FONDAZIONE ISTITUTO ITALIANO DI TECNOLOGIA

A TECHNOLOGY TEASER

TOTALLY ENDOGENOUS BIOENGINEERED TISSUE

ISTITUTO ITALIANO DI TECNOLOGIA

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HEALTH TECHNOLOGIES

Istituto Italiano di Tecnologia – Mission and History

The Foundation is intended to promote Italy's technological development and advanced education, consistent with national policies for scientific and technological development, thus strengthening the national production system.
For this purpose, the Foundation:

- helps and accelerates the development, within the national research system, of scientific and technological skills able to facilitate state of the art technological advancements of the national production system;

- develops innovative methods and know-how, in order to facilitate new high-level practices and positive competitive mechanisms in the field of national research;

- promotes and develops scientific and technological excellence, both directly, through its multi-disciplinary research laboratories, and indirectly, through a wide collaboration with national and international laboratories and research teams;

- carries out advanced training programs as a part of wider multi-disciplinary projects and programs;

- fosters a culture based on sharing and valuing results, to be used in order to improve production and for welfare-related purposes, both internally and in relation to the entire national research system;

- creates technological understanding about components, methods, processes and techniques to be used for the implementation and interconnection of innovative products and services, in strategic areas for the competitiveness of the national production system;

- pools research scientists operating in various research institutes and establishes cooperation agreements with high-level, specialized centers;

- promotes interactions between basic research and applied research facilities, encouraging experimental development;

- spreads transparent, merit-based selection mechanisms for research scientists and projects, in compliance with globally approved and established criteria.

CONTENTS

EXECUTIVE SUMMARY

The present technology refers to an *in vitro* 3D endogenous dermis equivalent and to a full skin model including both dermis and epidermis, composed of all endogenous extracellular matrix (ECM) components, without any exogenous ECM or synthetic materials. The dermis equivalent is composed by fibroblasts and neo-formed organized endogenous ECM including collagen, elastin and all components presented in native dermis. The whole human skin model presents a fully differentiated epidermis, well anchored to the dermis equivalent, including keratinocytes and melanocytes as cell population, and expressing all epidermal differentiation markers. Dermis equivalent alone, as well as the full skin model, can be used as *in vitro* testing system or for therapeutic purposes.

To date, several human skin equivalent (HSE) models have been developed to be used both as substitute in case of injured skin, and also as testing skin in order to assess, for example, potential skin toxicity or unwanted side effects of medications or cosmetics, or physical agents such as light or heat, in particular irritation, corrosion, toxicity, and inflammatory effects, as well as their skin biocompatibility. Moreover, such a system can also be used to answer many types of immunological, histological, and molecular biological questions. This includes, for example, studies about wound healing and of the penetration and absorption of substances through the skin. Compared with animal experiments or studies using human test subjects, the studies or tests of substances with such full skin models offer substantial advantages since the results obtained with them are more reproducible, and the studies less expensive and quicker. More importantly, the using of such skin models, in place of animal experiments, is in accordance with the commitments of the European Partnership for Alternative Approaches to Animal Testing (EPAA), that aims to pooling knowledge and resources to accelerate the development, validation and acceptance of alternative approaches to implement the reduction, refinement and replacement of animal use in regulatory testing.

In this context, the aim of the project “Tissue On Chip” (TOC) and the associated technology developed at IIT Center For Advanced Biomaterials for Healthcare (IIT@CRIB Napoli) was to provide an *in-vitro* 3D dermis equivalent and whole skin model mimicking the structural organization, function and composition of native human skin.

This technology represents a unique chance for companies which manufactures, or are willing to manufacture, skin equivalent tissues, or operates with complementary products. IIT assets appear well positioned for an out-licensing strategy, providing the licensee partner with the ability to take care of the late stage development, CE certification, scale-up and production process. The licensee should guarantee a high probability of market success based on consolidated marketing & distribution organization.

INTELLECTUAL PROPERTY

PCT International Publication #	WO 2015/166455– 30th October 2015
Priority Application #	US 61/986627 – 30th April 2014
Applicant	Fondazione Istituto Italiano di Tecnologia
Inventors	Giorgia Imparato, Costantino Casale, Francesco Urciuolo, Paolo Netti, Sara Scamardella
Title	Method For Producing A Totally Endogenous Bioengineered Tissue And Tissue Obtained Thereby

Short description

The present invention relates to a method for producing a totally endogenous bioengineered tissue including a first layer of connective tissue and a second layer of epithelial tissue, to a tissue equivalent obtained thereby and to a method for determining the effect of a chemical substance or an agent on skin employing the tissue equivalent.

IIT TECHNOLOGY

A biological tissue is not a combination of cells within a bundle of inert macromolecules, but it is rather an intricate complex of cell and cell-synthesized matrix that constitute a *unicum* element regulated by a multifaceted homeostatic equilibrium. Cells are regulated and controlled by the ECM composition and structure, which, in turn, is synthesized, assembled and remodeled by cells. Tissue pathology or dysfunction even when arisen from cellular components affects the extracellular space and *vice versa*. Therefore, a reliable thick and homologous tissue or part of organ to be used as biological substitute or as screening/testing platform should realistically reproduce the whole tissue/organ including the macromolecular organization and assembly of the ECM in order to faithfully mimic either the physiological and pathological status of a native tissue. The dermis equivalent developed by this technology mimics the structural organization, function and composition of the native tissue.

These features let it to be the ideal connective tissue on which keratinocytes can adhere, grow and differentiate generating an organized epidermis. In this way an organoid *in vitro* skin model comprising two-specific layers, i.e. a dermis equivalent and an epidermis equivalent, is formed in which cross talking and interplaying between the two-layers is guaranteed. It can be used in various fields of skin biology, as testing skin of defined size and shape for studying, for example, pharmacological and cosmetic effects of drugs as well as for cellular and molecular biology studies and also as a replacement for human skin in clinical applications.

In contrast to previously reported and already commercialized technologies, IIT@CRIB has developed an endogenous HSE model where a fully developed epidermis is formed on a thick dermis equivalent in which fibroblasts are embedded in their own ECM mimicking structural organization, function and composition of native ECM. The dermis equivalent, realized by modifying a process previously developed and patented by the IIT scientists guarantees the dermal local micro-environment allowing cell-matrix interactions and maintenance of a functional stem cell compartment, rendering the full HSE suitable for *in vitro* long-term studies.

