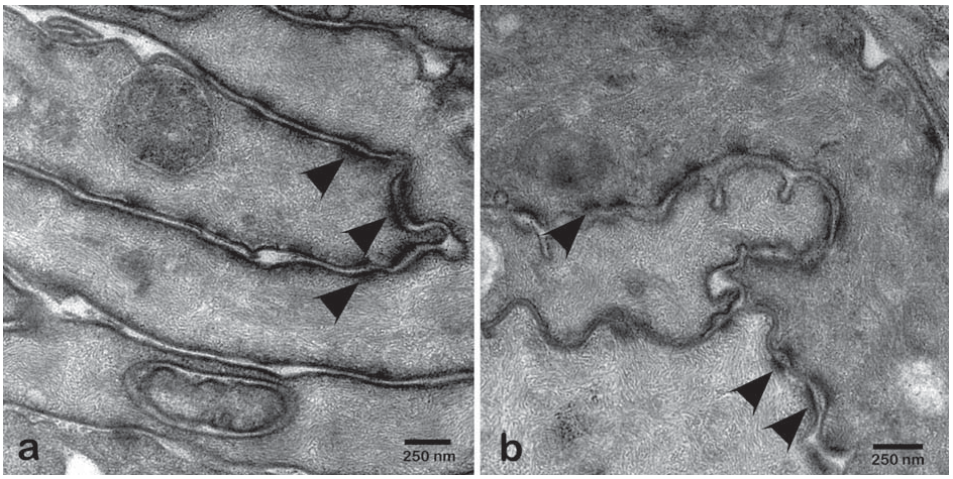

Fig. 10. Transmission electron microscopy images of KCFB constructs. Enucleated, flattened corneocytes which adhere to each other through corneodesmosomes (arrowheads) characterize the cellular part of the stratum corneum. The corneocytes are packed with highly organized keratin filaments.

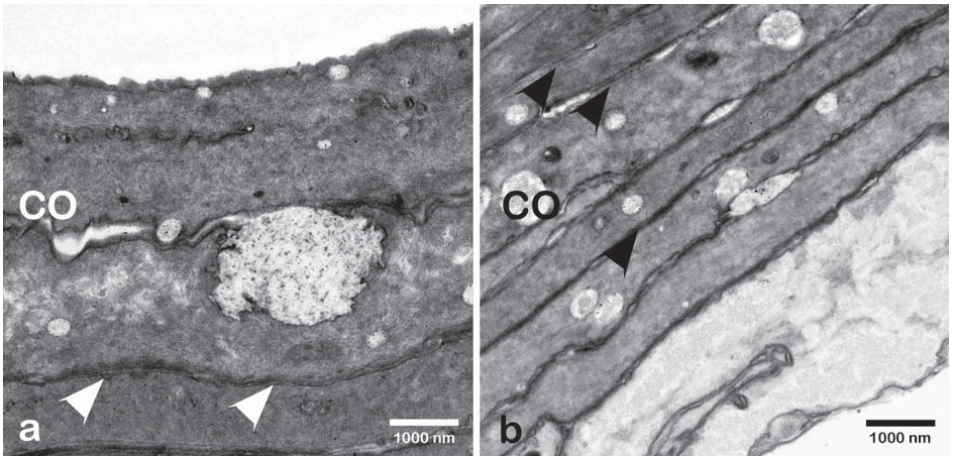

Fig. 11. Transmission electron microscopy images of KCFB constructs. In the intracellular compartment of the stratum corneum (CO) a lipid-film organized into lamellar lipid bilayers is detectable (arrowheads).

4. Discussion

In an attempt to optimize a 3D in-house skin construct by adding endothelial cells (EC), changes in the epidermal architecture of the endothelialized skin constructs were observed. The current study aimed at identifying the morphological characteristics and properties of the cells and layers in the endothelialized versus non-endothelialized constructs on a qualitative and quantitative basis. Results gained on a light-microscopical and ultrastructural level were also compared to findings of normal human skin.

