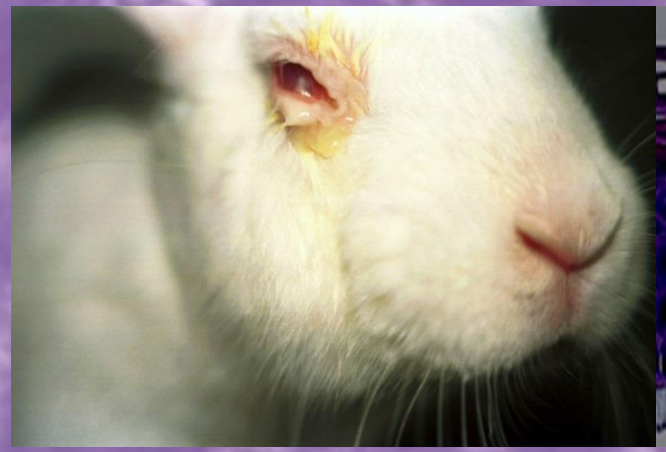
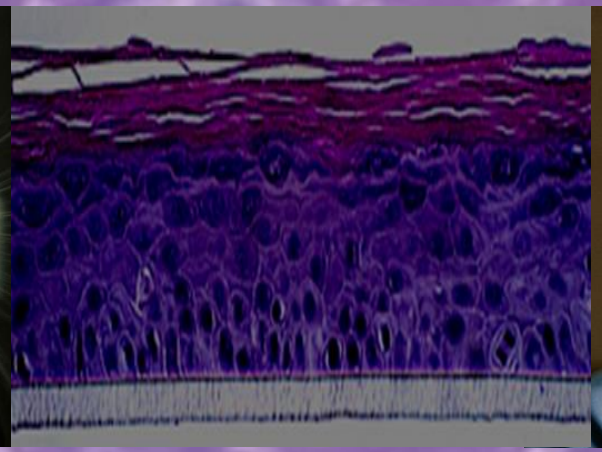
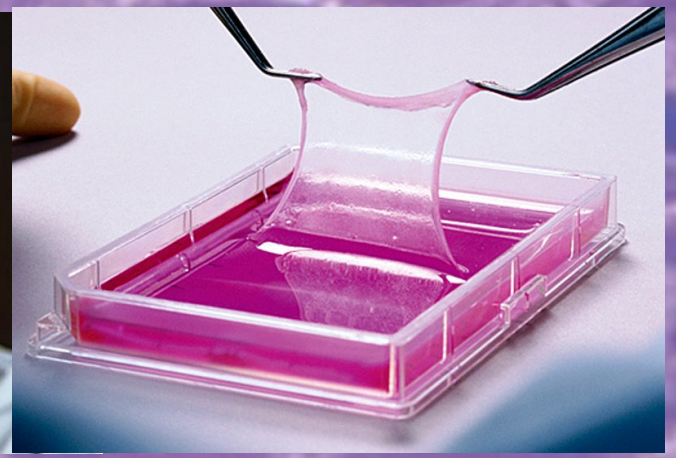
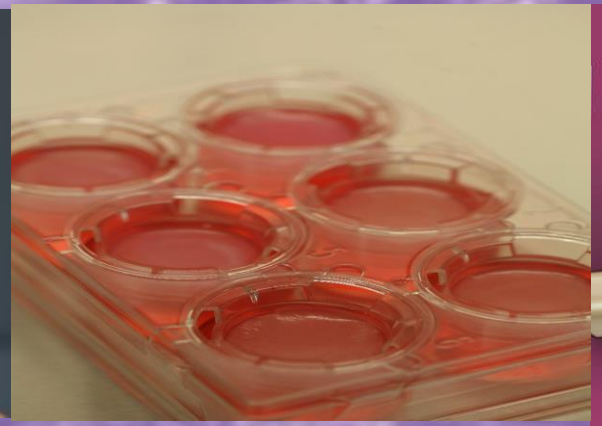
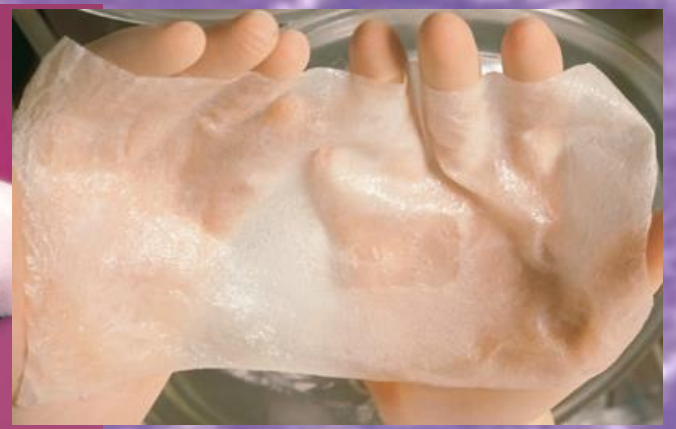





Artificial Skin for Cosmetic Testing



Summary

- Human Skin
  - Functions
  - Structure
  - Most relevant properties
- History and development of skin substitutes
- Cosmetic testing
- Commercially available artificial skin
  - SkinEthic
  - Epiderm
  - Episkin
- Future perspectives: generation of human epidermal equivalents (HEEs) with a functional permeability barrier

Skin

- The **human skin** is the outer covering of the body.
- It is the largest organ of the integumentary system (its surface is about 2 m^2) and it guards the underlying muscles, bones, ligaments and internal organs from external environment.
- It is the largest sense organ in the human body.
- It is composed of three primary layers: the epidermis; the dermis; the hypodermis *(subcutaneous adipose layer)*.

Functions

Skin performs the following functions:

- **Protection**: an anatomical barrier from pathogens and damage between the internal and external environment in bodily defense.
- **Control of evaporation**: the skin provides a relatively dry and semi-impermeable barrier to fluid loss. Loss of this function contributes to the massive fluid loss in burns.
- **Heat regulation**: the skin contains a blood supply far greater than its requirements which allows precise control of energy loss by radiation, convection and conduction. Dilated blood vessels increase perfusion and heatloss, while constricted vessels greatly reduce cutaneous blood flow and conserve heat. Excretion by **sweating** is at most a secondary function to temperature regulation.
- **Storage and synthesis**: acts as a storage center for lipids and water, as well as a means of synthesis of vitamin D by action of UV on certain parts of the skin.

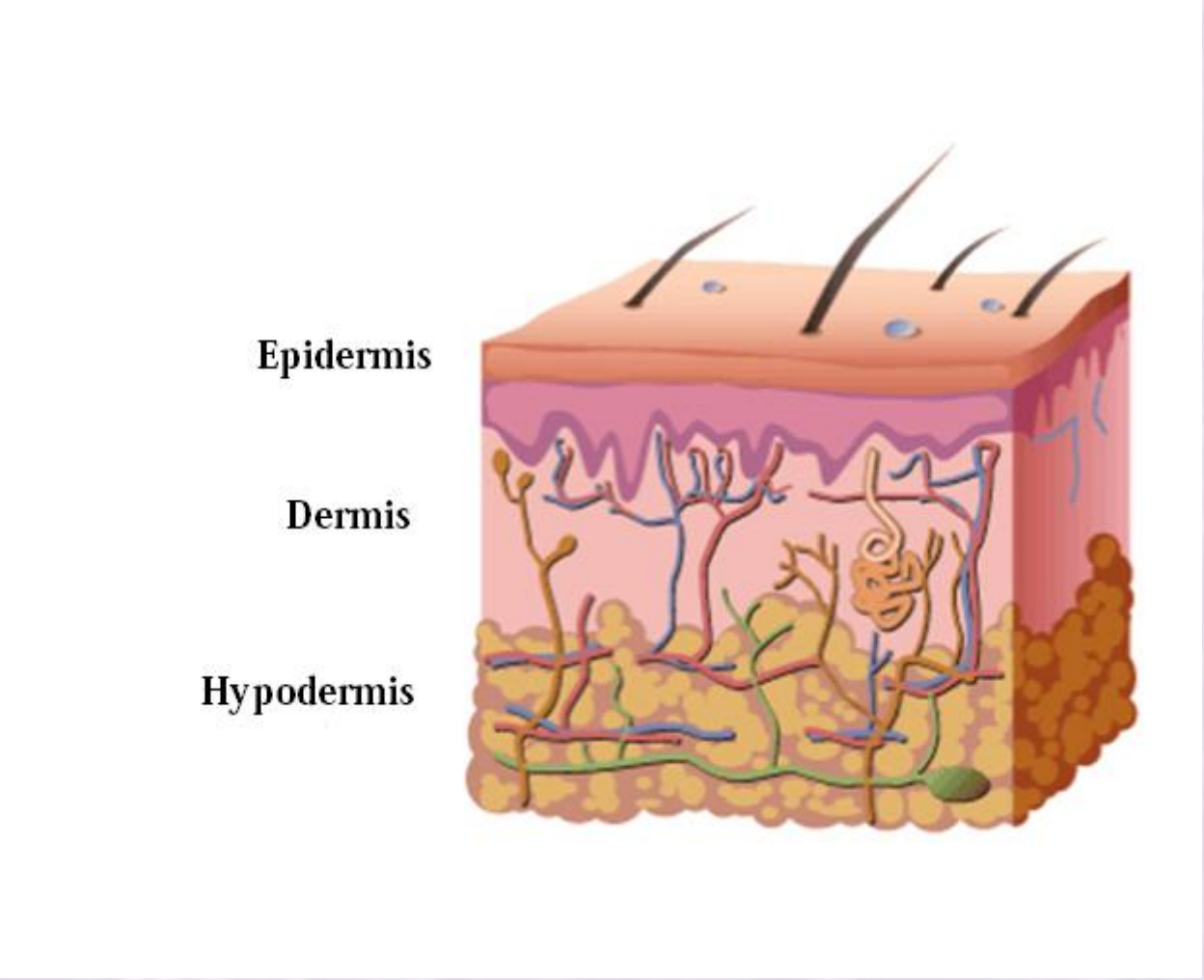
Structure

Skin is composed of three primary layers:

- **Epidermis:** is the outermost layer of the skin
- **Dermis:** connective tissue
- **Hypodermis**: its purpose is to attach the skin to underlying bone and muscle as well as supplying it with blood vessels and nerves. It consists of loose connective tissue, adipose tissue and elastin.

thickness: 2–4 mm

pH acid: 4,2 – 5,6

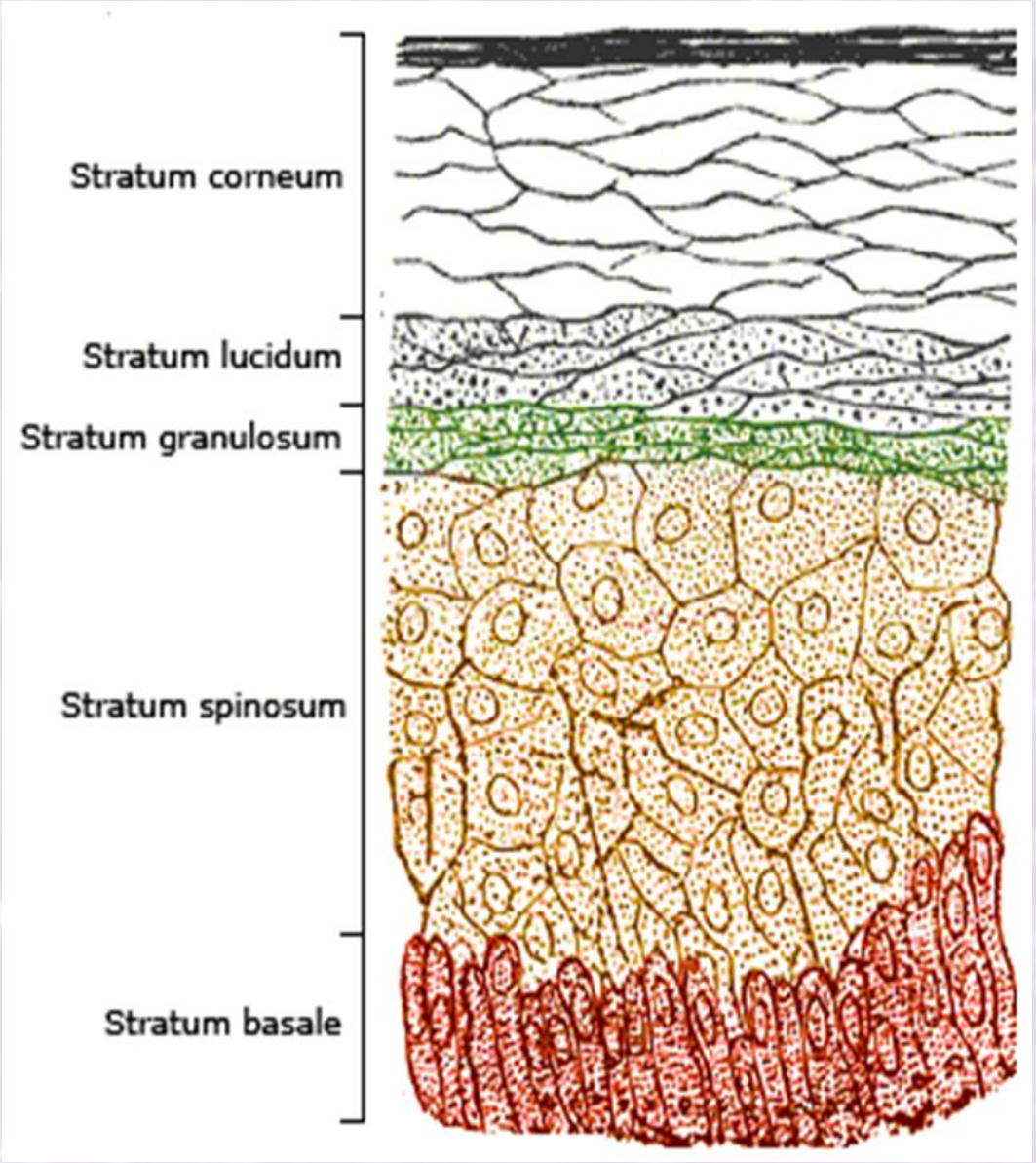


The epidermis can be further subdivided in five strata. Cells are formed by mitosis at the basale layer. The daughter cells move up the strata changing shape and composition as they die due to isolation from their blood source.