The whole human skin model so developed presents a fully differentiated epidermis, well anchored to the dermis equivalent, including keratinocytes and melanocytes as cell population and expressing all epidermal differentiation markers. A preferred implementation of the process for the production of the dermis equivalent and the full skin model includes the following steps:

a) dynamic seeding of fibroblasts on biodegradable porous microbeads with a controlled and tunable degradation rate, in order to obtain dermal micro-tissues;
b) placing and molding the micro-tissues in a maturation chamber;
c) culturing the maturation chamber to obtain an endogenous dermis equivalent tissue;
d) seeding epidermal cells on the top of dermal equivalent construct;
e) maintaining the culture submerged for 6 days to allow complete epidermal cells coverage, melanocytes organization along epidermal basal membrane, and early keratinocytes stratification;
f) raising the developing culture to the air-liquid interface thus promoting epidermal differentiation, stratification and forming a complete full skin model comprised of a dermal and epidermal layers.

The dermis equivalent produced in accordance with the invention may also be used for studying the wound healing process; in fact, by inducing a wound on the dermis equivalent, the cells are able to remodel their

own ECM and to synthesize neo-ECM in order to close the wound; in this way a process of self-repair occur, and the effects of wound on cell as well as ECM structure on gene expression can be studied. Analogously, the effects of substances on cell structures and on ECM assembly and composition can be studied too, using this dermis equivalent after UVA irradiation.

Either dermis equivalent as well as skin equivalents with defined diameter and uniform surface are obtained. In the testing of substances for pharmacological and/or cosmetic effects, the uniform size and uniform properties of the full skin model used as test surface permit higher quality results and more reproducible test results.

In addition, the human skin model obtainable in accordance with the technology also comprises functional melanocytes; in fact, few days after the submerged culture, brown zones can be observed on the top of the tissue equivalent. The pigmentation of human skin equivalent model, that is the synthesis of melanin by the melanocytes, has been confirmed by histochemical analysis (Masson-Fontana) that highlights the organization of the melanocytes at the level of basal layer and their ability to transfer melanosomes (melanin grains) to the keratinocytes in the upper layer.

In view of its complexity, the whole skin model produced can be specifically used for tackling various problems in the chemical/pharmaceutical industry and in the cosmetics industry. Since an endogenous ECM is present, it is possible to study not only the cellular response but also the ECM response in terms of change in assembly as well as composition to a chemical, pharmaceutical or cosmetics product for testing its effectiveness, unwanted side effects, for example, irritation, toxicity and inflammation or allergenic effects, or the compatibility of substances. In addiction the skin equivalent produced in accordance with the invention may also be used, for example, for studying the absorption, transport and/or penetration of substances. It is also suitable for studying other physical agents, such as light or heat, and photoxicity (i.e. the damaging effect of light of different wavelengths on cell structures as well as on ECM organization). Moreover the human dermis/skin model obtained by this technology may also be used for treating patients suffering from a wound to the skin, for example, burn patients; the dermis/skin model system may be applied to the wound, for example, by transplantation or grafting.

The main advantage of the human skin model optimized by this technology is based on the development of a full-differentiated epidermis on an endogenous dermis equivalent. This allows a strong adhesion and cross-talking between dermis and epidermis, demonstrated by the formation of a basal membrane having the characteristic rete ridge structures with typical epidermis appendages going deep through the underlying dermis resembling bulge-like structure. Taken together these results make the model of the invention closer to the native skin. Moreover, the presence of an endogenous dermis equivalent allow to study the effect of a chemical, pharmaceutical, cosmetic or physical external agent on cells structure as well as on ECM assembly.

Further developments are planned regarding:

- Inserting other kinds of cells in the human skin equivalent such as Langherans cells or Langherans precursor cells, endothelial cells and Merkel cells.
- Realizing another kind of epithelium by following the method of the invention such as bronchial epithelium, intestine epithelium and cervix epithelium.

Some examples illustrating the technology

Figure 1. On the left the maturation chamber designed to obtain a large endogenous dermis equivalent is shown, on the right a gross picture of the Endogenous dermis equivalent 25 cm^2 in size is shown.

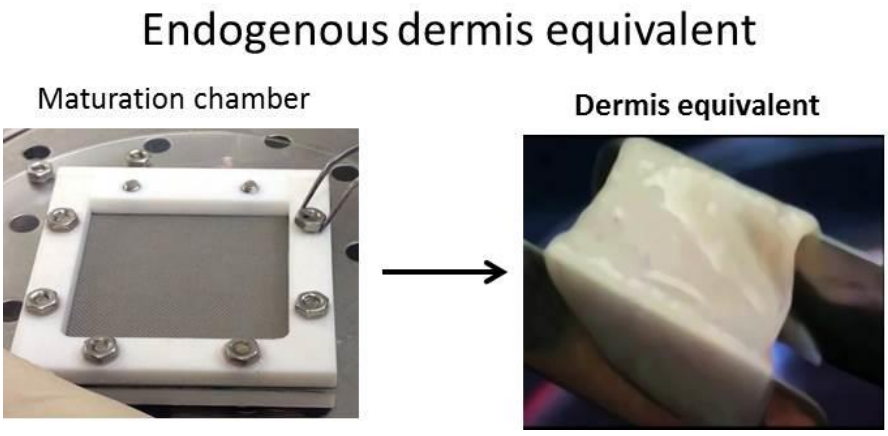

Figure 1

Figure 2. Shows mechanical properties of human endogenous dermis equivalent. The stress- curve at two deformation rate (0,5 mm/min at 10mm/min) is reported.

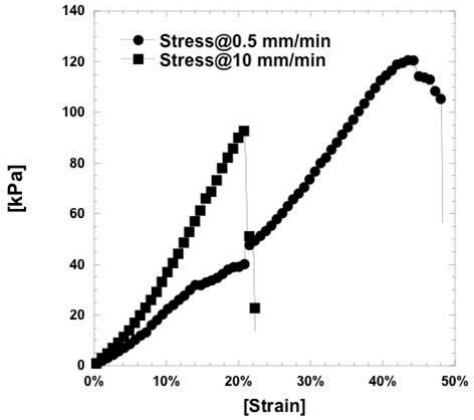

Figure 2

Figure 3. Second harmonic generation image shows the collagen bundle in the endogenous ECM of dermis equivalent (scale bar 40µm).