4.1. Distinctive signs of keratinocyte differentiation and keratinization in both skin constructs

The stratum corneum represents the last differentiation stage of a complicated maturation process in the epidermis. It provides an important barrier function that is impermeable to diverse harmful insults, by presenting a wall of flat, enucleated cells that are: 1. filled with highly organized keratin filaments which are cross-linked by epidermal specific proteins, forming a cornified envelope, 2. embedded in a precisely organized lipid matrix and 3. connected by corneodesmosomes [5, 27, 37]. In the present study of both skin constructs the final steps in keratinocyte differentiation were found. The keratinocytes of both constructs were transformed into flat, tightly packed corneocytes that adhered to each other through corneodesmosomes. Corneodesmosomes are derivates from desmosomes and appear firstly at the stratum granulosum/corneum interface. They ensure adhesion between the upper granular layer and the inner horny layer; they also establish links between the corneocytes themselves [7]. In both constructs studied in the present work, an intracellular network of filaments establishing a cornified envelope was detected. The cornified envelope is a protein/filament matrix, formed in the subcytolemmal space. Here, the keratinofilaments are cross-linked by specific proteins (e.g. filagrin, involucrin, loricrin), that give rise to cell rigidity and provide resistance against chemical insults [5, 19]. Moreover in our constructs, intercellular lipids, arranged in lamellar bilayers were observed between the corneocytes. Intercellular lipid lamellae in the stratum corneum are delivered by lamellar bodies which are epidermal specific organelles that occur firstly in the upper layers of the stratum spinosum and then in the cytoplasm of the granulosum layer [11]. Lamellar bodies are derived from the Golgi apparatus [12, 47] and are loaded with hydrolytic enzymes, phospholipids, cholesterol, ceramides, sphingomyelin, glucosylceramides and sterols. They have the appearance of lamellar vesicles and store their lipid contents as a continuous membrane [38]. After fusion with the plasma membrane, they extrude the membranous content through exocytosis into the space of the stratum granulosum/corneum interface and in the subsequent process, the lamellar bodies' lipids are modified and arranged into intercellular lamellae positioned parallel to the cell surface, creating a "secure wrap" over the body surface [11, 15, 37].

4.2. Excessive proliferation and differentiation processes characterize the endothelialized constructs

The first noticeable difference between the two skin constructs was the occurrence of significantly ($p = 0.013$) greater numbers of mitotic cells in the stratum spinosum of the KCFB-EC skin constructs, and consequently also significantly ($p = 0.025$) more cell layers in the stratum granulosum, than found in the KCFB constructs. Moreover, in the KCFB constructs the spinosus cells were uniformly ellipsoid in shape while those of the KCFB-EC constructs were mostly irregular in shape. Furthermore the KCFB-EC constructs had significantly more keratohyalin granules and more lamellar bodies than the KCFB constructs ($p = 0.01$). The rate of mitosis in normal uninjured epidermis in the human adult has

been examined *in vivo* [1] as well as *in vitro* [13, 30]. This rate seems to be considerably lower compared to the results obtained in our study on non-endothelialized versus endothelialized skin constructs *in vitro*.

These differences may be attributed to the supplementation of growth factors. Principally, mitosis of the normal epidermis is recognized as the mechanism upon which the other functional processes of the epithelium depend. In the normal uninjured epidermis, cell turnover may be defined as cell formation balanced by cell loss. While it appears to be an inherent property rather than one of repair, growth factors and hormones as well as other substances may influence this process [18]. While the cells of the KCFB construct have been incubated only in keratinocyte differentiation medium, the endothelialized skin constructs have been incubated in a 1:1 mixture of keratinocyte medium (50%) and endothelial growth & differentiation medium (50%), that contain a plethora of growth factors for both, keratinocytes and ECs. Basically growth factors for cells in the KCFB-EC constructs are derived from two sources, namely 1. the factors added by the supplier of the cells and media, and 2. the factors produced by ECs, fibroblasts or/and the keratinocytes themselves as a result of their cellular interactions in the co-culture.

The EC medium, used in our study was mixed 1:1 with the keratinocyte differentiation medium and contained FBS (fetal bovine serum), human epidermal growth factor (hEGF), VEGF (vascular endothelial growth factor), human fibroblast growth factor B (hFGF-B), insulin like growth factor (R3-IGF-1) and heparin. However, the exact composition of the growth media was and is not disclosed by the supplier as this is frequently done due to patents pending or other reasons. FBS for example, is the most widely used serum supplement for *in vitro* cell culture; it contains all components of bovine serum including growth factors. As serum is not a standardized product, the exact composition is unknown; for example seasonal variations in the serum composition, produce batch-to-batch variations. This, in turn, causes phenotypical differences in the cell cultures, resulting in variations in results [51]. The second source of growth factors present in the medium for KCFB-EC constructs, but not in the one for KCFB constructs are the ECs themselves as well as potentially the fibroblasts of the dermal equivalent and even the keratinocytes themselves, after activation by ECs. It is well known that ECs produce cytokines that activate keratinocytes such as fibroblast growth factor (FGF) [35], interleukin-1 (lL-1), interleukin-6 (IL-6), interleukin-8 (lL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF). The very same growth factors stimulate keratinocytes to differentiate. For example, IL-1 is an autocrine factor, produced by keratinocytes themselves, that is modulated by ultraviolet light and increases the proliferative potential of keratinocytes in culture. IL-1 alpha stimulates keratinocyte migration on collagen via a mechanism distinct from that of epidermal growth factor/transforming growth factor alpha (EGF/TGF-alpha) [6].

The observed elevated mitotic proliferation, the abundance of keratohyalin granules and lamellar bodies and the heterogeneity of cellular morphology of the keratinocytes in the KCFB-EC constructs are the morphological equivalent of unbalanced, i.e., uncontrolled or enhanced maturation processes.