They eventually reach the corneum and slough off (desquamation).

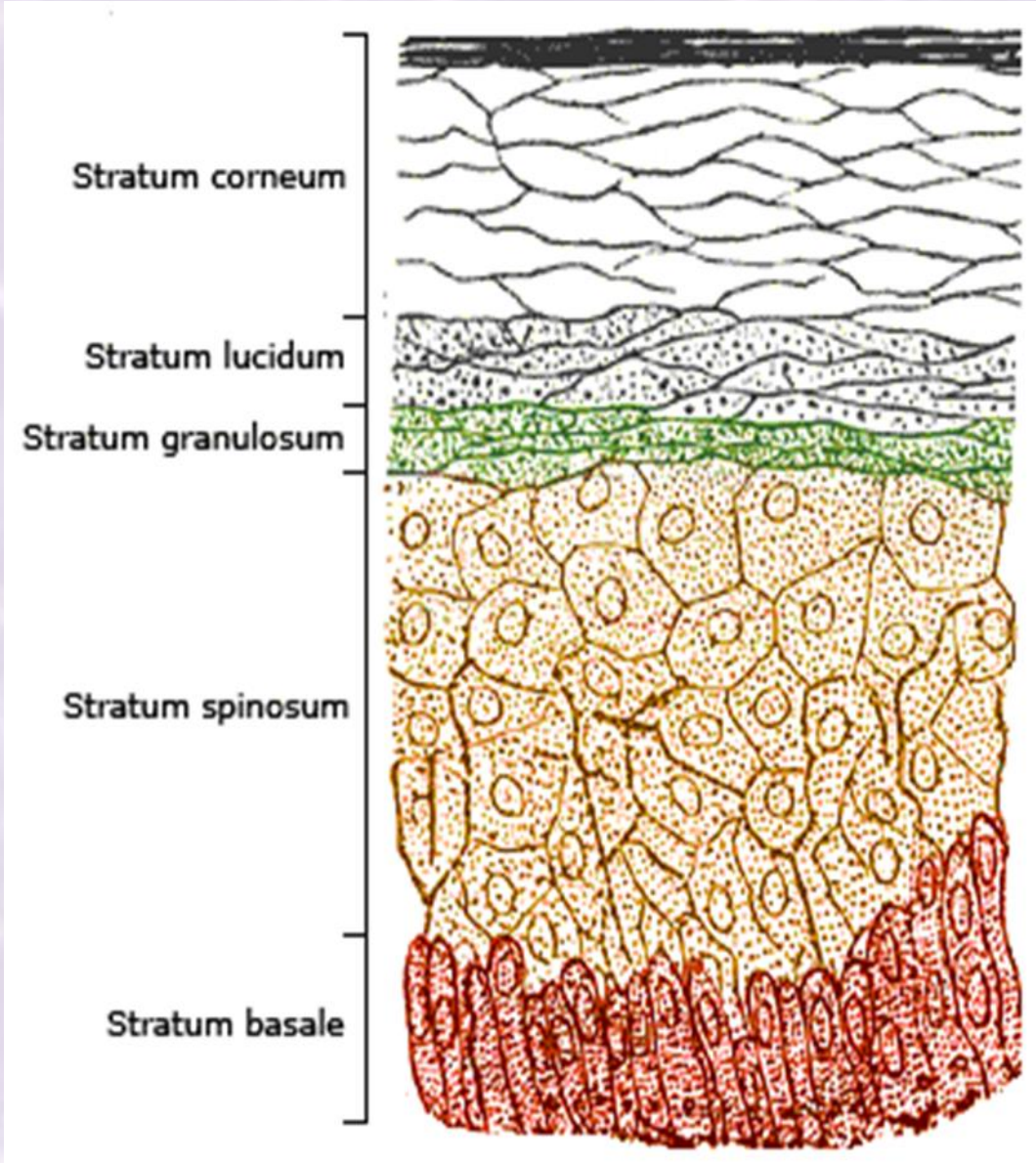
Epidermis

- **Stratum basale**: It is the deepest layer of the five epidermis layers, composed mainly of proliferating and non-proliferating keratinocytes, attached to the basement membrane by desmosomes. The adhesion with the basement membrane is mediated by integrins.
- **Stratum spinosum**: It is a layer of the epidermis found between the stratum granulosum and stratum basale. This layer is composed of polyhedral keratinocytes (5-10 rows).
- **Stratum granulosum**: Keratinocytes lose their nuclei and their cytoplasm appears granular. Lipids are contained into those keratinocytes within lamellar bodies. Those polar lipids are then converted into non-polar lipids and arranged parallel to the cell surface. For example glycosphingolipids become ceramides and phospholipids become free fatty acids is composed of 4 or 5 layers of cell of flattened form. This layer represents the last layer with living cells.



- **Stratum lucidum:** Layer with keratin filaments with 1-3 rows of cells rich in **eleidin** (rich of sulfur, glycogen and lipids). The cells are translucent. This layer contributes to the waterproofing of the skin.

- **Stratum corneum**: It is the outermost layer of the epidermis, consisting of dead cells (corneocytes). This layer is composed of 15-20 layers of flattened cells with no nuclei and cell organelles. This complex surrounds cells in the *stratum corneum* and contributes to the skin's barrier function. Intercellular space is occupied by the lipids released by the spinous layer. These cells are sometimes called corneus lamellae or corneocytes .

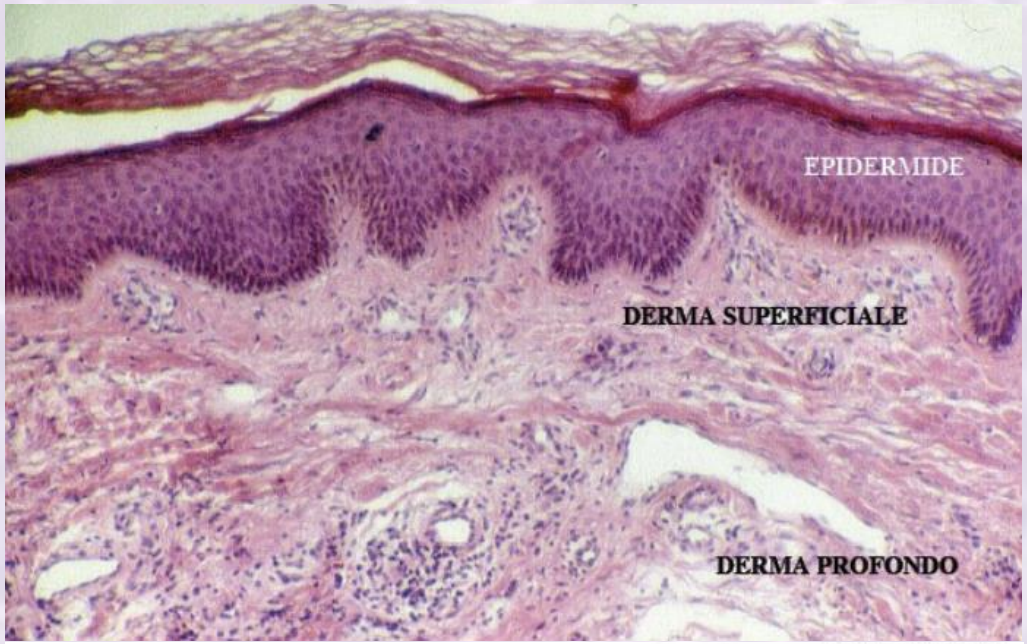


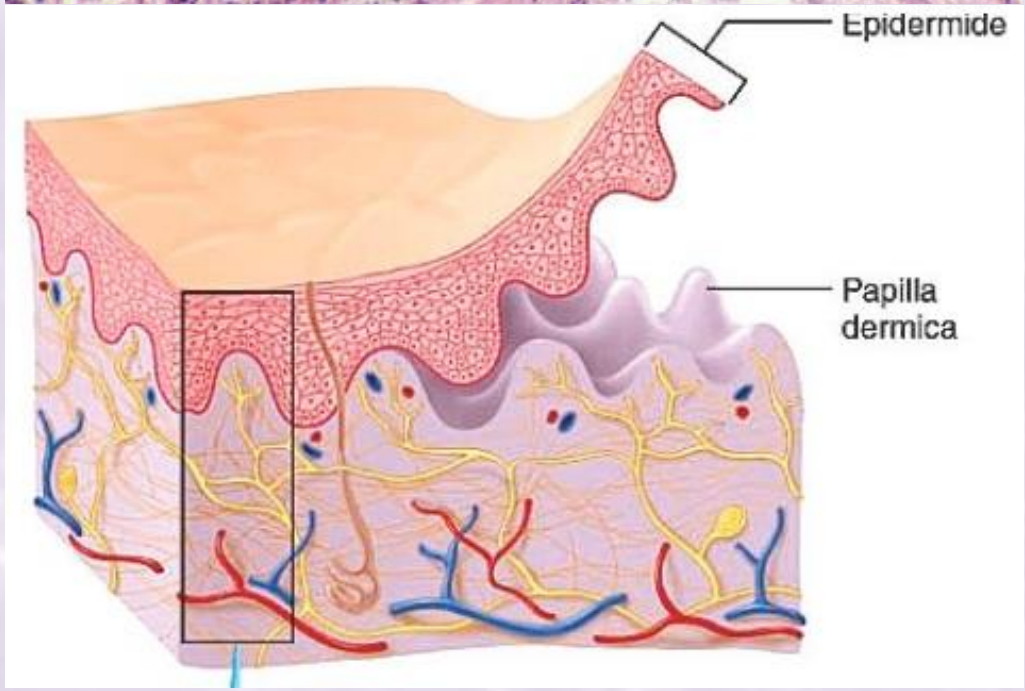
Dermis

The **dermis** is a layer of skin between the epidermis and subcutaneous tissues.

It is composed by two layers:

- **<u>Superficial Dermis</u> (papillary region)** consists of connective tissue, connected to the epidermis through a basement membrane. The papillary region is composed of loose areolar connective tissue. This is named for its fingerlike projections called *papillae*, that extend toward the epidermis and contain either terminal networks of blood capillaries or tactile Meissner's corpuscles.
- **<u>Deep Dermis</u> (reticular region)** consists of connective irregular tissue with protein fibers responsible of dermis properties (force, strength, extensibility, and elasticity).



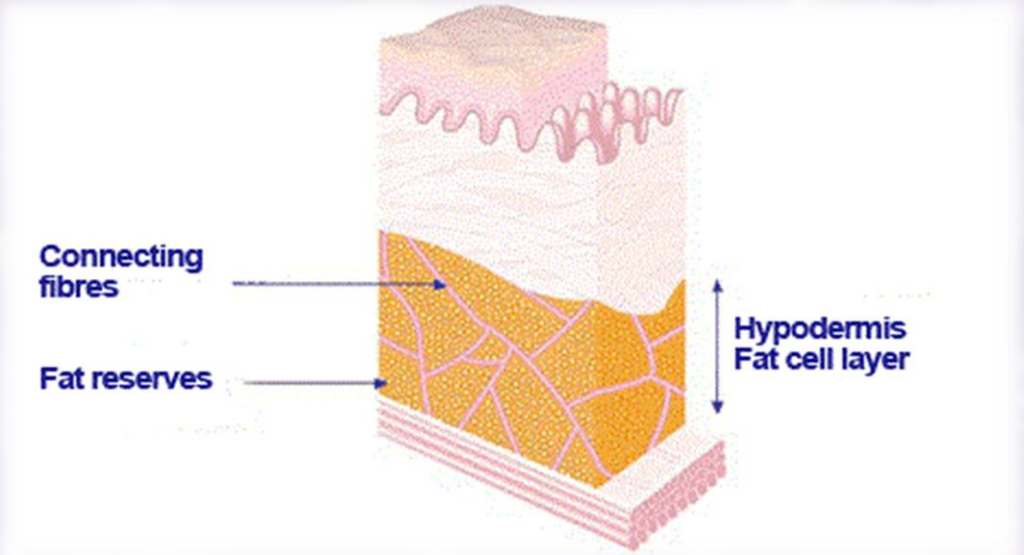


Hypodermis

It is the lowest layer of the integumentary system in human skin, whose function is to attach the skin to underlying bone and muscle. It is composed of lipid cells (**triglycerides**), called **adipocytes**. The thickness range is between 0,5 - 2 cm.

The types of cells found in the hypodermis are fibroblasts, adipose cells and macrophages

It is used mainly for insulating and fat storage.