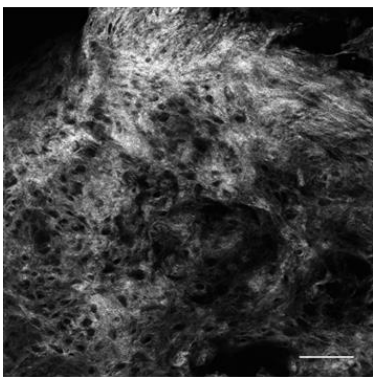

Figure 3

Figure 4. The evolution of a wound healing process is shown. On the left "wounded" endogenous dermis equivalent is shown, on the right after 3 days the complete closure of wound area is reached. The cells close the wound at rate of 0,3 mm^2/day (scale bar 200µm).

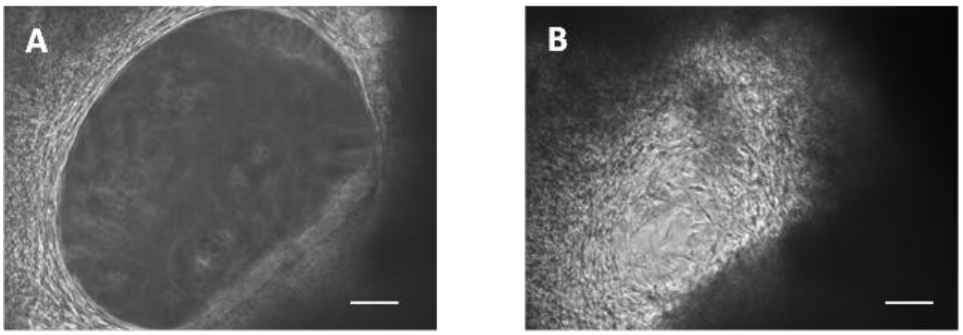

Figure 4

Figure 5. This figure shows the epidermal cells seeding on the top of dermal equivalent construct is shown. A) Epidermal cells are seeded drop by drop on the dermal equivalent surface. B) Histology of the longitudinal section stained by hematoxylin and eosin show the full the human skin equivalent morphology. C) Human skin equivalent surface show brown spot indicating that pigmentation is occurred.

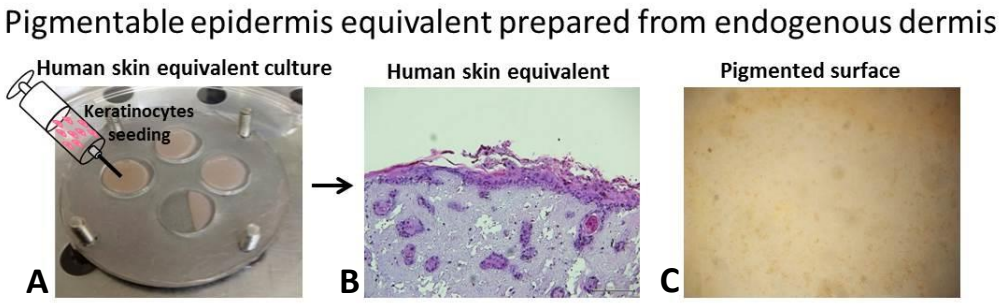

Figure 5

Figure 6. Shows longitudinal section of *in-vitro* full skin model that highlights the presence of melanocytes and its pigment of melanin by histochemical analysis such as Fontana Masson. In detail it is possible to observe that the melanocytes are homogeneously distributed along the basal layer of keratinocytes. Moreover, melanin (dark stained) was transferred also in keratinocytes on the upper layer, this phenomenon indicates a correct functionality of the melanin synthesis and transfer pathway. The number of melanocytes present in the skin model is similar to that found in native human skin (scale bar 50nm).

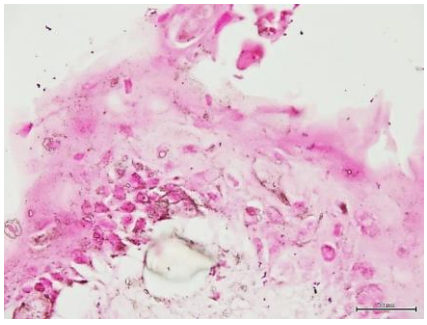

Figure 6

Figure 7. Shows the dermis–epidermis junction in the skin model observed by electron transmission microscopy. In particular, it is possible to observe that the dermal layer of the skin model is rich in collagen bundle having cross-striated patterns. Moreover, just below the basal layer a lot of smaller collagen fibrils, such as collagen IV and VII are present, as reported in literature they are localized close to lamina dense and are 50-80nm in thickness. In the same image it is possible to observe the melanocytes correctly localized along the basal membrane. In particular melanosomes and single pigments of melanin in the dendritics elongation of the melanocytes are well evident (scale bar 1µm). At higher magnification (see inset) a detail of the basal membrane zone is reported. It highlights hemidesmosomes composed of an the electron-dense inner plaque into which intermediate filaments (tonofibrills) are inserted, and an outer plaque that lies on the plasma membrane. Since hemidesmosomes are junctions constituting the main adhesion units of the basement membrane zone, the great quantity of hemidesmosomes found in our skin model, demonstrated the good adhesion and cross-talking existing between dermis and epidermis. Finally, lamina lucida having a thickness of 30-50 nm is correctly present between the plasma membrane of keratinocytes and lamina dense (scale bar 500nm).