"Uncontrolled maturation processes" suggest that the specific characteristics of the KCFB-EC construct are signs of an impaired maturation such as hyperproliferation and subsequent disturbed epidermal layer formation. In this study the corneocytes in 2 out of 4 of the KCFB-EC constructs were separated by wide intercellular spaces. In addition a spatial segregation between their stratum granulosum and corneum was obvious. Furthermore the cells of their stratum granulosum and spinosum were decidedly heterogeneously shaped and also displayed wider intercellular spaces. A cytokine potentially responsible for this is IL-6 that stimulates proliferation of cultured human keratinocytes and is expressed in high levels in psoriatic skin [16], i.e. a pathologic skin lesion that is characterized by the loss of intercellular cohesion between keratinocytes. The abundance of keratohyalin granules and lamellar bodies found in the endothelialized constructs may be interpreted as "enhanced maturation processes". These features

may lead to an improved barrier function, due to increased synthesis of keratin filaments, keratin filament associated proteins and lipid matrix components.

As the underlying mechanisms are unclear, molecular analysis of the lipid composition in the KCFB-EC constructs are under investigation in order to verify uncontrolled or enhanced maturation processes. In summary, despite the efforts made to optimize cell culture media for the constructs analyzed in the present study, as well as efforts made by other researchers e.g. [33, 42, 48], their specific composition is largely unknown and potentially far away from mimicking the situation *in vivo*. Further studies are in progress to measure the secretion of the critical growth factors by ECs into the growth media used in our experiments.

4.3. Are the membrane coated organelles, found in the lower epidermal layers, progenies of the lamellar bodies?

Both skin constructs contained membrane coated organelles whose number decreased in the upper epidermal layers. We propose that these membrane coated vesicles are lysosomes or autophagolysosomes.

Membrane coated organelles are mostly diagnosed as lysosomes, i.e., as cellular organelles, containing several classes of hydrolytic enzymes, which are assigned to the degradation of a large repertoire of cellular substances [45]. It is well known that lysosomes have important functions in the skin, particularly during the final steps of epidermal differentiation, when calcium entry stimulates exocytosis of lysosomes in keratinocytes [20]. Only recently Appelqvist et al. [2] found that lysosomal exocytosis is part of the keratinocyte response to ultraviolet A rays. Moreover, endolysosomal enzymes, the cathepsins, play a vital role during apoptosis when keratinization takes place [17, 21, 40]. When lysosomes swallow further vesicles, for example autophagosomes, which are double-membrane-surrounded organelles originating from several membrane sources (i.a. endoplasmatic reticulum, mitochondria, etc), they transform into secondary lysosomes, more precisely in this specific case, into autolysosomes [23]. As the membrane coated organelles found in the present skin constructs contained membranous structures, they are more likely to be autolysosomes than lysosomes.

The lamellar bodies which have been found in our constructs are specific organelles of the epidermis, specifically of organs that establish a barrier between the organism and the environment (e.g. lung epithelium). As lamellar bodies contain typical lysosomal enzymes they are considered to be lysosome-related organelles [38]. Although it seems to be proven that the lamellar bodies are generated from the Golgi apparatus, the pathways that mediate the entry of lipids into their internal compartment have not been clarified fully [12]. We propose that the membrane coated organelles (autolysosomes) are precursors of the lamellar bodies because they are found predominately in the lower but only rarely in the upper epidermal layers of the skin constructs. Additionally the ultrastructural characteristics of the lamellar bodies, i.e. the continuous membranes, are composed evidently of membranous parts of other cell organelles. This theory must be examined carefully in future experimental approaches.

4.4. Comparison and major differences between the skin constructs and native human skin

Despite the differences found between the KCFB-EC and KCFB skin constructs, light microscopic and ultrastructure results show that both resemble normal human skin. All laminae of stratified soft-cornified epithelium were established: stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Albeit some typical features of the human skin were not observable in the constructs, e.g. neither a basement membrane nor hemidesmosomes were present in the basal layer. There is a profound dependency

between the epidermis and the basement membrane, which is its extracellular matrix. Vice versa, when lacking a basement membrane, the basal cells have no inductive signal to develop hemidesmosomes [4, 9]. A basement membrane develops in reconstructed skin constructs exclusively in case the dermal component origins from de-epidermized dermis, which is typically obtained from normal skin. However, the dermal equivalent of the constructs examined in the present study was based on fibroblast populated collagen matrices. Marinkovich et al. [29] evaluated both epithelial and dermal/mesenchymal contributions to the basement membrane. They proposed that *in vivo* a specific population of differentiated fibroblasts of the dermis exists adjacent to epithelial tissues, which produces basement membrane components and assists in basement membrane assembly.