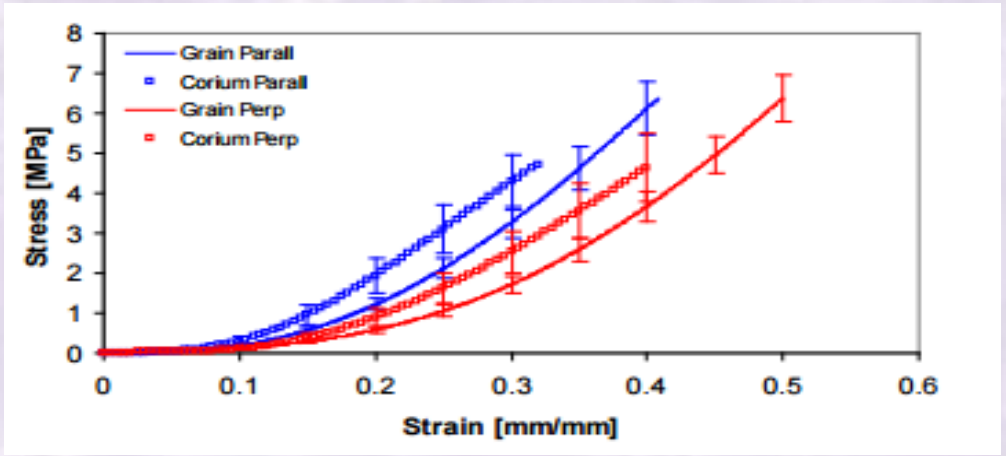


Composition and mechanical properties

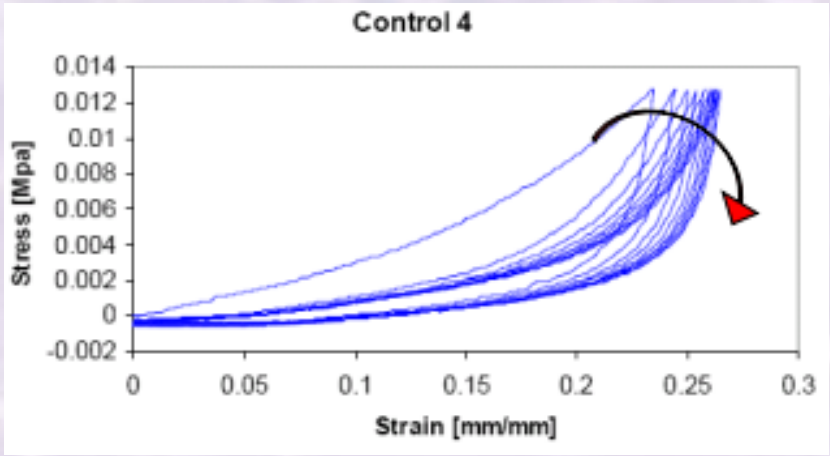
Constituted by

- **Cells**: Lymphocyte, Macrophage e Fibroblast (main component)
- **Collagen Fiber**: inextensible and very resistant to tension;
- **Elastic Fiber:** costituited by microfibrills tubular (consisting of fibrillin, inextensible) and by an amorphous matrix (costituited in elastin)

These components give to the skin properties of tensile and strength.



High extensibility for small stresses



Gradual adaptation to the load

Permeability of the Skin

Human skin has a **low permeability**; that is, most foreign substances are unable to penetrate and diffuse through the skin. Skin's outermost layer, the **stratum corneum**, is an effective barrier to most inorganic nanosized particles. This protects the body from external particles such as toxins by not allowing them to come into contact with internal tissues. However, in some cases it is desirable to allow particles entry to the body through the skin. Potential medical applications of such particle transfer has prompted developments in nanomedicine and biology to increase skin permeability. One application of transcutaneous particle delivery could be to locate and treat cancer. Nanomedical researchers seek to target the epidermis and other layers of active cell division where nanoparticles can interact directly with cells that have lost their growth-control mechanisms (cancer cells). Such direct interaction could be used to more accurately diagnose properties of specific tumors or to treat them by delivering drugs with cellular specificity.

CHARACTERISTICS OF THE IDEAL SKIN SUBSTITUTES

- Able to resist infection
- Able to withstand wound hypoxia
- Cost-efficient
- Easy to prepare
- Easy to store
- Easy to use
- Flexible in thickness
- Lack of antigenicity
- Offers long-term wound stability
- Provides permanent wound coverage
- Recreates dermal and epidermal components
- Able to resist shear forces
- Widely available

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WHY SKIN SUBSTITUTES?

SKIN SUBSTITUTES: A heterogeneous group of substances that aid in the closure of many types of wounds

Skin **defects** induce water, electrolytes and protein loss from the wound site and may allow bacteria to invade

- Burns
- Venous ulcer
- Diabetic ulcer
- Acute injury

The phrase "**tissue engineering**" was adopted by the Washington National Science Foundation bioengineering panel meeting in 1987: "*The application of the principles and methods of engineering and the life sciences toward the development of biological substitutes to restore, maintain, or improve tissue or organ function.*"

HISTORY OF SKIN SUBSTITUTES:

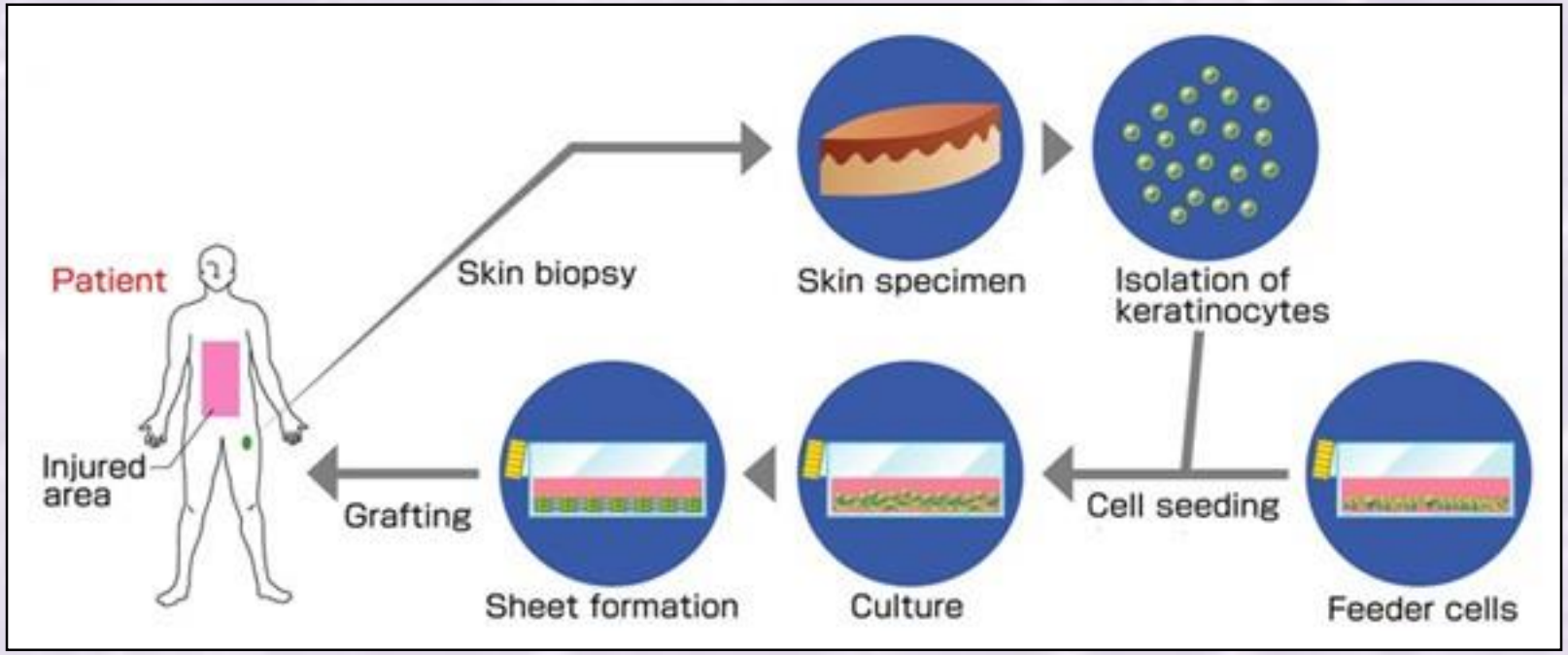
XENOGRAFTS → ALLOGRAFTS and AUTOGRAFTS → TISSUE-ENGINEERED SKIN SUBSTITUTE

Grafts of tissue transplanted between animals of different species

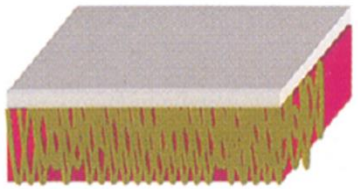
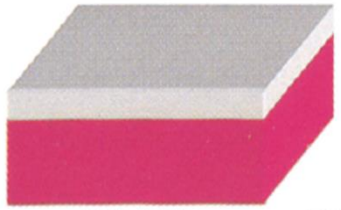
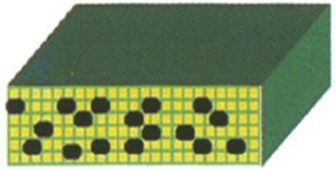
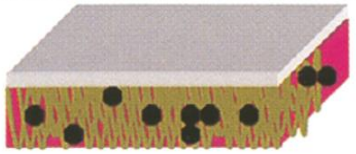
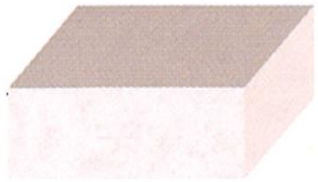
- Effective
- Limited by the availability of donor tissue
- Rejection

MILESTONES:

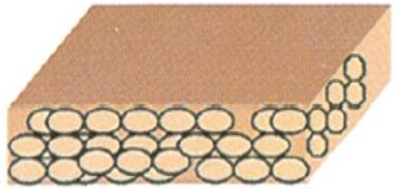
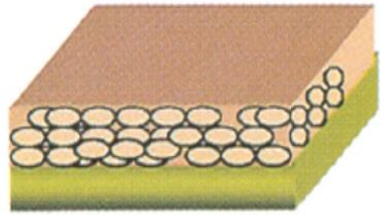
- **Karasek** demonstrated that keratinocytes could survive in tissue culture (1968).
- **Green** and **Rheinwald** published their work on the growth and proliferation of human keratinocytes on 3T3 cells (1975).
- **O'Conner** reported the first success in covering burn defects with cultured autologous epithelium (1981).



ONLY DERMAL COMPONENTS

Trade Name	Schematic Representation	Layers
Biobrane™ (Dow Hickam/Bertek Pharmaceuticals, Sugar Land, TX).		1. Silicone 2. Nylon Mesh 3. Collagen
Integra® (Integra Life Science Corporation, Plainsboro, New Jersey)		1. Silicone 2. Collagen and glycosaminoglycan
Dermagraft® (Advanced Tissue Sciences, Inc. La Jolla, California, USA)		1. Polyglycolic acid (Dexon™) or polyglactin-910 (Vicryl™) seeded with neonatal fibroblasts
Transcyte® (Advanced Tissue Sciences, Inc. La Jolla, California, USA)		1. Silicone 2. Nylon Mesh 3. Collagen seeded with neonatal fibroblasts
Alloderm® (LifeCell,Woodlands, Texas.)		1. Acellular de-epithelialised cadaver dermis

ONLY EPIDERMAL COMPONENTS

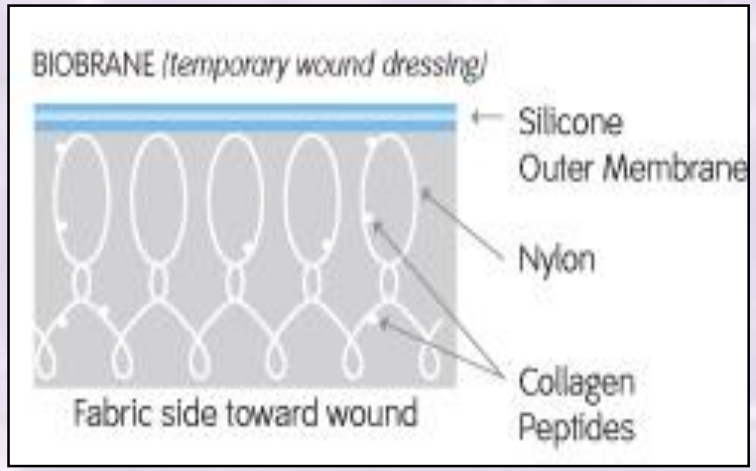
Trade Name	Schematic Representation	Layers
Epicel™ (Genzyme tissue repair corporation, Cambridge, MA.)		1. Cultured autologous keratinocytes
Laserskin™ (Fidia Advanced Biopolymers, Italy) also marketed as Vivoderm™ by ER Squibb & sons Inc,		1. Cultured autologous keratinocytes 2. Hyaluronic acid with laser perforations

COMPOSITE GRAFTS

Trade Name	Schematic Representation	Layers
Apligraf® (Organogenesis Inc, Canton, MA and Novartis Pharmaceuticals Corporation, East Hanover, NJ)		1. Neonatal keratinocytes 2. Collagen seeded with neonatal fibroblasts

ONLY DERMAL COMPONENTS (1)

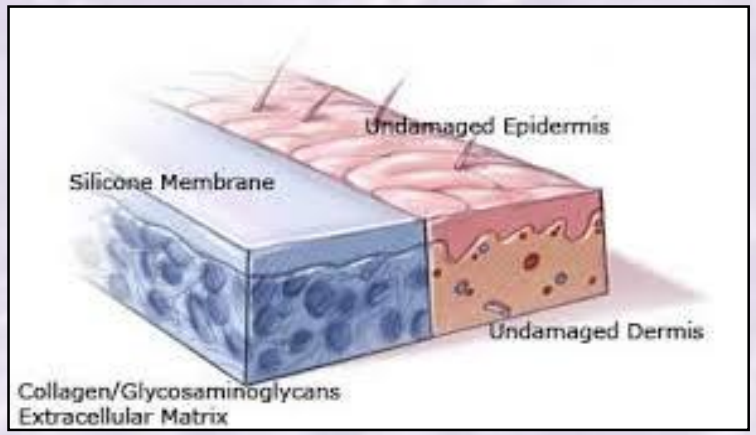
➢ BIOBRANE



- Nylon mesh bonded to a thin layer of silicone.
- The mesh is coated with porcine type I collagen-derived peptides (dermal analogue).
- Small pores are present to make Biobrane semipermeable to allow transudates to escape.