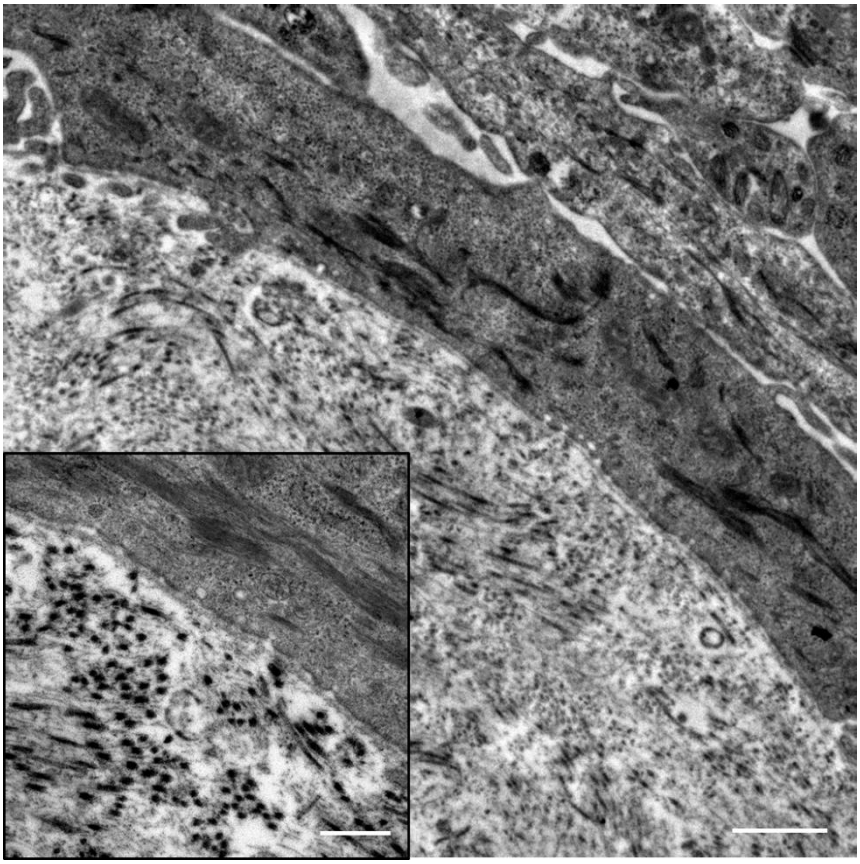

Figure 8. Shows a histology of longitudinal section of in-vitro full skin model made of both dermis and epidermis and devoided of exogenous extracellular matrix stained by hematoxylin and eosin. In particular, the picture highlights the epidermis appendage delimited by a basal membrane similar to the vitrea membrane delimiting the hair bulge in the native human skin (scale bar 100 µm).

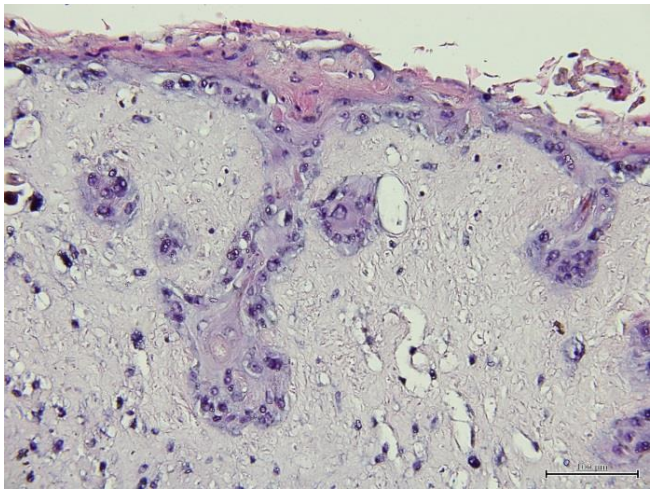

Figure 9. Shows immunofluorescent images indicating that Keratinocytes seeded on dermis equivalent, proliferated and when raised at the air-liquid interface, differentiated to produce a stratified, cornified epidermis. Moreover, in the upper part of dermis it is possible to observe epidermal appendages and cyst-like structure. Positive immuno staining with Keratin 10 assess the presence of suprabasal layer in the epidermis as well as in the appendages and cyst-like structure (scale bar 100µm). P63 positive immunostaining identifies keratinocytes stem cell in the in basal layer of epidermis as well as in the appendeages and cyst-like structure. Positive immuno staining with filaggrin assess the presence of the terminal layer of epidermis (stratum corneum) while DAPI stain all kinds of cells. Skin equivalent is featured by a well-organized epidermis, which expresses the characteristic differentiation markers observed in normal human skin (scale bar 50µm).

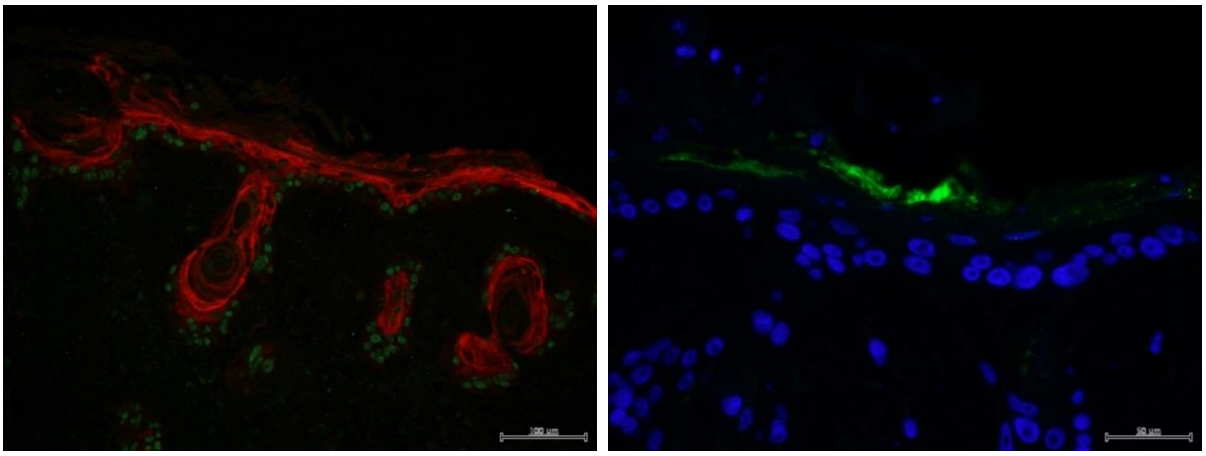

Figure 9

MARKET ANALYSIS

Human skin not only serves as an important barrier against the penetration of exogenous substances into the body, but also provides a potential avenue for the transport of functional active drugs/reagents/ingredients into the skin (topical delivery) and/or the body (transdermal delivery).

In the past three decades, research and development in human skin equivalents (HSEs) have advanced in parallel with those in tissue engineering and regenerative medicine. The HSEs are used commercially as clinical skin substitutes and as models for permeation and toxicity screening. Several academic laboratories have developed their own HSE models and applied these models for studying skin permeation, corrosion and irritation, compound toxicity, biochemistry, metabolism and cellular pharmacology. Various aspects of the state of the art of HSEs, including commercialization, are reviewed and discussed in a couple of recent papers by Zhang *et al. Pharmaceutics* 2012, *4*, 26-41, and by De Wever *et al. Household & Personal Care Today* 2013, *8(1),* 18-23, which the following paragraphs are largely taken and/or adapted` from.