Furthermore cell shapes as well as strata architecture of the skin constructs were different to the ones of the normal human skin. In both construct types the cells of the upper spinosum layers and of the granulosum cell layers were flat, while the ones in human skin cells are only flattened in the upper granulosum layers and above. Moreover, the shape of the keratohyalin granules in the constructs was seldom star-shaped like those found in the normal human skin. Additionally lipid droplets, that were found in all skin constructs and throughout all cell layers, do not occur in the same frequency in adult human skin. In summary, our study corroborates the findings of Ponec et al. [36] and other scientists [14] who conclude, that skin constructs reproduce many but not all characteristics of normal human epidermis. Despite the differences between constructs and normal human skin, the formation of many features of a competent permeability barrier to perform skin-related studies seems to be possible. Further studies will concentrate on optimization of the cocktail of growth factors for *in vitro* constructs in order to activate angiogenesis of endothelial cells in the dermal compartment.

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6. Discussion

6.1. Skin in situ

With the shortage of human skin, alternative biological replacements are in demand to meet the needs of researchers and pharmaceutical companies. Skin sourced from many different mammalian species has been used as a substitute for human skin. Of these, porcine skin has been claimed to be the most suitable replacement for human skin because of its anatomical and physiological similarities (Haque et al., 2017; Jung et al., 2013; Parra et al., 2016; Todo, 2017). Even so, porcine skin morphology varies in different body regions, in many ways it corresponds to the one of human skin. (Kakasheva-Mazhenkovska et al., 2011; Turner et al., 2015)

The first part of the dissertation study aimed to identify the region of German Landrace pig skin which was used as the best possible substitute for human abdominal skin. The results showed that certain parameters of the four regions of porcine skin (ear, flank, back and caudal abdomen) were comparable to those of human abdominal skin. Parameters such as the thickness of the epidermis and its stratum basale, spinosum and granulosum, the number of cell layers in the stratum corneum, as well as the shapes of keratinocytes, the structure of cell contacts, and the presence of Weibel Palade bodies in endothelial cells were similar in the human abdominal skin and all four regions of porcine skin.

However, there were also significant differences, especially in the thickness of the stratum corneum, the length of the dermo-epidermal interface, and the density of blood vessels. The thickness of the stratum corneum from four regions of the porcine skin was significantly thinner than that of human abdominal skin, whereas the whole epidermal thickness did not show any significant difference. Similar regional differences also existed in dermal thickness from the porcine caudal abdomen, back, ear, and flank regions compared to human abdominal skin. In particular, the caudal abdominal porcine skin was much thinner than that of the human skin. The epidermal and dermal thickness in humans and pigs studied in the present dissertation resembled that reported earlier (Jung & Maibach, 2015; Liu et al., 2010a; Takeuchi et al., 2011; Turner et al., 2015). Possible explanations for the differences in stratum corneum and dermal thickness were age and level of sun-exposure. A previous study reported that the human epidermal thickness was high at 20-50 years of age but decreases and becomes very thin in the elderly (Manimegalai et al., 2015). A study of corneocyte size revealed an age-dependent increase in cell area up to 70 years of age (Stucker et al., 2015). The epidermal and the dermal thickness of 4 months old pigs was found to be close to those of human adults (Liu et al., 2010a). Skin examined in the present dissertation study was obtained from pigs younger than 3 months of age, whilst the typical age of pigs for use in research is 3-6 months (Gutierrez et al., 2015; Swindle et al., 2011). Therefore, the present

dissertation study's skin samples may not have reached full maturity and thus would likely be thinner than human skin.

It is supposed that the difference in epidermal thickness between body sites is related to the level of sun-exposure. Sunlight exposure has been shown to induce thickening of the stratum corneum through enhance keratinocyte proliferation (D'Orazio et al., 2013; Liu et al., 2010b; Scott et al., 2012; Tian et al., 2014). This may be the reason that the porcine back skin was found to be thicker compared to the abdominal or caudal ear skin of pigs (Khiao In et al., 2019).

The length of the epidermal-dermal junction depends on the undulated line of rete ridges. The rete ridges represent the contact area of the epidermis and dermis and provide the mechanical strength of adhesion between the epidermis and dermis (Burgeson & Christiano, 1997; Lawlor & Kaur, 2015; Vázquez et al., 1996). The stem cells of keratinocytes in adult skin appear at the tip of the rete ridges (Lawlor & Kaur, 2015). Developing rete ridges into engineering skin presents significantly increased epidermal proliferation and stratum corneum thickness (Blackstone et al., 2019; Kumamoto et al., 2018). The result of studies in the frame of my doctoral thesis showed that the dermo-epidermal length of the porcine skin caudal to the ear was significantly shorter than human abdominal skin.

It is known that the number of blood vessels in the dermis of human abdominal skin, which is significantly lower than in all four porcine skin areas, maybe since the donor was an older person. Natural aging and photo-aging of the skin are associated with a reduction of the number of blood vessels, especially in the upper dermis (Bentov & Reed, 2015; Rittié & Fisher, 2015; Výbohová et al., 2012). The study by Gunin et al. (Gunin et al., 2015) confirmed this when they reported that in human skin the greatest number of the blood vessels was observed in antenatal age (gestation weeks 20-40), then gradually decreased with age and was the lowest in the group of 61–85 years old subjects.