Since its development in 1979, Biobrane has become the standard for skin substitute coverage. It was first introduced as a low-cost alternative to cadaveric skin (allograft) for temporary coverage of wounds.

➢ INTEGRA

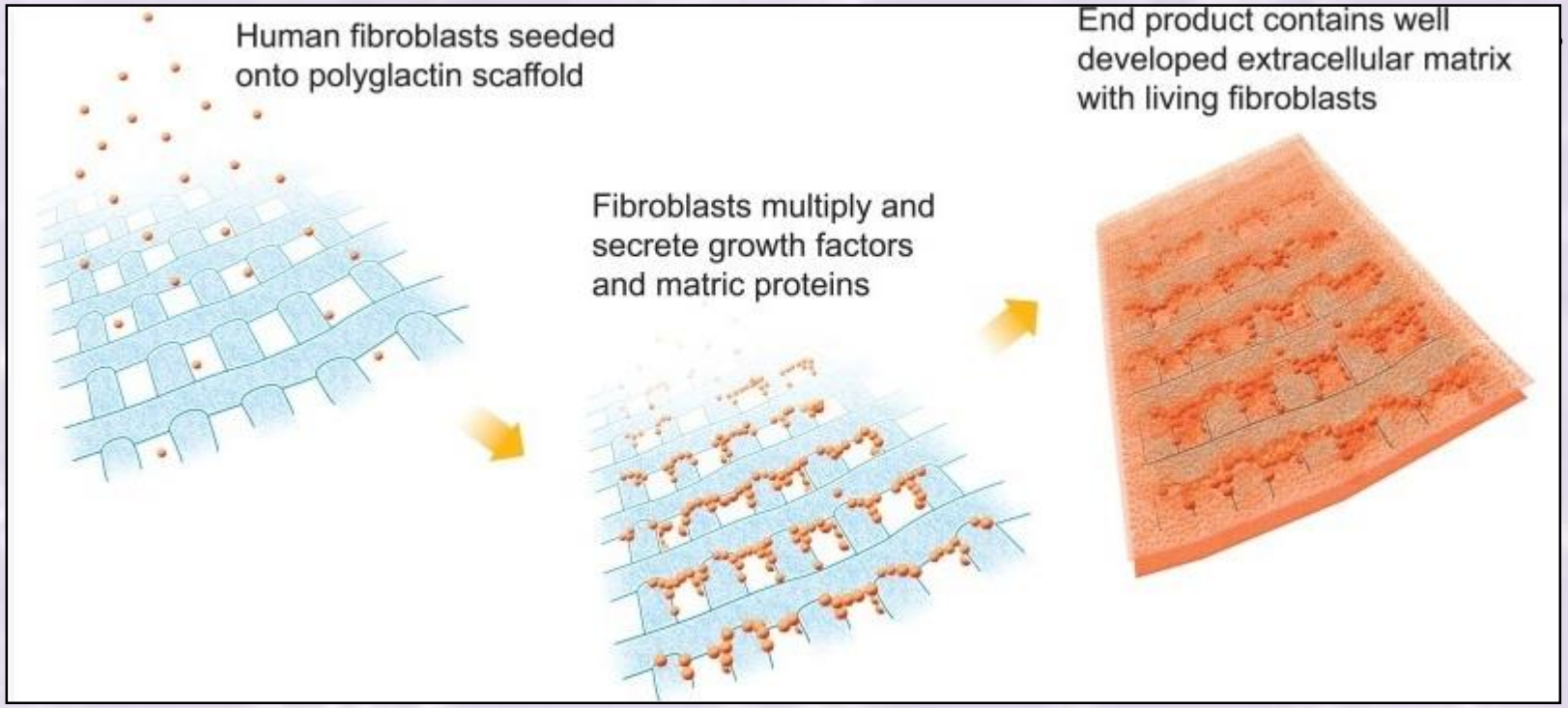


- Synthetic bilayer acellular skin substitute.
- Outer silastic sheet and a matrix composed of bovine collagen and glycosaminoglycan (dermal analogue).
- The dermal matrix is engineered to have a pore size of 20 to 50 micrometers to promote fibroblast and endothelial cell ingrowth by the host wound bed.

The wound gradually remodels and resorbs the matrix to create neodermis (it takes 3 to 6 weeks). After adequate vascularization of the matrix has occurred and the neodermis has formed, the silastic sheet may be removed.

ONLY DERMAL COMPONENTS (2)

- **DERMAGRAFT**



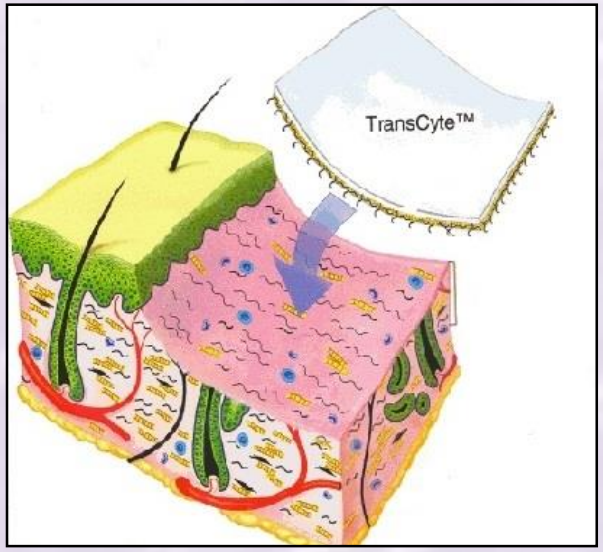
Sterile, **cryopreserved**, human fibroblast–derived dermal substitute generated by the culture of neonatal dermal fibroblasts onto a bioresorbable polyglactin polymer (Vicryl) scaffold.

Human fibroblasts proliferate to fill the interstices of this scaffold and secrete collagen, other extracellular matrix proteins, growth factors, and cytokines, creating a 3D human-derived dermal substitute containing metabolically active, **living cells**.

The process lasts about 14-17 days to produce a dermis-like matrix.

ONLY DERMAL COMPONENTS (3)

➢ **TRANSCYTE (Dermagraft-TC)**



- Newborn human fibroblast cells cultured under aseptic conditions in vitro on a nylon mesh bonded to a polymer membrane (silicone). Prior to cell growth, this nylon mesh is coated with porcine dermal collagen.

As fibroblasts proliferate within the nylon mesh during the manufacturing process, they secrete human dermal collagen, matrix proteins and growth factors. Following **freezing**, no cellular metabolic activity remains; however, the tissue matrix and bound growth factors are left intact.

➢ **ALLODERM**

- Made of salt processed human cadaveric skin: the patented process removes all of the cells from the donated skin, while retaining all of the important biochemical and structural components.
- Decellularized.

Use of human donor tissue → Risk of infectious disease transmission

However, with more than one-million implants and grafts to date, **the safety of AlloDerm has been proven**.

ONLY EPIDERMAL COMPONENTS

➢ EPICEL

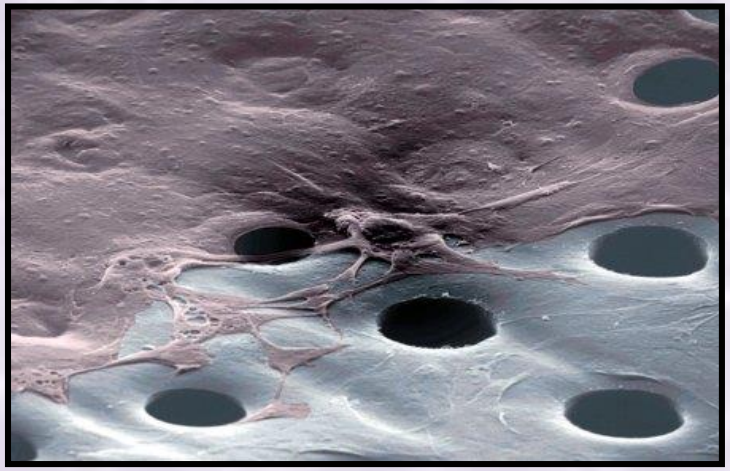
- Autologous cultured keratinocytes product.

A small skin biopsy sample can generate a large area of cultured epidermis. Even cultured keratinocytes allografts was developed to avoid the 2-3 weeks occurred to the creation of right quantities of autologous epidermis.

Both keratinocytes autografts and allografts are thin, **fragile** and lack a dermal component.

Dermal elements play an important role in the wound healing process

➢ LASERSKIN



- Autogenous keratinocytes from the patient are cultured in a lab and seeded onto membrane consisting of 100% esterified hyaluronic acid, which is microperforated.

Scaffold made of an innovative biomaterial:

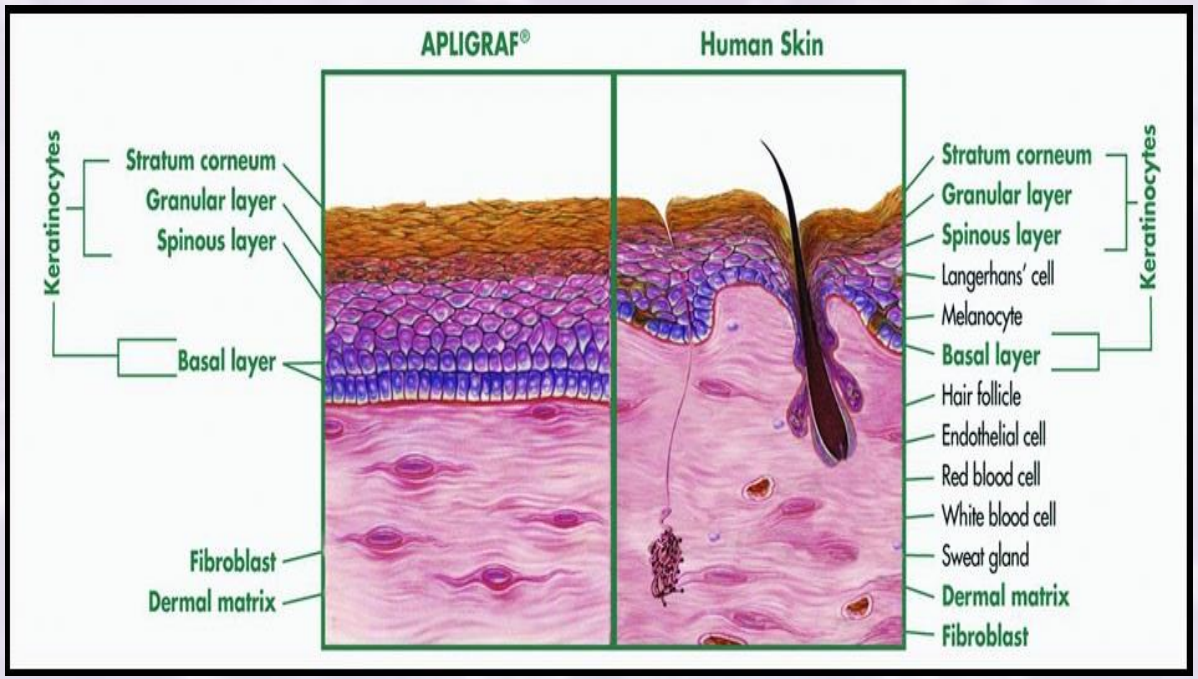
HYAFF →
- Controlled release of hyaluronic acid
- Structural function
- Biodegradable

COMPOSITE GRAFTS

- **APLIGRAF**

Like human skin, Apligraf consists of **living cells** and **structural proteins**.

- The lower dermal layer combines bovine type 1 collagen and human fibroblasts (dermal cells), which produce additional matrix proteins.
- The upper epidermal layer is formed by a suspension of keratinocytes (epidermal cells) that are allowed first to multiply and then **<u>to differentiate</u>** to replicate the architecture of the human epidermis.



Exposure to air to promote keratinocyte differentiation, and the formation of a stratum corneum

NOT CONTAINED:
melanocytes, Langerhans' cells, macrophages, lymphocytes, structures such as blood vessels, hair follicles or sweat glands.

NOT ADVERSE HOST RESPONSE

Definition of **cosmetic**:

“Articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body...for cleansing, beautifying, promoting attractiveness, or altering the appearance“.

The definition includes products for makeup, shampoos, soaps etc

Is it fair to use animals in cosmetic testing?

In most countries outside UE and a few years ago also in Europe, every new ingredient of a cosmetic and every finished product had to go through a series of animal tests (on dogs, rats, mice, guinea pigs and rabbits mainly).