The most significant and costly problem in healthcare today is the loss or failure of a tissue or organ. In recent years, tissue/organ transplantations of bone, tendon, cornea, heart valve, vein, skin, kidney, heart, lung, liver, pancreas, and intestine, *etc*., have been used clinically to save lives. Tissues and organs that are transplanted within the same person's body are called autografts; transplants that are performed between two individuals are called allografts. In very limited cases, the patients have suitable autografts for transplantation. For allografts, the demand for tissues and organs by far exceeds the supply, creating a substantial waiting list; moreover, the immune system tends to reject the foreign tissue or organ. Most organ recipients take immunosuppressive drugs for the rest of their lives; further, immunological imbalances caused by transplantation often lead, on the long term, in tumor growth. Therefore, there is a significant need for tissue engineering and regenerative medicine approaches aiming at generation of implantable or *in situ* forming tissues and organs.

HSEs are bioengineered substitutes composed of primary human skin cells (keratinocytes, fibroblasts and/or stem cells) and components of ECM (mainly collagen). In the last three decades, tremendous efforts have been devoted to the research and development of HSEs, resulting in a number of clinical products and skin models for pharmaceutical and cosmetic companies. In general, HSEs are applied in two major categories:

(a) as clinical skin replacements and grafts;

(b) as models for drug permeability tests and toxicity screening.

Skin injuries (from burn, accident, acute trauma or chronic wounds and diseases, etc.) can compromise skin barrier and lead to permanent disability or death of the injured person depending on the severity of the wound. Wound dressings that cover the site of the wound allow the re-epithelialization, remodeling of granulation tissue, and formation of scar tissue. However, full-thickness skin is not generated in this way. Clinical skin replacements and grafts are in high demand for the treatment of skin injuries: they represent approximately 50% of tissue engineering and regenerative medicine market revenues. In 2009, the potential US market for tissue-engineered skin replacements and substitutes totaled approximately USD 18.9 billion, based on a target patient population of approximately 5.0 million. By the year 2019, the total

potential target population for the use of tissue-engineered skin replacements and substitutes is expected to increase to 6.4 million, resulting in a potential US market of approximately USD 24.3 billion.

The other field of application for HSEs is as model for drug/ingredient permeability testing and toxicity screening. Animal testing of cosmetic ingredients is strictly limited in the EU; even if no alternative tests are available, the majority of the animal tests are banned. Human cadaver skin and excised animal skin have been traditionally used as topical and transdermal permeation models. Although human cadaver skin replicates *in vivo* permeation performance to some extent, there is a high sample to sample variation. Animal skin, though easily procured, is morphologically different to human skin. Therefore, there is a commercial need for better HSEs that serve as a suitable model for various skin tests.

For pharmaceutical companies, HSEs can provide specific skin models for diseases such as vitiligo, melanoma, squamous cell carcinoma, psoriasis, and blistering skin disorders as well as models for healthy skin; this is a unique advantage over the cadaver skin and animal skin specimens.

HSEs can be designed as epidermis only, dermis only, or full thickness (both dermis and epidermis) depending on the application. The ideal HSEs should have differentiated epidermis morphology, appropriate protein expression, similar lipid contents and lipid multi-lamellar structures as those of human skin; the HSEs should be easy to handle and transport, and should be easy to package and ship. When used as a permeation model for drugs/ingredients, the HSEs should produce consistent data that predict the permeation behavior of the tested compounds through human skin.

Table 1 summarizes the commercially available HSEs products.

Commercially Available Human Skin Equivalents for in Vivo Applications: Clinical Skin Replacements and Skin Grafts

Tissue engineered HSEs are the first commercially available and clinically applied organ substitutes. The success was achieved due to major advancements in keratinocyte cell biology, ECM biology, production of collagen scaffolds and polymeric scaffolds, and stem cell biology. When skin is wounded, a cascade of biological responses occurs after homeostasis: it begins with immune cells migrating to the site of injury, followed by fibroblasts generating a new tissue matrix; concurrently, re-epithelialization and re-vascularization occur.

Without a graft, a full-thickness skin damage of diameter larger than 4 cm is difficult to heal. Skin autografts are harvested from uninjured areas and then applied to the excised or debrided areas of the wounded skin of the same individual. Upon application of the skin graft, the capillary network of the wound will merge with the skin graft. However, several problems arise from skin autografts: significant scarring and pigmentation, often the dermis is not replaced, harvesting skin causes a new wound at the donor site, and extensive skin damage cannot be treated using skin autografts. Allogeneic skin grafts harvested from cadavers can also be used; however, they face immunogenic rejection and must be replaced. Therefore, bioengineered HSEs could be used to provide a more permanent solution.

Currently, several HSEs are commercially available for clinical applications. Examples include Apligraf®, Epicel®, Dermagraft®, Alloderm®, Transcyte®, Orcel®, Integra® DRT, and Epistem®; a few others are currently under clinical trial, one example is StrataGraft® developed by StrataTech Corp.

These HSEs can be divided into three major categories: epidermal, dermal, and full-thickness models.

Table 1. Summary of commercially available human skin equivalents.

Brand	Company	FDA Approval	Product Description
1. Clinical skin replacements and grafts			
Integra® DRT (Dermal Regeneration Template)	Integra Lifesciences	1996	Thin silicone film covering a porous matrix of cow collagen and glycosaminoglycan
Apligraf®	Organogenisis	1998	Fibroblasts and collagen combined in dermal matrix onto which keratinocytes are seeded to form an epidermal layer
Epicel®	Genzyme	2007	Autologous keratinocytes grown *ex vivo* in the presence of proliferation-arrested mouse fibroblasts
Transcyte®/Dermagraft®	Advanced Tissue Sciences/Advanced Biohealing	1997/2001	Cryopreserved dermal substitute: human fibroblast seeded onto polymer mesh and cultured *ex vivo*
Orcel®	FortiCell Bioscience	2001/2008	Human epidermal keratinocytes and dermal fibroblasts are cultured in separate layers into a Type I bovine collagen sponge
Alloderm®/Strattice®	LifeCell Co.	None	Acellular cadaver skin matrix
StrataGraft®	StrataTech	None	Full thickness skin substitute where a near-diploid human keratinocytes cell line, NIKS, was utilized.
2. *In Vitro* Permeation and Toxicity Screening Models			
SkinEthic Rhe (Reconstructed Human Epidermis)	SkinEthic		Human keratinocytes cultured on an inert polycarbonate filter at the air-liquid interface in chemically defined medium
Episkin	SkinEthic		Human keratinocytes cultured on a collagen base which permit terminal differentiation and reconstruction of the epidermis with a functional stratum corneum
Epiderm	MatTek		Neonatal human-derived epidermal keratinocytes (NHEK) cultured to form a multi-layered, highly differentiated model of the human epidermis
EpidermFT	MatTek		Neonatal human-derived dermal fibroblasts (NHFB) and NHEK co-cultured to form a multi-layered, highly differentiated model of the human dermis and epidermis
StrataTest	StrataTech		Full thickness skin model where a near-diploid human keratinocytes cell line, NIKS, was utilized.
Epidermal Skin Test 1000 (EST1000)	CellSystems Biotechnologie GmbH		Reconstructed epidermal model made from primary human keratinocytes; it comprises a fully differentiated epidermis with viable and cornifiedcell layers
Advanced Skin Test 2000 (AST2000)	CellSystems Biotechnologie GmbH		It comprises a dermal equivalent with embedded fibroblasts as a basis and epidermal layer of keratinocytes on top; it is a full thickness model.