It is well known that the density of the hair follicles in humans varies at different body sites (Otberg et al., 2004). Similarly, the hair follicle distribution of German Landrace pig's skin differs with the sample location. The back, flank and ear region of the porcine skin have a higher number of hair follicles than that of the human abdominal skin. However, the number of hair follicles from the caudal abdomen of pigs corresponded to that of human abdominal skin. In correlation with the distribution of hair follicles, the number of arrector pili muscles varied considerably across the different body sites of pigs. Sweat glands in human abdominal skin seemed to be lower than that found in all porcine skin areas, but the difference was not significant. The mean hair follicle and sweat gland density is already determined in the early fetal period. After birth, no new follicles and glands can grow (Baker, 2019; Rishikaysh et al., 2014).

The results on porcine skin thickness provide essential information for studies of percutaneous absorption, where regional differences in skin morphology may influence absorption rates (Monteiro-Riviere et al., 1990; Scott et al., 1990; Takeuchi et al., 2012; Yamaguchi et al., 2008). It is established that skin appendages, hair follicles and sweat glands, play a subordinate role in absorption processes (Otberg et al., 2004). By the transfollicular route, substances are absorbed through the skin. The hair follicle accelerates percutaneous absorption (Mangelsdorf et al., 2014; Otberg et al., 2008; Patzelt & Lademann, 2015). Liu et al. (Liu et al., 2011) reported a constant absorption rate of caffeine for hair follicles that was nearly ten times higher than that of the stratum corneum.

It is possible that differences in the thickness of the dermis and epidermis from different body sites in the pig may influence the production of biologic scaffolds derived from the porcine dermis. A thick dermis has been reported to be important in the selection of donor area for recurrent split-thickness grafts (Greco et al., 2015; Oltulu et al., 2018). It has been claimed that a high dermal thickness is associated with increased wide scar formation (Kim et al., 2018; Oltulu et al., 2018). Knowing the approximate dermal thickness prior to surgery might help to reduce the extent of hypertrophic scarring (Kim et al., 2018).

Limitations and conclusion

The principal limitation of this study is that it was restricted to investigating only one single breed of pig, the German Landrace. It is possible that the regional variations in skin morphology in this breed may differ from that of other pig breeds commonly used for ex vivo studies. Another limitation of this study was that only four anatomical regions were sampled from each pig.

In conclusion, the pig is arguably the most suitable experimental model for research studies on human skin. This study's results showed specific skin regions of domestic pigs were suitable for human skin replacement. According to the results, the back region, particularly of the German Landrace breed, had superior skin characteristics to those from the flank, the abdominal and the caudal ear regions. The dermal thickness of the back region was comparable to the thickness of human abdominal skin. It is possible that the caudal ear skin could be used as a model in studies of chemicals utilizing the trans-follicular route for their absorption.

6.2. Skin constructs in vitro

The second part of the study aimed to investigate the morphology and ultrastructure of two types of human three-dimensional skin constructs and to assess morphologically the effect of endothelialization on the epidermal differentiation in two constructs.

The two variations of human full-thickness skin constructs consisted of keratinocytes and fibroblasts only (type 1, KCFB) or of keratinocytes as well as fibroblasts and vascular endothelial cells (type 2, KCFB_EC).

In general, the overall morphology and ultrastructure of the epidermis in both the KCFB and KCFB-EC skin models were similar to that of native skin. All major epidermal strata, including stratum basale, stratum spinosum, stratum granulosum, and stratum corneum, were present. In the skin constructs, corneodesmosomes and the lamellar lipid bilayer was seen in the stratum corneum. Membrane coated granules and lamellar bodies were found in the stratum spinosum and stratum granulosum, respectively. However, there were several differences when the KCFB-EC and KCFB models were compared.

The KCFB- EC constructs had significantly more mitotic cells in the stratum spinosum than the KCFB constructs suggesting a higher rate of keratinocyte proliferation in the constructs with endothelial cells. This is probably because the KCFB-EC culture media used in the study (EGM-2 medium by Lonza) included VEFG for supporting endothelial cells´ growth. This finding supports earlier evidence that VEGF stimulates epidermal thickening and enhances keratinocyte proliferation (Li et al., 2014; Wise et al., 2012). Furthermore, Brown et al. (Brown et al., 1992) found that keratinocytes in culture can secrete VEGF the same as that secreted by the normal epidermis. In the normal epidermis, the level of VEGF was found to be low (Johnson et al., 1997). However, there is a significant elevation of VEGF in epidermal keratinocytes in many skin disorders with aberrant keratinization, such as psoriasis, contact dermatitis, and delayed wound healing (Bae et al., 2015; Man et al., 2008). This suggests that increased VEGF may play a key role in these skin problems. In the present dissertation study this reason could account for the excessive epidermal differentiation and the unorganized epidermal architecture in KCFB-EC skin constructs.