Type of tests - short-term tests

- long-term tests

Short-term tests

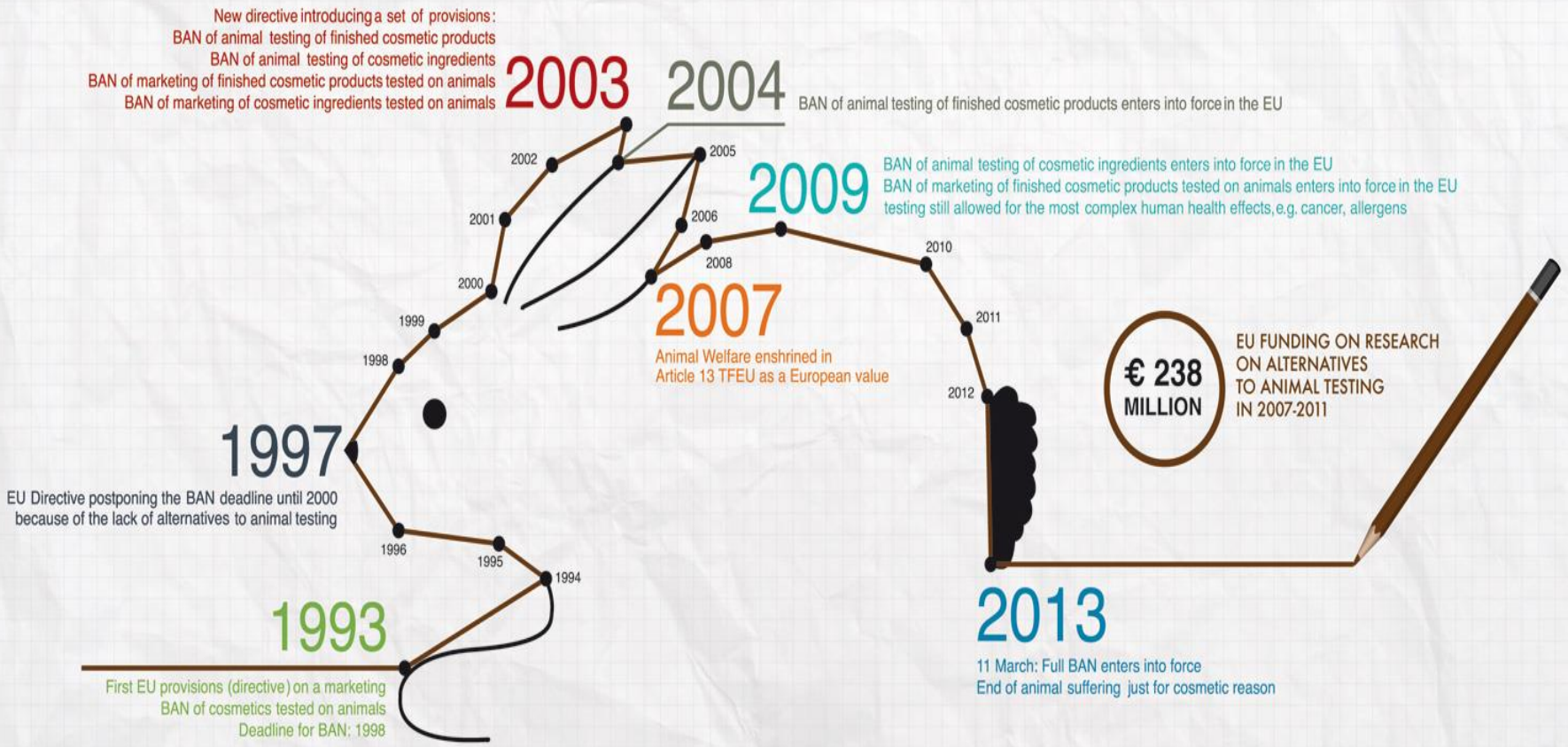
1. Skin sensitization (32 guinea pigs or 16 mice)
2. Skin irritation/ corrosion (1-3 rabbits)
3. Eye irritation/ corrosion (1-3 rabbits)
4. Acute oral toxicity (7 rats)
5. Acute dermal toxicity (20 rats, rabbits or guinea pigs)
6. Acute inhalation toxicity (20 rats)

Long-term tests

1. Repeat dose (28 day) and subchronic (90 day) toxicity (40 rats or 80 rats)
2. Carcinogenicity or combined carcinogenicity/ chronic toxicity (400 mice or rats)
3. Toxicokinetics (4-12 rats)
4. Reproductive/ developmental screen (675 rats)
5. Reproductive toxicity in two generations (2,600 rats)
6. Developmental toxicity (480 rabbits or 1,300 rats)
7. Genotoxicity/ mutagenicity (12-500 mice or rats)

CONNECTING THE DOTS FOR ANIMALS:

HISTORY OF THE EU BAN ON ANIMAL TESTING FOR COSMETICS



UE Cosmetic Regulation (2013)

Specifically, it establishes:

- **Testing ban** – prohibition to test finished cosmetic products and cosmetic ingredients on animals;
- **Marketing ban** – prohibition to market finished cosmetic products and ingredients in the EU which were tested on animals.

Motivations:

1.Public opinion

2. Relative safety of cosmetics (topical use)

3. Cosmetics are not essential

4. Innovation is not necessary for cosmetics

Not all that glitters is gold…

Advantages:

1.3R approach

2. Economic investment in the development of alternative methods

3. Acceleration in the processes of validation

4. Ethical implications

5. Long-term cost reduction

Disavantages:

-The regulation requires something that research has not yet reached

Critical issue: there are no alternative methods validated by long-term exposure

Difficulties in the development of new cosmetic products

Validation process

The process of validation of an alternative method lasts about ten years (three years for the validation, two years for the compilation of the report, two years for the acceptance of the legislation).

-All in vivo, in vitro and in silico methods used for regulatory purposes, new or updated, must go through the validation, which checks:

1. **Reliability**: reproducibility of the method in different time and locations
2. **Relevance**: usefulness of the procedure for the intended purpose.

The European institute responsible for validation processes is the ECVAM

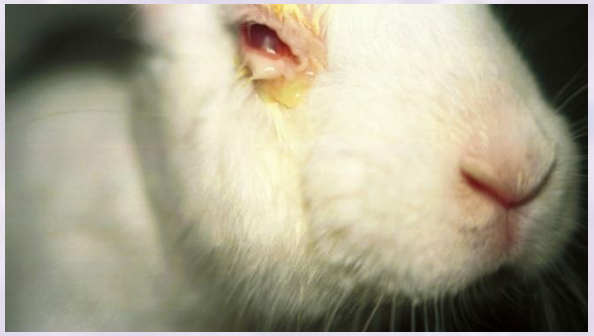
(**European Union Reference Laboratory for alternatives to animal testing**)

VALIDATED & ACCEPTED ALTERNATIVE METHODS

- **Skin irritation**

In vitro human skin model

EpiSkin SIT model, Epiderm, SkinEthic RHE, LabCyte EPI-MODEL

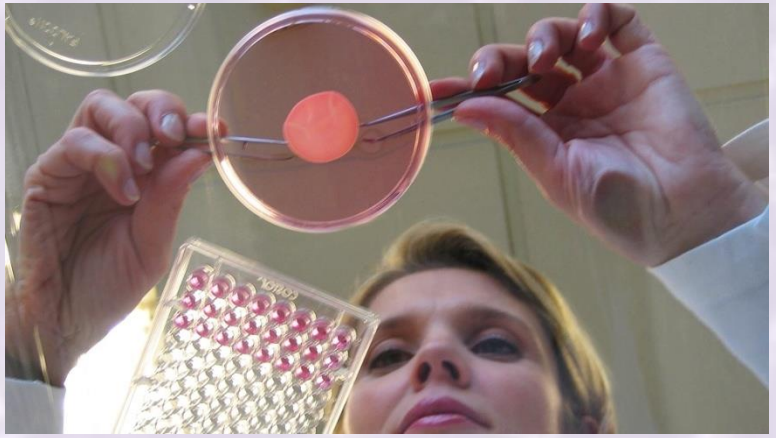


VS

- **Skin corrosion**:

In vitro human skin model

Epiderm, Episkin, SkinEthic, Vitrolife-Skin RHE



Definitions:

Lipid composition: The lipid composition of skin determines its permeability, flexibility,the partitioning of drug into the skin and many other aspects of skin biology. The metods usud to determine lipid profles is analytical TLC or HPTLC.

Biochemical markers: The biochemical markers are a proteins indicative of the differentiation process.

Photoxicity testing: phototoxic substance are applied to the model and are then irradiated with UV-light of different wavelengthd and intensities to trigger a phototoxic reaction.

Irritancy testing: irritants are applied to the model in order to see if the model shows the biochemical and histological signs of irritation.

Transport data: drug formulations are applied to the surface of the model and the amount of drug which has been transported through the model is measured as a function of time.

Corrosivity testing: the model is exposed to corrosive substance and the reaction is assessed

SkinEthic

About the company:

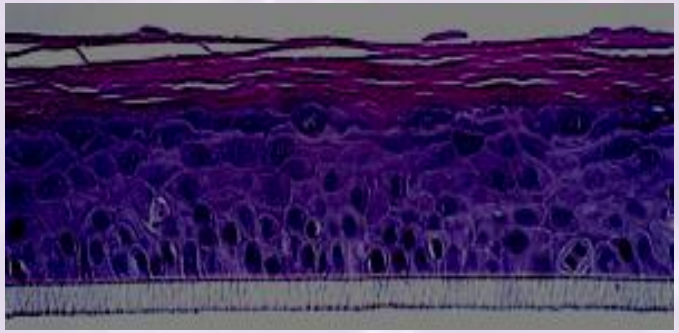
Skinethic laboratories was founded in 1992 by Martin Rosdy in Nice (France) to develop and produce the artificial human skin model.
The company offers reconstituted human corneal, oral gingival, esophageal epithelium and other model also.
SkinEthic Laboratories is an affiliated company of Episkin SNC, part of L'Oreal Research.

General features:

The human epidermis is reconstructed from normal keratinocytes cultured on an inert polycarbonate filter at the air-liquid interface in a chemically defined medium for 17 days.

Morphology:

The general epidermal structure of the model is highly similar to human epidermis.
In the skinethic model can be found :



- Stratum corneum
- Stratum granulosum
- Stratum spinosum

In the model also desmosomes, keratohyalin granules and lamellar granules can be found.

Hemidesmosomes, anchoring filaments and a structure which looks closely like a basament membrane were identified by electron microscopy.

The number of viable epidermal and stratum corneum cell layers in the skinethic model seems to differ from native tissue. Through all layers of the model there is a highest frequency of lipid droplets which are found only in stratum basale of native tissue.

Lipid composition:

The general lipid composition of the model comes close to that of native tissue.

The major subclasses of ceramides and their precursors,the glucosylceramides, are present in the model

The model have higher ceramide 2 content than the native tissue but ceramide 7 is missing

Lipid class	SkinEthic® mean±SD (*n*=4)	Native tissue mean±SD (*n*=3)
Phospholipids	17.0±10.6	36.5±4.1
Sphingomyelin	2.8±1.3	8.9±1.6
Phosphatidylcholine	6.4±3.8	11.2±0.8
Phosphatidylserine	1.1±0.7	3.9±0.3
Phosphatidylinositol	1.8±1.2	2.2±0.8
Phosphatidylethanolamine	4.9±4.0	10.3±0.8
Cholesterolsulfate	3.8±2.0	5.0±1.6
Glucosphingolipids	3.0±1.7	5.0±0.4
Ceramides	26.5±12.2	12.1±1.8
Free fatty acids	6.9±3.9	7.8±1.2
Cholesterol	19.5±9.5	17.7±3.2
Lanosterol	4.3±3.1	–
Di-/triglycerides	12.6±8.6	8.9±3.7
Cholesterolester	6.5±4.4	7.0±0.4

Biochemical markers:

Keratin 1, Keratin 10, loricrin, involucrin and transglutaminase are all present in the skinethic model. In particular, in analogy to human skin, loricrin is present in the stratum granulosum , while involucrin and transglutaninase were encountered in suprabasal layer.

Keratin 6 and SKALP are not found in human skin. Skalp is present only in psoriasis and headling wounds.

Loridrin and involucrine are prtotein precursors of the cornified envelope, which are cross linked by a transglutaminase in the final stages of keratinization.

The proper expression of these enzymes and their substrates is a condiction sine qua non for the formation of a competent barrier.

Applications:

The skinethic epiderm is a validated model for screnninig skin irritation for cosmetic testing.

Irritancy testing:

1)Sodium lauryl sulfate (SLS), calcipotriol and trans-retinoic acid were applied to both human skin and in vitro model for 24h. Afterwards the level of cytokinen expression and inlammatory skin reaction was misured.

2)Protocol is based on measurement of cytotoxicity using MTTassay .the release of proinflammatory mediators and enzymes after different times of exposure to SLS were quantified. Skinethic is the most sensitive model to SLS.

3)The release of interleukin1alfa and interleukin8 by the model can be used to classify sensitizing and irritating compounds.

Other applications of this model are:

⟶ Phototoxicity testing

⟶ Transport data

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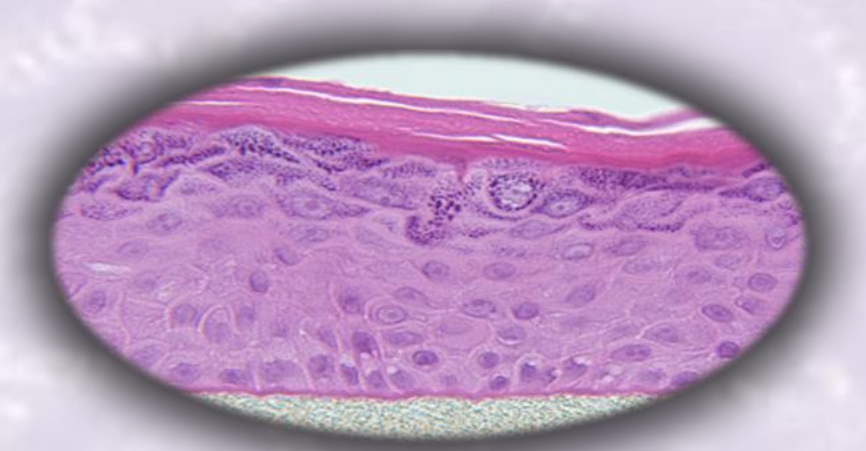
1993: Introduction of EpiDerm artificial skin to the market



EpiDerm Tissue Model

Described as
'normal, human
derived epidermal keratinocytes (NHEK) which have been
cultured to form a multilayered, highly differentiated model
of the human epidermis'

Comparison with other commercial products was made considering the percuteneous absorption culture and the hydrocortisone-free culture including epidermis and stratum corneum.