Epidermal Models

Epidermal skin replacements require a 2-5 cm^2 skin biopsy from which the epidermis is separated and the keratinocytes are isolated and cultured on top of fibroblasts. Several companies offer epidermal HSEs including Genzyme's Epicel® (Cambridge, MA, USA). Epicel® is based on the use of a cultured epithelium prepared from autologous epidermal cells on grafts of burn wounds.

Dermal Models

Dermal skin replacements add greater mechanical stability and prevent the wound from contracting. Transcyte®, a product made by Advanced Tissue Sciences, Inc. (La Jolla, CA, USA), utilizes seeded neonatal human dermal fibroblasts in a polymeric scaffold that is then cryopreserved, making it a non-living wound covering. Transcyte® has been successfully used as a temporary wound covering after the burn wound has been excised. A derivative of this product, Dermagraft® by Advanced Biohealing, utilizes a biodegradable polygalactin mesh and has shown limited success in diabetic foot ulcer treatment.
LifeCell (Branchburg, NJ, USA) developed Alloderm® and Strattice®, intact acellular matrices produced from cadaver skin by removing epidermis and the antigenic cellular elements in the dermis. Often, autologous keratinocytes were seeded and cultured on Alloderm® to form epithelium, and the epithelium-Alloderm® structure can be applied for wound and burn closure.

A composite skin graft composed of an outer layer of thin silicone film and an inner layer constructed of a complex matrix of crosslinked fibers is marketed under the product name Integra® Dermal Regeneration Template (Integra® DRT, Integra Life Sciences Corp., Plainsboro, NJ, USA). Once the dermal layer is regenerated, the silicone film on the dermal layer can be removed and replaced with an epidermal autograft. Integra® DRT has been successfully shown to treat burns.

Full-Thickness Models

Full-thickness models of HSEs are composed of both epidermal and dermal layers; keratinocytes and fibroblasts, either autologous or allogeneic, are utilized to prepare the bilayer structures.
Histological data indicated that keratinocytes cultured for one week on fibroblasts that had been seeded and cultured in a sponge resulted in optimum proliferation and differentiation of keratinocytes and most closely resembled the histology of the epidermis *in vivo.* In addition, the fibroblasts in the dermal layer provided an ample support matrix for the keratinocytes.

An early example of full-thickness HSEs was bioengineered from a fibroblasts laced collagen lattice covered with epidermal cells. Based on this pioneer work, Organogenesis (Canton, MA, USA) became one of the first tissue-engineering companies and developed the bilayered skin model Apligraf®. Apligraf® is made of living allogeneic human skin fibroblasts that are obtained from human foreskins and soluble type I bovine collagen in the form of a gel seeded with keratinocytes. Apligraf® has been used in surgical wound healing, and venous ulcers, but not with major burns.

PermaDerm® (Regenicin, Inc) is a promising new product that can act as a permanent cover of large burns and injuries. It uses keratinocytes and fibroblasts seeded into a collagen sponge.

Commercially Available Human Skin Equivalents for in Vitro Applications: Models for Drug Permeability Tests and Toxicity Screening

HSEs are used as models for *in vitro* testing, and can demonstrate fundamental biological processes of the skin such as the examination of different stimuli that lead to the formation of the epidermis, stem cell maintenance, wound healing processes, the effect of corrosiveness of various chemicals on the skin, phototoxicity of substances, and toxicity of various chemicals without the need for animal testing.

Today, *in vitro* reconstructed skin models are used globally in both industrial and academic research laboratories. In general terms, the implementation of 3D *in vitro* reconstructed human tissue models makes it possible for pharmaceutical, chemical and consumer product companies to:

- test the efficacy of newly developed formulations and products and thus to use them for claim support;
- reproducibly differentiate compounds that cause reversible and irreversible toxicity without resorting to in vivo animal testing;
- provide accurate and measurable mechanistic information that can be utilized to determine whether a molecule or compound can be altered to reduce irritation or toxicity, without loss of efficacy;
- objectively compare the toxicity and efficacy of newly-developed compounds with those already in use in the market place for the same purpose;
- reveal the precursor steps which lead to toxic reactions, thereby making reformulations possible, based on human response;
- evaluate the long term stability or shelf-life of finished products or raw materials;
- determine the potential health risks of employees exposed to chemicals (worker safety).

Furthermore, the use of 3D skin models enables groups to conduct safety studies at a fraction of the cost and time required for animal or human testing. Additionally, the methodology is user and implementation friendly, does not require a large staff, extensive facilities, or supplies.

In recent years national and European legislation has placed considerable constraints on industry to totally abandon animal-based test systems when assessing the toxicological potential of chemicals and finished products. Both the “EU REACH directive (Registration, Evaluation, Authorization and Restriction of Chemicals), the “EU Classification, Labeling and Packaging (CLP) directive” and the “EU Cosmetics directive” demand for alternatives to animal testing. Hence they are creating a growing need for human reconstructed skin models, accelerating the adoption of these models in skin-related testing strategies required for regulatory purposes.

HSEs can be utilized to test the permeability of skin to various topically applied cosmetics/personal care agents and drugs. It is important for cosmetic and pharmaceutical companies to have a reliable *in vitro* screening system to test the amount of drugs/active ingredients that permeate into the epidermis, dermis, and across the membrane. In recent years, companies such as L’Oreal and SkinEthic have invested heavily in the development of skin models for pharmaceutical, cosmetic and chemical compound testing.