Ultrastructure assessments of the skin constructs showed them to be devoid of basement membrane structures. In other studies, on cocultures between keratinocytes and fibroblasts, basement membrane components were found by using immunohistochemistry to detect the genes of cell adhesion (El Ghalbzouri et al., 2005; Okamoto & Kitano, 1993). The basement membrane encourages the development of a well-stratified epithelium (Wojtowicz et al., 2014). Basement membrane structure defects affect skin integrity and skin barrier functions (Breitkreutz et al., 2013; Fisher & Rittié, 2018). The lack of basement membrane structures in the KCFB-EC constructs could explain their unpredictable differentiation of keratinocytes.

Cocultured fibroblasts, keratinocytes, and endothelial cells exhibit epidermal layer features with tubulogenesis, according to the literature (Gibot et al., 2010; Marino et al., 2014; Supp et al., 2002; Tonello et al., 2003). One of the most important results of this dissertation

study was the absence of vasculogenesis in the fibroblast matrix of the KCFB-EC constructs (Khiao In et al.,2015). Endothelial cells failed to form capillaries, presumably due to a lack of interaction between endothelial cells and fibroblasts. Consequently, in a later study from our Institute of Veterinary Anatomy, Kaessmeyer et al. (Kaessmeyer et al., 2016) directly cocultured endothelial cells and fibroblasts and were able to show that the endothelial cells can form tubes inside the extracellular matrix.

The extracellular matrix (ECM) produced by the fibroblasts is the cellular microenvironment that forms a complex 3D network consisting of many components, including collagens, non-collagenous glycoproteins, elastin, proteoglycans, and matricellular proteins in an organ-specific manner (Tracy et al., 2016). Importantly, the endothelial cell function and vascular growth are critically dependent on interactions with the surrounding ECM (Eming & Hubbell, 2011). However, the ECM needs living fibroblasts for some of its critical functions. Berthod et al. (Berthod et al., 2006) found that endothelial cells do not form capillary-like tubes in ECM in the absence of living fibroblasts or cultured with fibroblast-conditioned medium. In the first days of coculture, fibroblasts and endothelial cells grow filopodia, which interconnect fibroblasts, endothelial cells as well as fibroblasts and endothelial cells (FBs-FBs, ECs-ECs, and FBs-ECs). Fibroblasts are found adjacent to the endothelial tubular structures and physically contact the endothelial cells. In this location, the ECM starts to degrade, and the endothelial cells start sprouting tubular structures (Kaessmeyer et al., 2016). This observation confirms that fibroblasts influence angiogenesis probably by their production of angiogenic factors. Here it is believed that there are two groups of protein factors: one group supporting sprouting and another group facilitating lumen formation. Group one consists of angiopoietin-1, angiogenin, hepatocyte growth factor, transforming growth factor-α, and tumor necrosis factor (TNF), all promoting endothelial cell sprouting. While group two consists of collagen I, procollagen C endopeptidase enhancer 1, secreted protein acidic and rich in cysteine (SPARC), transforming growth factor-β–induced protein (βig-h3), and insulin growth factor–binding protein 7 (IGFBP7) that are all necessary for lumen formation. However, only the factor that promotes sprouting needs the presence of living fibroblasts. The factors that promote lumen formation can be extracted from fibroblast conditioned medium (VEGF included) (Newman et al., 2011).

It is desirable that the formation of dermal vascular elements in artificial skin constructs closely mimic that found in human skin. For this the interaction between fibroblasts and endothelial cells is essential. The skin constructs examined in this dissertation study lacked the dynamic interaction between fibroblasts and endothelial cells because the endothelial cells were seeded separately to the fibroblasts. Consequently, the dermal vascularization did not develop. Subsequently Kaessmeyer et al. (Kaessmeyer et al., 2016), in a later study from the

Institute of Veterinary Anatomy, directly cocultured fibroblasts and endothelial cells showing that both, the physical and chemical contact is necessary for tube formation.

Conclusion

In future studies two important changes should be made to create a more accurate model of human skin: it is necessary to optimize the numbers of fibroblasts and endothelial cells to form a stable dermis. Then seeding keratinocytes on top of this optimized dermis may initiate the epidermis to develop.

7. Summary / Zusammenfassung

7.1 Summary

From porcine skin samples in situ to three-dimensional human skin constructs in vitro. Studying skin with a focus on the 3R principles.

Chapter 1 gives a general introduction to the importance of animal models, focusing on porcine models as well as on in vitro models in skin research.

Chapter 2 presents a literature review of research on pigs as animal models for human skin in skin research and of in vitro models of skin research. Furthermore, the principle of the 3Rs is reviewed.

Chapter 3 states the objectives and hypotheses of this thesis.