All strata are present in both models (no rete ridges). Number of viable cell layers range is 6-8 (28–43 µm) for penetration model and 7-14 (83-100 µm) for irritation model.

Absence of dermis negatively influences the barrier function of EpiDerm skin model, causing problems in irritancy tests.

A full thickness skin has been made (EpiDermFT) good for wall to wall structure and an in vivo-like basement membrane developement.

Good similarities with natural epiderm considering structure, lipid composition (analytical TLC or HPTLC) and marker has been prooved

Lipid composition of EpiDerm^{w} model compared to native human tissue

Lipid class	EpiDerm^{w} irritation model meanGSD (nZ5)	EpiDerm^{w} penetration model meanGSD (nZ3)	Native tissue meanGSD (nZ3)
Phospholipids	36.5G2.7	30.4G1.1	36.5G4.1
Sphingomyelin	8.2G1.5	6.3G0.3	8.9G1.6
Phosphatidylcholine	13.6G2.4	10.7G0.2	11.2G0.8
Phosphatidylserine	3.2G0.7	2.3G0.1	3.9G0.3
Phosphatidylinositol	4.3G0.8	3.7G0.9	2.2G0.8
Phosphatidylethanolamine	7.1G1.6	7.4G0.3	10.3G0.8
Cholesterolsulfate	5.8G1.2	5.7G1.6	5.0G1.6
Glucosphingolipids	9.5G1.3	5.8G0.1	5.0G0.4
Ceramides	18.5G3.5	28.9G0.3	12.1G1.8
Free fatty acids	2.6G0.5	3.1G0.6	7.8G1.2
Cholesterol	14.8G1.3	17.9G0.9	17.7G3.2
Lanosterol	1.2G0.5	1.0G0.1	–
Di-/triglycerides	10.5G2.2	6.9G0.8	8.9G3.7
Cholesterolester	2.7G1.1	2.1G0.4	7.0G0.4

Issues:

-Basement membrane patchy for both models and hemidesmosomes (in 50% of the cultures)

-Intracellular lipid droplets absent from native epidermis

EpiDerm behaviour reproduction has been validated for phototoxicity, irritancy, corrosivity and transport data tests

IRRITANCY
Studies were conducted to identify
biochemical markers
of skin irritation that are measurable before
physiological
signs of irritation occur
Irritant:SLS plus tritiated water
Markers: IL-1$^{\alpha}$ and mRNA levels

Results: significantly different responses between EpiDerm and natural skin for concentation levels due to suboptimal barrier function of the model

The model is suitable for screening possibly irritating substances

Irritants used in the EpiDerm^{w} irritation prevalidation study

Nr	Chemical name
1	1-Bromohexane
2	Tetrachloroethylene
3	2-Ethoxyethylmethacrylate
4	n-Butyl propionate
5	Alpha-terpineol
6	Heptanal
7	Tallow polypropylene polyamine
8	1,6-Dibromohexane
9	Sodium metasilicate (10%)
10	Sodium bisulphite
11	Methyl palmitate
12	1-Bromopentane
13	3,3^{0} -Dithiodipropionic acid
14	4,4^{0} -Methylene bis (2,6-ditert-butyl)phenol

CORROSIVITY

The use of EpiDerm skin model for corrosivity testing has been validated

Validation test realized by the convertion of a study based on ECVUM corrosivity test for EPISKIN
EpiDerm predictin model is based on assessment of cell viability using the MTT assay after exposure to test chemical for 3 minutes and 1 hour

CORROSIVITY CONDITION

- Viability<50% for a 3-minutes treatment
- Viability=50% for a 3-minutes treatment but<15% for a 1-hour treatment

Comparison of corrosivity classifications obtained from the epiderm test

	EpiDerm assay
Sensitivity(%)	88
Specificity(%)	86
Predictivity(%)	
C	86
NC	87
Accuracy(%)	87

C=corrosive, NC=non corrosive

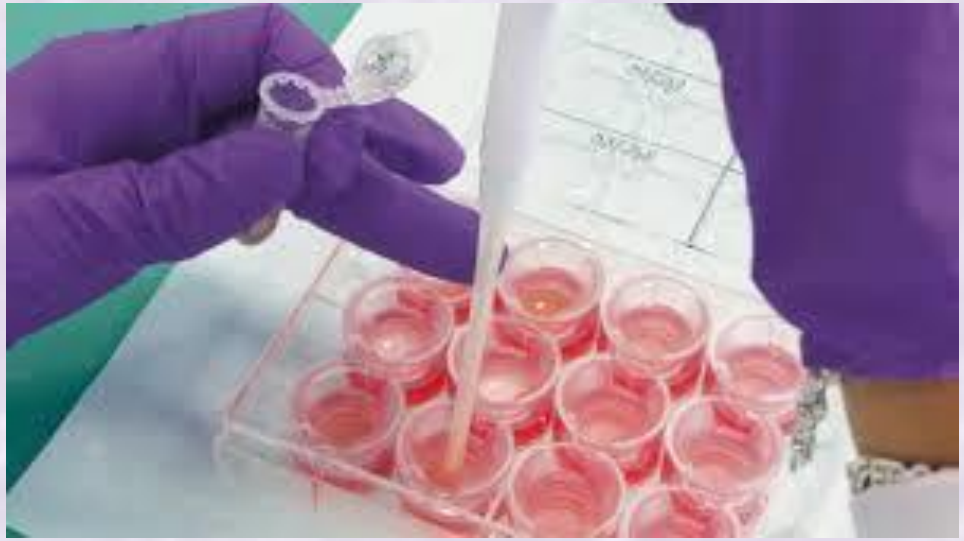
TRANSPORT DATA

Testing drugs: penetration of caffeine and a-tocopherol acetate, mannitol
Results: the rank order of permeability was the same in the EpiDerm model and human skin

Permeability for drugs, transport rate and influence by the different vehicles higher in the model

EPISKIN

Developed by E. Tinois , bought by L'Oréal in April 1997



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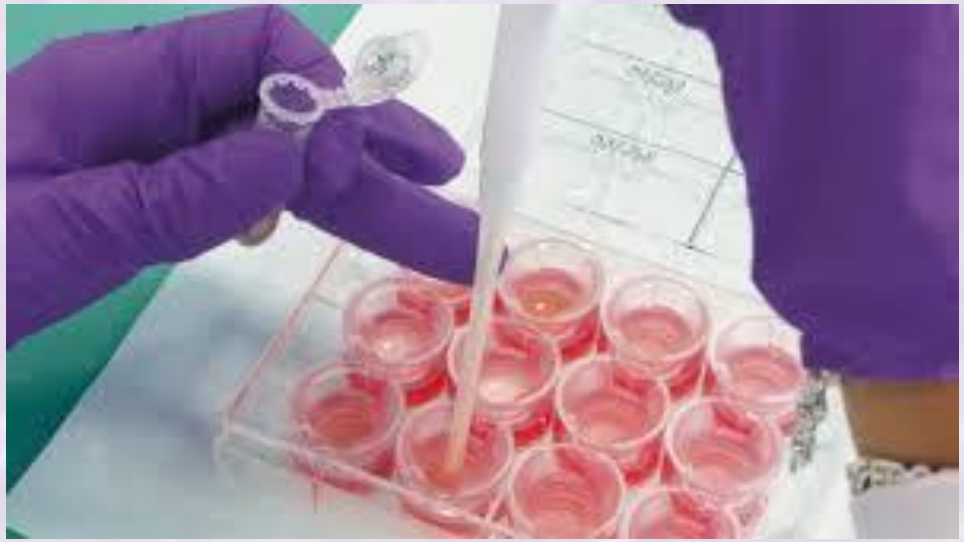
The EpiSkin kit is currently marketed in the form of 12 well plates.



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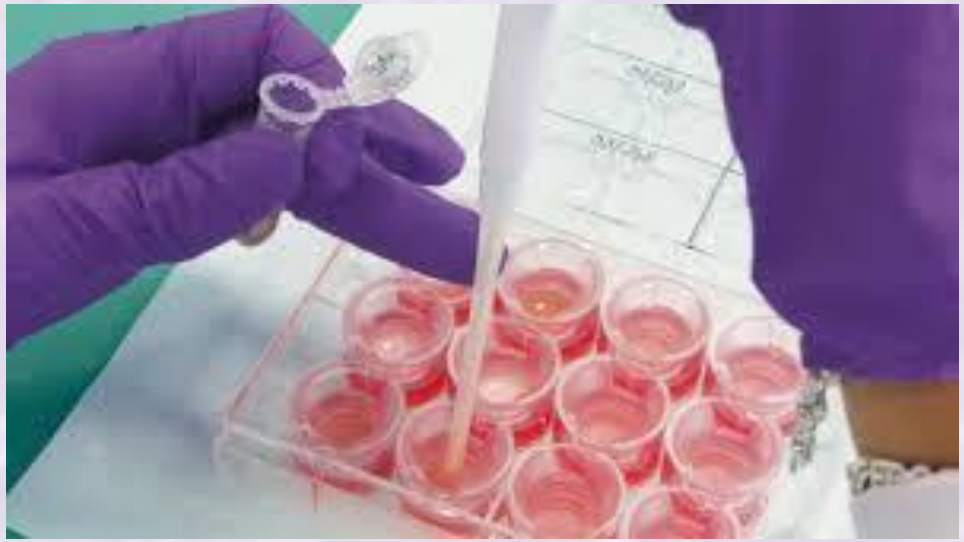
Morphology :

-type I bovine collagen matrix : the dermis

-film of type IV human collagen, upon which is laid, after 13 days in culture, stratified differentiated epidermis.



LIPID COMPOSITION

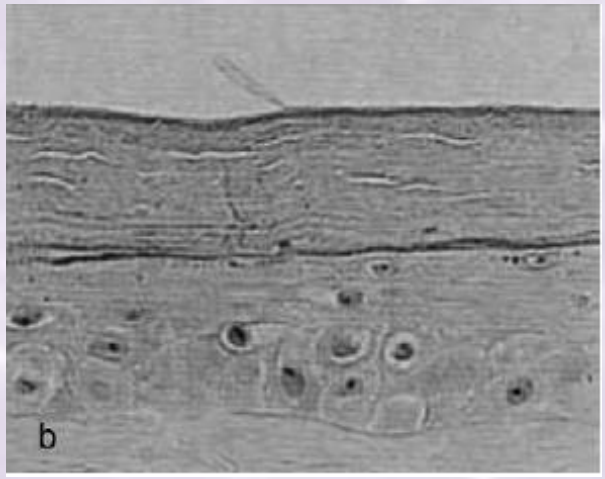
Lipid class	EpiSkin® irritation model mean ± SD ($n = 5$)	Native tissue mean ± SD ($n = 3$)
Phospholipids	33.0 ± 12.5	36.5 ± 4.1
Sphingomyelin	4.7 ± 1.3	8.9 ± 1.6
Phosphatidylcholine	10.8 ± 2.7	11.2 ± 0.8
Phosphatidylserine	2.6 ± 1.6	3.9 ± 0.3
Phosphatidylinositol	3.6 ± 2.5	2.2 ± 0.8
Phosphatidylethanolamine	11.2 ± 5.4	10.3 ± 0.8
Cholesterolsulfate	2.0 ± 0.5	5.0 ± 1.6
Glucosphingolipids	3.4 ± 1.4	5.0 ± 0.4
Ceramides	18.5 ± 6.9	12.1 ± 1.8
Free fatty acids	1.8 ± 0.5	7.8 ± 1.2
Cholesterol	17.6 ± 4.2	17.7 ± 3.2
Lanosterol	1.3 ± 0.5	–
Di-/triglycerides	20.9 ± 2.9	8.9 ± 3.7
Cholesterolester	1.5 ± 0.5	7.0 ± 0.4

- Phospholipid content very close to that of human epidermis
- Precursors of ceramides, glucosphingolipids in comparable amount
- Free fatty acids and of cholesterol esters were lower than in native tissue

Biochemical markers

Penetration model (b) :

- Keratin 1 and 10 are present in stratum spinosum and stratum granulosum
- Keratin 6 is present in all layers
- SKALP (skin-derived antileukoproteinase)is found in the upper stratum spinosum
- Loricrin is absent while involucrin and transglutaminase are present in all suprabasal layers.