HSEs for *in vitro* permeation and toxicity applications can be divided into two major categories: namely epidermis-only models and full thickness models. In US, MatTek Corp. developed a serial of HSE models,

e.g., Epiderm® (human keratinocytes-derived multi-layered model of human epidermis), EpidermFT (human keratinocytes and dermal fibroblasts derived multi-layered model of human epidermis and dermis), MelanoDermTM skin model (based on co-culture of human keratinocytes and melanocytes), and Melanoma skin model (melanoma cells combined with EpidermFT). Also, StrataTech Corp. (Madison, WI, US) developed a full thickness StrataTest® skin model where a near-diploid human keratinocytes cell line, NIKS, was utilized.

In Europe, SkinEthic/L'Oreal (France) developed epidermis models SkinEthic® Rhe (Reconstructed human epidermis) and Episkin®, and full thickness model RealSkin®. Epidermal-skin-test 1000 (EST1000, an epidermis model) and Advanced-skin-test 2000 (AST2000, a full thickness model) were developed by CellSystems Biotechnologie GmbH (Germany). Very recently the name of the EST1000® model was changed into epiCS®.

For these HSEs, the differentiation of epidermis is a key factor determining their barrier properties. These validated alternative methods are listed in Table 2.

Table 2. Validated alternative methods where human skin equivalents (HSEs) can be applied as models in the skin corrosivity test and skin irritation test.

Brand of HSE Models	Company	US Regulatory Acceptance/Endorsement by NICEATM-ICCVAM [58]	EU Regulatory Acceptance/Endorsement by ECVAM [59]
Skin Corrosivity Test			
EpiSkin™	SkinEthic	OECD Test Guideline 431 accepted in 2004	Commission Regulation (EC) No 440/2008; OECD Test Guideline 431 (April 1998)
Epiderm™	MatTek	OECD Test Guideline 431 accepted in 2004	Commission Regulation (EC) No 440/2008; OECD Test Guideline 431 (March 2000)
SkinEthic™ Rhe	SkinEthic	OECD Test Guideline 431 (meets performance standards 2006)	Commission Regulation (EC) No 440/2008; OECD Test Guideline 431 (November 2006)
EST1000	CellSystems Biotechnologie GmbH	OECD Test Guideline 431 (meets performance standards 2009)	Commission Regulation (EC) No 440/2008; OECD Test Guideline 431 (June 2009)
Skin Irritation Test			
EpiSkin™	SkinEthic	OECD Test Guideline 439 accepted in 2010	Commission Regulation (EC) Nr 761/2009; OECD Test Guideline 439 (April 2007)
Epiderm™	MatTek	OECD Test Guideline 439 accepted in 2010	Commission Regulation (EC) Nr 761/2009; OECD Test Guideline 439 (April 2007; modified Skin Irritation Test Method validated in November 2008)
SkinEthic™ Rhe	SkinEthic	OECD Test Guideline 439 accepted in 2010	Commission Regulation (EC) Nr 761/2009; OECD Test Guideline 439 (November 2008)

NICEATM-ICCVAM: The National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM); ECVAM: European Center for the Validation of Alternative Methods; OECD: The Organisation for Economic Co-operation and Development.

Other Skin Models

In addition to the skin models described above which can be considered the most widely used models in industry today, several other in vitro models of human skin have been developed.

The Phenion® Full-Thickness Skin Model and the OS-REp model. The proprietary Phenion® Full-Thickness Skin Model (Phenion® FT model) was originally developed by scientists of the Henkel AG & Co. KGaA (Düsseldorf, Germany) in collaboration with the Universities of Frankfurt and Munich, and was introduced commercially in 2006. Human fibroblasts isolated from biopsies obtained from healthy donors are grown in a specially-produced stable matrix equivalent. After the development of this dermal equivalent, keratinocytes originated from the same donor are seeded on top. Within several days an epidermal tissue featuring all typical epidermal layers including a multilayered stratum corneum develops. The Phenion® Full-Thickness Skin Model exhibits structural and physiological properties comparable with native human skin. It is primarily used for efficacy studies, skin irritation, phototoxicity, dermal absorption, metabolism and genotoxicity studies. Henkel also launched the so-called 'Open Source Reconstructed Epidermis', or OS-REp model. The Open Source concept supports the idea of free public access to information and systems, currently best known in the computer software market. When applied to skin models "Open Source" means that, once a production method has been established, the know how becomes openly available in order to empower scientific groups throughout the world to produce the model 'internally', independent of any commercial supplier. To expand the scientific knowledge base of this model, it is intended to be used for the toxicological assessment of substances, thus making it a viable alternative to animal testing. Clearly, especially for regulatory purposes, validated SOP's and quality criteria for both OS model production as well as testing protocol have to be strictly respected. Recently the OS-Rep model underwent a successful catch-up validation for skin irritation assessment, which has been submitted to EURL-ECVAM for official regulatory acceptance.

The StratiCELL® skin model. The company Straticell, Les Isnes, Belgium, founded in 2005 as spin-out of the University of Namur, Belgium, has developed two proprietary in vitro skin models. The StratiCELL® reconstituted human epidermis with and without pigmentation, is composed of normal human keratinocytes and melanocytes cultured in a well-defined serum-free medium on a polycarbonate filter at the air-liquid interface. In addition, the company provides epidermal models from young/old & normal/diseased individuals (atopic dermatitis and psoriasis). Currently, the StratiCELL® skin model is preparing for catch-up skin irritation validation and the models were also evaluated for usefulness in nanotoxicology testing. Additional specific safety applications of the StratiCELL® skin model, for assessing genotoxicity and phototoxicity are currently being evaluated.

The LabCyte™ skin model. The LabCyte™ skin model (EPI-model) is produced by Japan Tissue Engineering Co., Ltd., Gamagori, Japan, by culturing human epidermal cells on inserts. After human epidermal cells have proliferated, exposure to the air-liquid intereface causes it to keratinize, creating a cultured epidermis model similar to the human epidermis. The EPI-model has undergone validation for skin corrosion in accordance with the OECD test guideline 431. More recently the LabCyte™ model has undergone a formal catch-up validation study for skin irritation and has been included into a new draft version of the OECD TG 439.