Chapters 4 and 5 cover the two main publications for this thesis.

Chapter 4 reports on the comparison between abdominal human skin and four different areas of pig skin (the ear, the flank, the back, and the caudal abdomen). The skin samples were examined by light microscope (LM), transmission electron microscope (TEM) and immunohistochemistry. In addition, skin permeability studies were performed. The results of this study show both the similarities and differences in histological and ultrastructural features. The epidermis of the examined regions in both pigs and humans, except for the stratum corneum, is of similar thickness. Although the number of corneocyte layers was not different in the two species, the human corneocyte layer was thicker than that of the pig. The dermal structure of humans and pigs is similar, the thickness of the human one is more similar to the one of the porcine flank and back region. The ear and caudal abdomen of the pig showed a thinner dermis when compared with those of humans. The epidermal-dermal thickness ratio of the porcine back, flank and abdomen was similar to that of humans. The dermo-epidermal length was not found to be different in humans and pigs. This is significant since the skin´s barrier function depends on this interface. The number of hair follicles and arrector pili muscles of the porcine abdominal and ear skin was different from that of the human skin. The number of sweat glands of the four areas of pig skin was not significantly different from that of the human skin. The number of blood vessels in the human dermis was significantly lower than in the pig dermis.

Chapter 5 reports on the comparison between the two types of in-house skin constructs and human skin as examined by light and transmission electron microsopy. Data were analyzed by semi-quantitative and qualitative methods. The two types of skin constructs included a construct consisting of keratinocytes and fibroblasts only (type 1, KCFB) and another construct made of keratinocytes as well as fibroblasts and vascular endothelial cells (type 2, KCFB_EC). Both types of skin constructs showed all strata as known from human skin. When compared with the skin constructs without EC or with normal human skin, the skin

constructs with endothelial cells were characterized by unorganized epidermal layers, significantly more mitotic cells in the stratum spinosum, more layers of the stratum granulosum and more keratohyaline granules.

Chapter 6 discusses whether specific skin regions of domestic pigs were suitable for human skin replacement. According to the results reported in the first section, the back region, particularly of the German Landrace breed, had superior skin characteristics to those from the flank, the abdominal and the caudal ear regions. The dermal thickness of the back region was comparable to the thickness of human abdominal skin. It is possible that the caudal ear skin could be used as a model in studies of chemicals utilizing the trans-follicular route for their absorption.

The second section addressed the morphology and ultrastructure, and the effect of endothelialization on epidermal differentiation in two types of skin constructs. The epidermis in both the KCFB and KCFB-EC skin models had a similar overall architecture and ultrastructure to native skin. When the KCFB-EC and KCFB models were compared, however, there were several differences. The KCFB-EC constructs featured more mitotic cells, a considerable epidermal differentiation, and a significantly more disorganized epidermal architecture. The data supported the hypothesis that VEGF promotes epidermal thickness and keratinocyte proliferation. VEGF was obtained from both culture medium and keratinocyte culture. Increased VEGF levels may play a crucial role in these issues. In addition, no vasculogenesis was observed in the fibroblast matrix of the KCFB-EC constructs. Due to a lack of interaction between endothelial cells and fibroblasts, endothelial cells failed to form capillaries. To build a stable dermis, fibroblasts and endothelial cells quantity must be optimized. Thus, the epidermis may be initiated by seeding keratinocytes on top of the optimal dermis.

7.2 Zusammenfassung

Von porzinen Hautproben in situ zu dreidimensionalen in vitro Konstrukten der menschlichen Haut. Eine Untersuchung der Haut mit Schwerpunkt auf dem 3R-Prinzip.

Kapitel 1 stellt eine allgemeine Einleitung zur Bedeutung von Tiermodellen dar und konzentriert sich besonders auf Modelle vom Schwein sowie in vitro Modelle aus der Hautforschung.

Kapitel 2 präsentiert eine Literaturübersicht von Forschungsarbeiten zu Schweinen als Tiermodelle für menschliche Haut in der Hautforschung, sowie von in vitro Modellen der Hautforschung. Weiterhin wird das 3R-Prinzip vorgestellt.

Kapitel 3 führt die Zielstellungen und die Hypothese der vorliegenden Arbeit auf.

In den **Kapiteln 4 und 5** werden die zwei für die Arbeit wichtigsten Publikationen besprochen.