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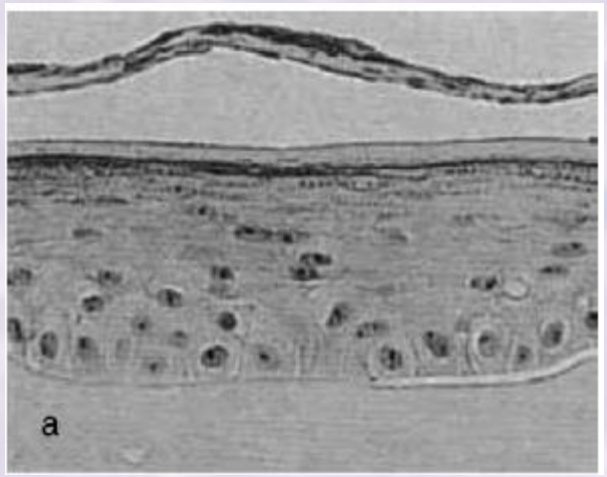
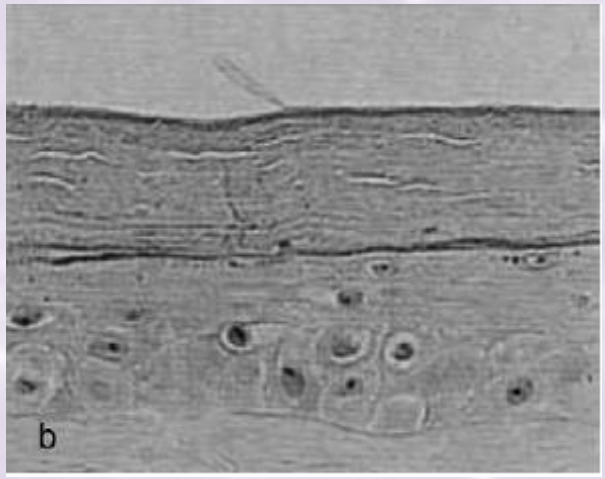
Irritation model (a):

- Loricrin is present
- Thinner and wider packed stratum corneum than the previous model





Phototoxicity testing

- The effects of several weak phototoxic, 6-methylcoumarin and ofloxacin, were compared to the effect of chlorpromazine
- SLS (Sodium laureth **sulfate**) and sulisobenzone served as negative controls
- MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-didiphenyltetrazolium bromide) viability test
- IL1-α (Interleukin-1 alpha)released

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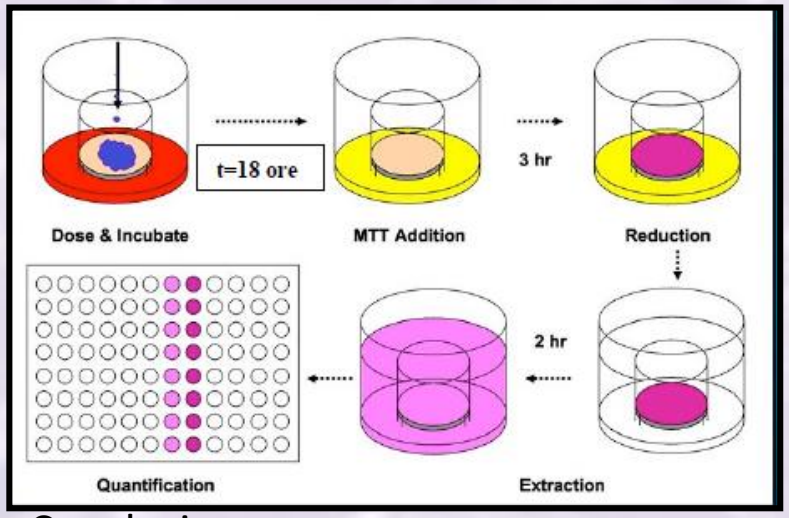
Procedure:



Conclusions:

Model can be used for identification of phototoxic substances.

Irritancy testing

Study's authors	Test	Results
Faller et Al.	22 different cosmetic products were tested	good correlation with in vivo skin
ECVAM	Acute skin irritation triggered by chemicals	sensitivity, specificity and accuracy
Cotovio et al.	Irritation in human skin	susceptible to oxidative stress induced by air pollutants
R.Roguet	Concentrations of surfactants	concentration necessary to trigger a reaction was smaller than required in vivo

Comparison

- In contrast to EpiDerm and SkinEthic, which are cultivated on inert filter membranes, the EpiSkin model is cultured on a layer of collagen

- EpiSkin is only delivered in a diameter of 12 mm or smaller; Epiderm and SkinEthic are also available in a 24 mm format

- EpiSkin and SkinEthic are similar for characteristic electron microscopic structures as alternating electron-dense and electron-lucent lipid lamellar sheets in the intercellular space of the stratum corneum

- General structure, composition and aspects of biochemistry bear a close resemblance to human skin

- Skin models are useful in toxicity testing

- More permeable than human skin on average but the culture models appear to be more consistant in permeability and responsiveness than human skin, which is highly variable.

3D in vitro model of a functional epidermal permeability barrier from human embryonic stem cells and induced pluripotent stem cells

In 2014 an international team led by King's College and the San Francisco Veteran Affairs Medical Center (SFVAMC) has developed the **first lab-grown epidermis with a functional permeability barrier**, whose main function are to prevent water from escaping and microbes and toxins from entering.

This new study, published in the journal Stem Cells Report, describes the **use of induced pluripotent stem cells (iPSCs) and human embryonic stem cells (hESCs) to produce an unlimited supply of pure keratinocytes** (the predominant cell type in the outermost layer of skin), that closely match normal primary human keratynocites (NHKs). These keratinocytes were then exposed to a **sequential high-to-low humidity environment** in an air/liquid interface culture and the resulting human epidermal equivalents (HEEs) had all the strata of the human epidermis, with skin barrier properties similar to those of normal skin.

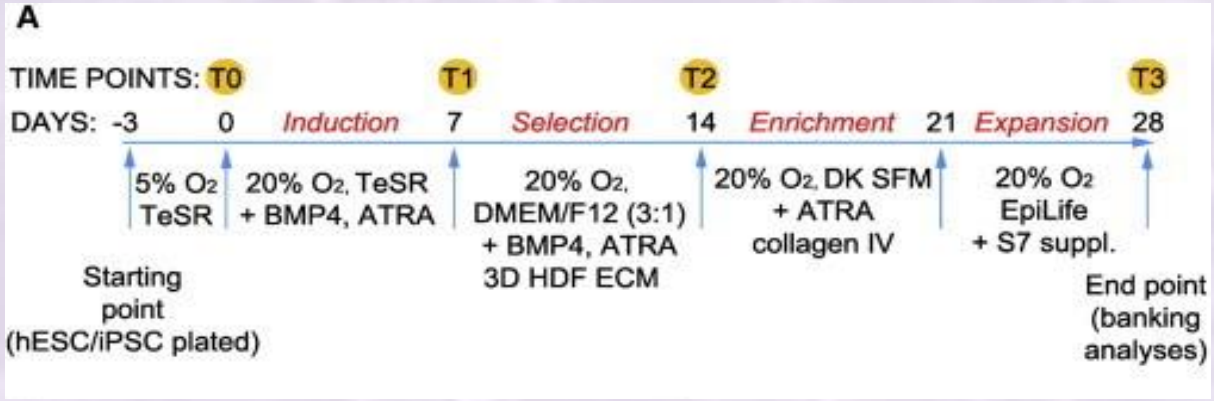
Before:

- previously engineered HEEs do not form a fully develop epidermal barrier
- in vitro studies have been limited by the fact that only a limited number of HEEs can be generated from one sample of epidermis and the primary keratinocytes generated from this sample may contain previously unidentified polymorphism in genes that modify epidermal growth, differentiation of barrier development

Now:

- the HEEs generated from hESC/iPSC-derived keratinocyteshave a **functional permeability barrier** similar to native human skin
- the use of hESCs and iPSCs makes tissue engineers able to produce an **unlimited number of genetically identical units**

Four-step protocol for manufacturing in vitro HEEs



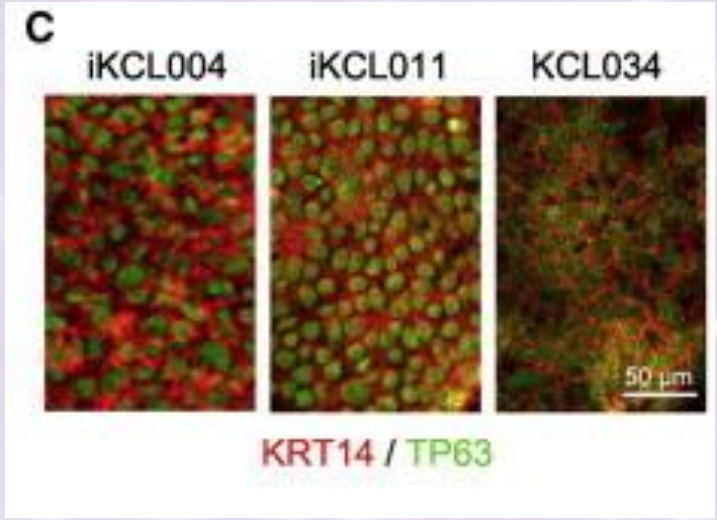


Figure C. Diversity is lost at later stages, and cell colonies at the enrichment step are uniformly positive for keratinocyte markers K14 (red) and p63 (green).

The **differentiation protocol** consists of four sequential steps:

1) **INDUCTION** : a clinical-grade hESC line (KCL034) and two iPSC lines (iKCL004 and iKCL011) are transferred from 5% O_2 to atmospheric O_2 and then exposed to 25 ng/ml BMP4 and 1 μM of all-trans retinoic acid (ATRA) for 7 days

2) **SELECTION:** native 3D decellularized human dermal fibroblast ECM are used as a growth-supporting platform and, a week later, epidermal progenitor stem cells are purified based on their preferencial adherence to collagen IV

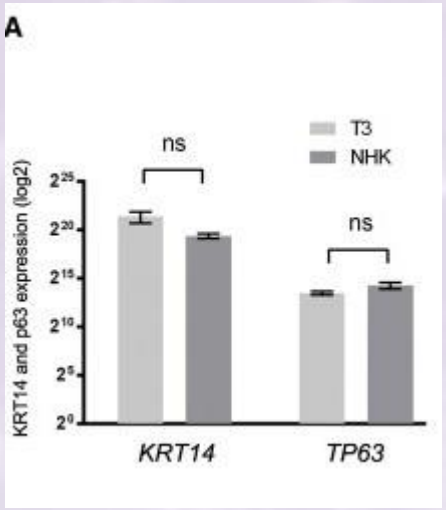
3) **ENRICHMENT**: the purified cells give rise to a homogeneous population of $(K14)^+p63^+$ colonies

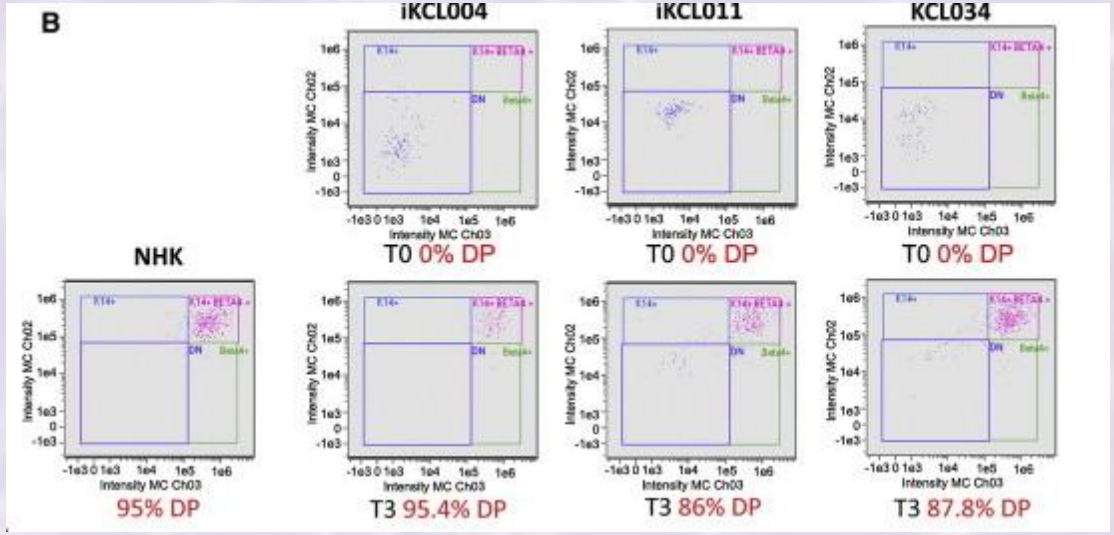
4) **EXPANSION** : $(K14)^+p63^+$ colonies are amplified.

This pure-population of hESC/iPSC-derived keratinocytes is then exposed to a sequential high-to-low humidity environment to gener the HEEs.