The Vitrolife-SKIN™ skin model. The Vitrolife-SKIN™ skin model, produced by Gunze Corporation Ltd., Kyoto, Japan, is a commercially available 3D reconstructed skin model, which is supplied as a kit containing 24 collagen sponges without cells and culture medium. The models need to be prepared in the laboratory (cultivation of cells in sponges) resulting in a 3D skin model composed of a dermis and an epidermis with cornified layers as described in the literature. The model was recently validated for corrosivity, and obtained the JACVAM (Japanese Centre for the Validation of Alternative Methods) validity statement that the Vitrolife-SKIN™ corrosivity assay complies with the OECD guideline TG 431.

BIOalternatives, Gençay, France, has developed an in-house reconstructed epidermis model, called EPI-Ba, however this skin model is mostly used for pre-clinical efficacy studies.

The Swiss-based company CellNtech Advanced Cell Systems AG, Bern, commercializes a 3D epidermal 'kit' which contains all necessary materials and reagents, including epidermal cells, to reconstitute a 3D epidermal model in the laboratory. More recently, Sterlab, a tissue engineering laboratory based in Sophia Antipolis, Nice, France, provides 3D tissue models 'on demand', including skin models using its proprietary chemically defined medium. Both epidermal, dermal-epidermal models with or without the presence of melanocytes, or Langerhans cells are proposed for research and preclinical testing applications, including toxicity and efficacy evaluations. Finally, the UK-based biotech company Evocutis has developed a full-thickness skin equivalent called LabSkin™, consisting of a fully differentiated epidermis on top of a dermal compartment composed of fibroblasts embedded in a bioartificial matrix. Originally developed as a platform to study the microbial system of human skin in vitro and eventually to develop anti-microbial products, the applications of LabSkin™ have extended to wound healing studies, basic and applied skin research, structural, metabolic and physiological studies and the testing of ingredients and product efficacy.

Development of Non-Commercial Human Skin Equivalents

Several academic laboratories have developed their own HSEs models. The Stark Group from the German Cancer Research Center (Heidelberg, Germany) has developed a model for *in vivo* study of long-term skin reconstruction and epidermal function. The effect of fibroblasts and microenvironment on epidermal regeneration and tissue function was investigated. The results indicated that: (a) the presence of fibroblasts, and the keratinocyte-fibroblast interactions play a critical role in epidermal tissue regeneration; (b) maintaining a correct microenvironment for epidermal tissue function is important. The HSEs model was also used to study the epidermal homeostasis and to provide experimental conditions for establishing a stem cell niche *in vitro*.

The Ponec, Bouwstra, and EI-Ghalbzouri groups in The Netherlands have developed the Leiden Human Epidermal (LHE) model, and have utilized it for the evaluation of skin corrosion of chemical compounds in accordance to European Center for the Validation of Alternative Methods (ECVAM) guidelines for testing the corrosive characteristics of chemical compounds. In a recent study performed by EI-Ghalbzouri and Bouwstra groups, the barrier properties of two novel HSEs, the fibroblast-derived matrix model (FDM) and the Leiden epidermal model (LEM), were compared with the full-thickness collagen model (FTM) and human skin. The results demonstrated that the barrier function of the FDM and LEM improved compared with that of the FTM, but all HSEs were more permeable than human skin.

The Morgan group (Boston, Massachusetts) has analyzed the effect of growth factors on cell proliferation for tissue engineering applications. Their findings have indicated that keratinocyte growth factors (KGF)

delayed differentiation and induced hyperproliferation. The KGF led to hyperthickening, crowding, and elongation of basal cells without disrupting the barrier function of the epidermis.

Recently, the Michniak group developed a full-thickness HSE model to serve as a permeation model for topical and transdermal formulations. The dermis of the model consists of human fibroblasts and bovine collagen; on top, keratinocytes are seeded and cultured at air-liquid interface, and differentiate into a highly differentiated epidermal layer. Michniak *et al.* reported that by adding clofibrate, ascorbic acid, and fatty acids into the growth media, lipid composition was improved with values obtained closer to that of human skin. The model overestimated (as all the other HSEs at present do) the permeation of a number of compounds including caffeine, hydrocortisone, ketoprofen, DEET, malathion, and paraoxon by 2-7 fold when compared to human skin and matched the permeation profiles ($p < 0.05$) of EpidermFT®.

In summary, whereas the skin has been successfully reproduced using tissue engineering techniques, its barrier properties are still low, resulting in an overestimation of compound permeability. Most models still lack appendages and a blood supply although melanocytes have been successfully added. The regeneration of hair follicles and sebaceous glands will be a challenge. There is evidence that introducing sebaceous glands into HSEs is a possibility. Another interesting aspect is the regeneration of blood vessels and nerves. The use of stem cells for epidermal differentiation has been explored, but it is far from commercialization and clinical applications. Many more studies have to be performed to optimize the current skin models for both clinical use as well as compound permeability testing.

COMPETITIVE SCENARIO

Leading Companies in the Human Skin Equivalent (HSE) Market

- Shire Regenerative Medicine Inc. (USA, https://www.shire.com/)
- Advanced Tissue Sciences Inc. (USA, http://www.advancedtissue.com/),
- BIOalternatives (France, http://www.bioalternatives.com/),
- CELLnTECH Advanced Cell Systems AG (Switzerland, http://cellntec.com/),
- CellSystems Biotechnologie GmbH (Germany, http://cellsystems.de/),
- Evocutis (UK, http://www.evocutis.com/),
- Genzyme (USA, http://www.genzyme.com/),
- Gunze Corporation Ltd. (Japan, http://www.gunze.co.jp/english/corporate/outline/index.html),
- Henkel AG & Co. KGaA (Germany, http://www.henkel.com/index.htm),
- Integra Life Sciences Corp. (USA, http://www.integralife.com/),
- Japan Tissue Engineering Co.Ltd. (Japan, http://www.jpte.co.jp/english/),
- LifeCell Corp. (USA, http://www.lifecell.com/),
- MatTek Corp. (USA, http://www.mattek.com/),
- Organogenisis Inc. (USA, http://www.organogenesis.com/),
- L'Oreal (France, http://www.lorealparisusa.com/),
- Regenicin Inc. (USA, http://www.regenicin.com/),
- SkinEthic (France, http://www.skinethic.com/),
- Sterlab (France, http://www.sterlab.com/),
- StrataTech Corp. (USA, http://www.stratatechcorp.com/),
- Straticell (Belgium, http://www.straticell.com/en).

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