Kapitel 4 beschreibt den Vergleich menschlicher Bauchhaut mit vier verschiedenen Bereichen der Haut vom Schwein (Ohr, Flanke, Rücken und caudales Abdomen). Die Hautproben wurden lichtmikroskopisch (LM), elektronenmikroskopisch (TEM) und immunhistochemisch untersucht. Zusätzlich wurden Studien zur Durchlässigkeit der Haut vorgenommen. Die Ergebnisse dieser Studien zeigen sowohl die Ähnlichkeiten als auch die Unterschiede der histologischen und ultrastrukturellen Eigenschaften. Die Epidermis ist beim Menschen und beim Schwein ähnlich dick, abgesehen vom Stratum corneum. Obwohl sich die Anzahl der Hornzellschichten der beiden Spezies nicht unterschied, war die menschliche Hornzellschicht dicker als die des Schweins. Die Hautstruktur von Menschen und Schweinen ist ähnlich, die Dicke der menschlichen Haut ähnelt allerdings mehr der Haut an der Flanken- und Rückenregion des Schweins. Das Ohr und die Abdominalregion des Schweins zeigten im Vergleich zur menschlichen eine dünnere Lederhaut. Das epidermale-dermale Schichtdickenverhältnis des Rückens, der Flanke und des Bauches vom Schwein war ähnlich dem des Menschen. Die dermo-epidermale Länge war bei Menschen und Schweinen gleich. Das ist eine wichtige Erkenntnis, da die Barrierefunktion der Haut von dieser Schnittstelle abhängt. Die Anzahl der Haarfollikel und Haarbalgmuskeln der Haut vom Bauch und Ohr des Schweins unterschied sich von der menschlichen Haut. Die Anzahl der Schweißdrüsen in den vier Arealen der Schweinehaut unterschied sich nicht signifikant von der in der menschlichen Haut. Die Anzahl der Blutgefäße in der menschlichen Haut war beträchtlich geringer als in der Schweinehaut.

Kapitel 5 stellt den Vergleich zwischen den zwei Typen der Hautkonstrukte und menschlicher Haut dar, wie licht- und elektronenmikroskopisch untersucht. Die vorliegenden Daten wurden mittels semiquantitativen und qualitativen Methoden erhalten. Unter den beiden Hautkonstrukten war eines, das nur aus Keratinozyten und Fibroblasten bestand (Typ 1, KCFB). Das zweite bestand aus Keratinozyten und Fibroblasten sowie vaskulären Endothelzellen (Typ 2, KCFB_EC). Beide Hautkonstrukte zeigten alle Schichten, die auch vom Aufbau menschlicher Haut bekannt sind. Verglichen mit den Hautkonstrukten ohne EC oder mit normaler menschlicher Haut, wiesen die Hautkonstrukte mit Endothellzellen unorganisierte Hautschichten auf, hatten deutlich mehr Mitosezellen im Stratum spinosum und mehr Schichten des Stratum granulosum sowie mehr *Keratohyalingranula.*

Kapitel 6*,* die Diskussion, befasst sich mit der Frage, ob spezielle Hautareale von Hausschweinen geeignet sind, menschliche Haut zu ersetzen. Laut der Resultate, die im ersten Abschnitt dargestellt wurden, übertrafen die Eigenschaften der Rückenregion die Eigenschaften der Flanke, der Bauchregion und der kaudalen Ohrregion. Die Hautdicke am Rückenareal war vergleichbar mit der Hautdicke menschlicher Bauchhaut. Es ist möglich, dass Haut des Ohrs als ein Modell in Studien zu Chemikalien, die den transfollikularen Weg zur Resorption nutzen, dienen kann.

Der zweite Abschnitt diskutiert die Morphologie und Ultrastruktur sowie die Auswirkung der Endothelialisierung auf die epidermale Differenzierung in zwei Hautkonstrukten. Die Epidermis in sowohl der KCFB als auch der KCFB-EC Hautmodelle hatte einen ähnlichen Aufbau und Ultrastruktur wie ursprüngliche Haut. Beim Vergleich der KCFB-EC und KCFB Modelle zeigten sich jedoch mehrere Unterschiede. Die KCFB-EC Modelle wiesen mehr Mitosezellen auf, eine deutlichere epidermale Differenzierung sowie einen signifikant schlechter organisierten epidermalen Aufbau. Die Daten unterstützten die Hypothese, dass VEGF sowohl epidermale Dicke als auch die Vermehrung der Keratinozyten begünstigt. VEGF stammte aus Nährmedium und kultivierten Keratinozyten. Erhöhte VEGF Werte könnten eine entscheidende Rolle bei diesen Vorgängen spielen. Außerdem wurde in der Fibroblastenmatrize der KCFB-EC keine Vaskularisierung festgestellt. Durch ein fehlendes Zusammenspiel von Endothelzellen und Fibroblasten bildeten die Endothelzellen keine Kapillaren. Um eine stabile Dermis zu formen, muss eine optimale Anzahl an Fibroblasten und Endothelzellen vorliegen. Daher kann die Bildung der Epidermis durch das Auftragen von Keratinozyten auf eine optimierte Dermis ausgelöst werden.

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11. Declaration of academic honesty

I hereby confirm that the text at hand is solely my own work. I assure that I only used the cited sources, and the thesis has not been submitted in any form for another degree at any university or other institute.

Berlin, 18.02.2022
Maneenooch Khiao-in

I declare that there is no conflict of interest in my dissertation.

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