Comparison between enriched and expanded population of hESC/iPSC-derived keratinocytes with NHKs

Molecular characterization suggested that the differentiation protocol yelds a **fairly pure population of cells that are very similar to NHK.**





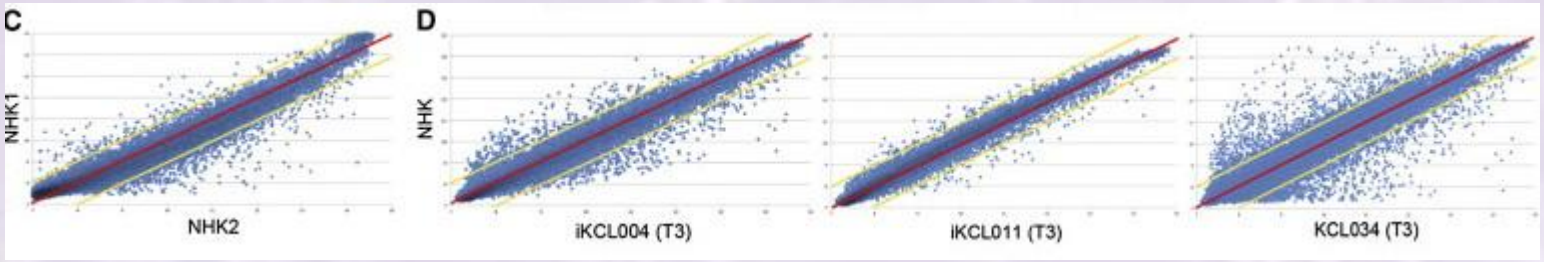


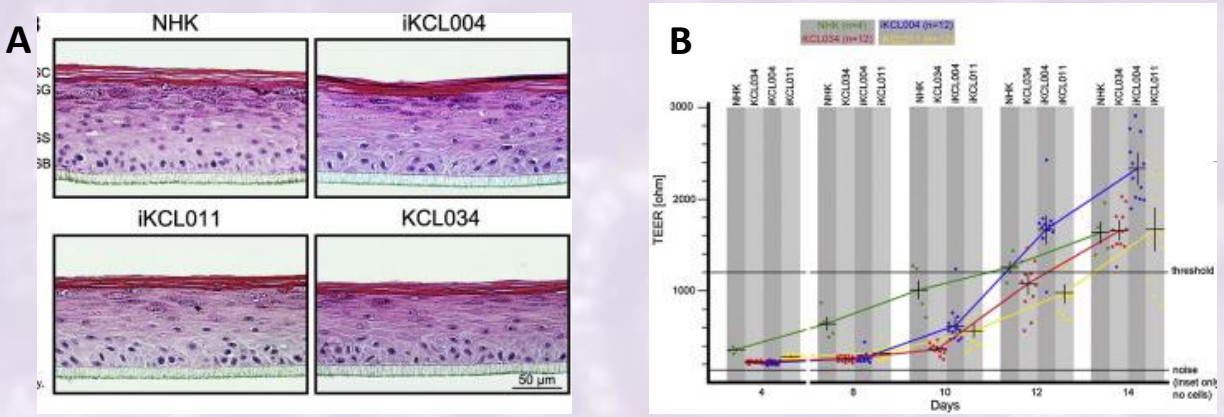
Figure A. qPCR analyses measuring expression of *KRT14* and *p63* in hESC/iPSC-derived keratinocytes at T3 (n = 9; three rounds of differentiation for each of three lines) and NHKs (n = 2) found no significant difference between the two groups. Multiple t tests. Statistical significance was determined using the Holm-Sidak method. For *KRT14*, p = 0.120881; for *p63*, p = 0.155472.

Figure B. Populations of hESC/iPSC-derived keratinocytes at T3 have a similar percentage of $K14^+$/integrin $\beta4^+$ cells as NHKs. The percentage is an average from three rounds of differentiation for each of the three lines. At T0, no $K14^+$/integrin $\beta4^+$ cells were detected. Fluorescence-activated cell sorting (FACS) images represent one of the three rounds.

Figure C,D. Scatterplots showing the relative expression of all genes on the chip.

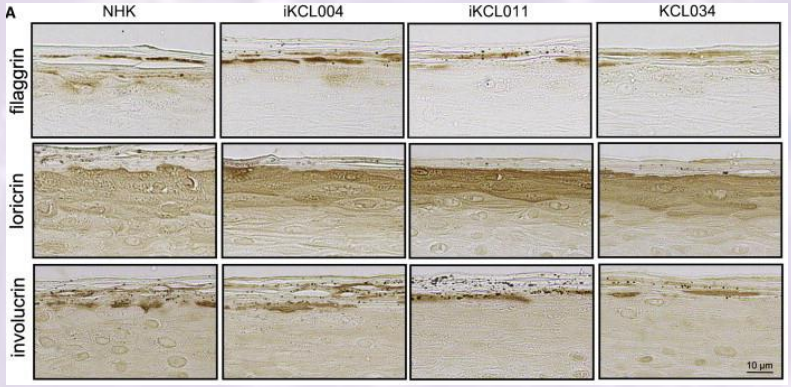
Comparison between HEEs derived from hESCs and iPSCs and human epidermis

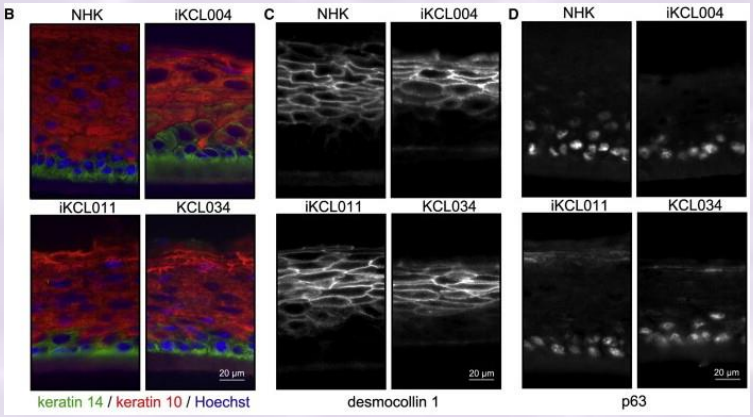
- The HEEs generated using this protocol developed in vitro all of the cellular strata seen in the human epidermis
- The integrity of the stratifying cultures was assested by measuring the transepithelial electric resistance (TEER), which indicates changes in the transcellular and paracellular permeability of epidermal in vitro cell cultures: within 2 weeks, the TEER in all cultures reached >1,000 Ω , indicating the formation of a permeability barrier and multilayered epithelia



(A) H&E staining demonstrates normal morphology with the presence of all epidermal layers regardless of the source of keratinocyte population. SB, stratum basale; SS, stratum spinosum.
(B) The TEER during HEE formation over a period of 14 days reflects permeability barrier formation (n = 4 for NHK, n = 12 for each of lines; each point represents an average of measurements from three different spots). Error bars represent SD. Only 14-day-old HEE cultures that displayed a TEER of >1,200 ohm cm^2 were used for subsequent analyses unless indicated otherwise

- Differentiation markers were normally expressed and located

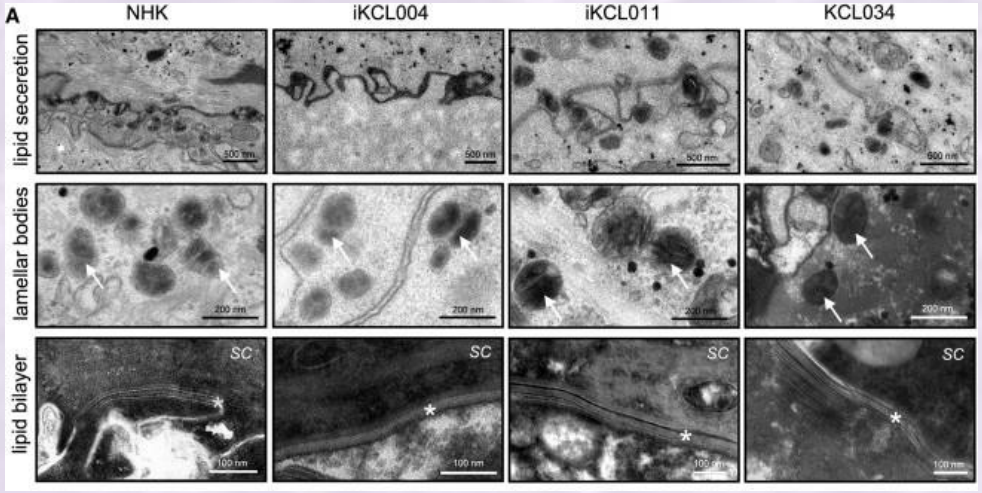




(A) Immunostaining for three markers of keratinocyte terminal differentiation: filaggrin, loricrin, and involucrin. Each of these markers is expressed at the appropriate site, denoting normal epidermal differentiation.
(B–D) Immunostaining for K14 (basal layer) and K10 (suprabasal layers) (B), desmocollin 1 (suprabasal layers) (C), and p63 (basal layer in general) (D) demonstrates normal epithelial stratification in all HEE cultures.

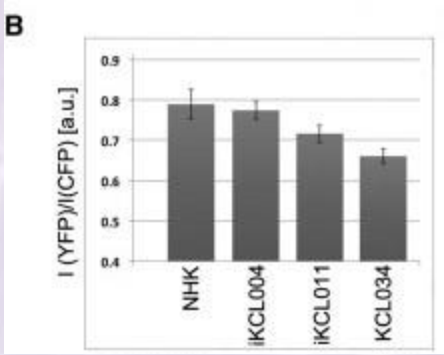
Comparison between HEEs derived from hESCs and iPSCs and human epidermis

- Lipid secretion appeared normal
- Lamellar bodies and extensive intercellular bilayers were detected in all cultures



(A) Lipid bilayer formation assessed with TEM. Arrows, lamellar bodies; asterisks, lipid bilayers. Upper row: normal lipid secretion between the SG and SC is seen in all cultures. Middle row: LBs are seen in the SG of all cultures (arrows). LB morphology was normal in all cultures, although it appeared slightly smudged in KCL034. Bottom row: lipid was successfully processed into lipid bilayers (asterisks) in all cultures. Like LB morphology, lipid bilayer morphology was normal in all cultures, although it seems slightly disrupted in KCL034.

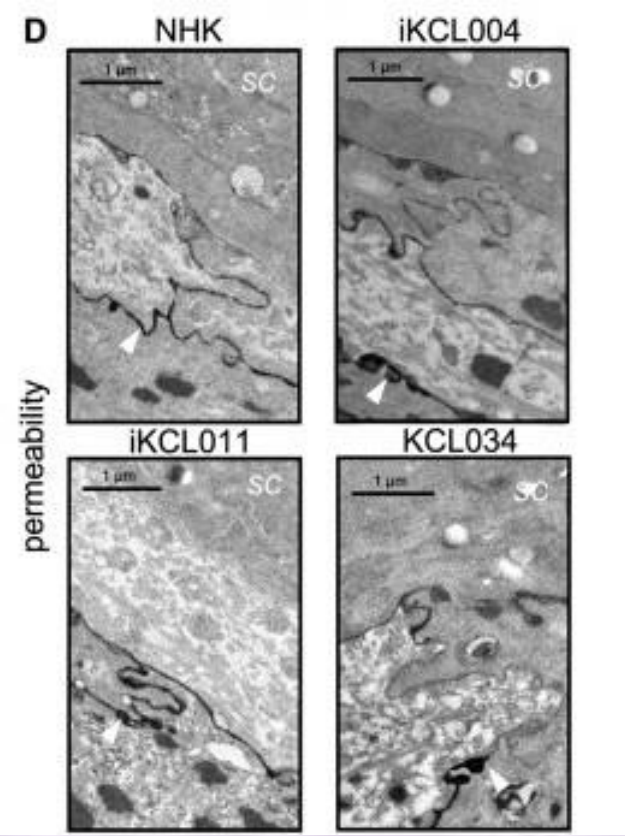
- The endoplastic reticulum Ca^{2+} store, which is essential for normal keratinocyte signaling and differentiation, was detectable at day 8 of the culture



(B) ER Ca^{2+} sequestration at day 7 of HEE culture in cells transfected with the ER-targeted Ca^{2+} sensor D1ER. Data are presented as the mean of the intensity ratio (I) between the yellow-channel (higher Ca^{2+}) and blue-channel (lower Ca^{2+}) images (I(yfp)/I(cfp)) ± SEM. Higher ratios denote higher Ca^{2+}stores in the ER. n = 10–14 cells from two biological replicates in each group. Significance was calculated using a one-way ANOVA. Distributions with $p < 0.05$ were assumed to be statistically different based on unpaired t tests between the populations.

Comparison between HEEs derived from hESCs and iPSCs and human epidermis

- The lanthanum perfusion assay, which is used to depict pathways of water movement through epidermis, demonstrated a functional permeability barrier in all cultures: the passage of electron-dense lanthanum tracer was blocked by the epidermal lipid barrier at the SG/SC interface and was not detected in the SC



(D) Permeability barrier integrity assessed by lanthanum perfusion. Lanthanum is visualized as electron-dense deposits in the extracellular spaces of the viable SG (arrowheads), demonstrating that lanthanum and, by extension, water and other small ions can pass between keratinocytes in this stratum. In contrast, lanthanum spreads along the base of the SC, but cannot penetrate further into the SC because a functioning lipid barrier is blocking its movement upward. All cultures demonstrated a functional permeability barrier.

Future perspectives

Dr Theodora Mauro, leader of the SFVAMC team, said:

‘ The ability to obtain an unlimites number of genetically identical units can be used to study a range of conditions where the skin’s barrier is defective due to mutations in genes involved in skin barrier formation, such as **ichtyosis** or **atopic dermatitis**. We can use this model to **study how the skin barrier develops normally, how the barrier is impaired in different diseases and how we can stimulate its repair and recovery**’.

Dr Dusko Ilic, leaader of the team at King’s College London, said:

‘This is a new and suitable model that can be used for **testing new drugs and cosmetics** and can **replace animal model**. It is cheap, easy to scale up and it is reproducible’ ;

he also added:

‘When you produce a new cream you need to know how much of it goes through the skin, because if there is too much it can cause damage. If you imagine the skin as a three or four-storey building with a roof on top, other people have made the roof but their roofs are always leaking. They could not get their tiles together. We are the first to achieve this’.

The Humane Society International, which works to protect animals, including those in laboratories, welcomed the research and its toxicology director Troy Sedle said:

‘This new human skin model is **superior** scientifically **to killing rabbits, pigs, rats or other animals** for their skin and hoping that research findings will be applicable to people - which they often aren’t, due to species differences in skin permeability, immunology and other factors’.