Artificial skin at the USP Laboratory of Skin Biology reconstructed from human cells

Laboratory Skin

Brazilian researchers create human skin models to study diseases and replace animal testing for cosmetics and drugs

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Just three years from the enforcement of a resolution by Brazil's National Council for the Control of Animal Experimentation (CONCEA), which will require cosmetic manufacturers and pharmaceutical laboratories to adopt alternative, animal-free research methods, Brazil has already made significant advances in the development of reconstructed skin in the laboratory. Also known as artificial skin, three-dimensional skin, and skin equivalent, this biological material is similar in structure and physiology to human tissue and may be used to replace animals in testing new cosmetics and personal hygiene products, studying diseases such as melanoma and cervical cancer, and treating chronic skin ulcers and burns. Businesses, research institutes, and universities in Brazil are in a race against time to develop local models of human skin *in vitro*.

Artificial skin is reconstructed from human cells and takes 10 to 30 days to develop (*see infographic on p. 85*). The tissue lasts approximately 7 to 10 days, during which it is ready for use. In the case of cosmetic testing, the new product is applied to the skin. When the product is in cream or powder form, the material is spread over the skin with the aid of a spatula or flexible wand; when the product is in liquid form, it is dripped over the tissue. After a few hours, the *in vitro* skin is washed to remove the substance. The next day, researchers in the laboratory count the number of living and dead cells to ascertain the new product's potentially irritating or corrosive effects. Measuring 1.5 to 3 centimeters (cm) in diameter, each fragment of reconstructed skin can be used only once.

The artificial skin market is currently led by the French multinational company L'Oréal, a cosmetic

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Skin sample preparation developed by USP professor Silvya Stuchi Maria-Engler

industry giant. The company owns the EpiSkin and SkinEthic models, which are distributed in Europe in kits containing 24 units of artificial human skin reconstructed in the laboratory. In addition to full-thickness skin—which is composed of the epidermis, or outermost layer, and the dermis, which lies just beneath the epidermis—L'Oréal markets six other skin models abroad, including a reconstructed human epidermis; a pigmented epidermis, which mimics different skin tones; and various types of epithelium, such as those that line the mucous membranes of the mouth, gums, and vagina and cover the cornea. Another big player in this market is MatTek, a US firm that markets various models of skin equivalents, which are similar to those made by L'Oréal. The companies charge \$50 to \$80 per individual sample. In Germany, the Fraunhofer Institute for Interfacial Engineering and Biotechnology devised an automated system that can produce 12,000 skin patches from a single sample of human tissue. Since 2014, the German institute has been marketing the system to businesses that want to ensure that their beauty products do not cause allergies or irritation.

The characteristics of cell structures produced in the laboratory have much in common with human skin

While Brazilian law allows the importation of artificial skin, this option is not always viable; hence, there is a push to develop tissue in Brazil. "Because it's living material, and therefore perishable, the skin fragments that come in the kits are good for only a few days. We often run into trouble at customs, which effectively makes importing unfeasible," says biologist Silvya Stuchi Maria-Engler, professor at the School of Pharmaceutical Sciences at the University of São Paulo (FCF-USP), a leader in research on skin equivalents. "With the ban on animal testing of cosmetics and raw materials going into effect in 2019, it's very important for kits to be produced in Brazil" (*see box on p. 88*).

In late 2015, the Brazilian cosmetics company Grupo Boticário, which controls the business units O Boticário, Eudora, and Quem disse, Berenice?, announced that it had successfully created a material equivalent to human skin at its Research and Development Center in São José dos Pinhais, in the state of Paraná. The tissue is used as an animal-free alternative in testing raw materials and finished products, such as makeup, lotions, and creams, and in safety and toxicity tests. "To make our 3D skin, we use cells isolated from skin tissues that have been discarded during plastic surgery, with donor consent and the approval of the Ethics and Research Committee at our R&D Center," explains Márcio Lorencini, manager of Biomolecular Research at the firm. The new tissue is formed in the laboratory, cell by cell and layer by layer, similar to human skin. The result is a patch up to three centimeters in diameter that is ready for use in testing.

During *in vitro* reconstitution, the epidermis—the outermost layer of the skin—is obtained by culturing both keratinocytes, the cells that synthesize keratin and are responsible for provid-

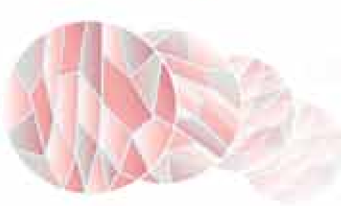
PHOTO LÉO RAMOS CHAVES **INFOGRAPHIC** ANA PAULA CAMPOS **ILLUSTRATION** FABIO OTUBO

Reconstructed tissue

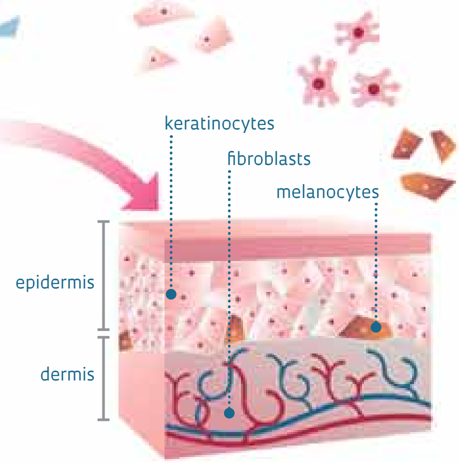
Method enables testing of new cosmetics and drugs and reduces the number of human trials

SOURCE: GRUPO BOTICÁRIO AND SILVYA STUCHI MARIA-ENGLER

1 TISSUE ORIGIN
Artificial skin is reconstituted from human cells that were discarded during plastic surgery or from samples taken from the foreskins of newborns.

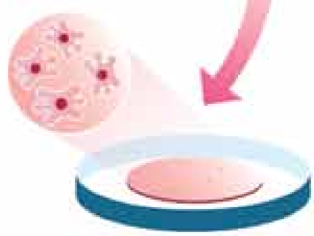


2 STERILIZATION AND CLEANING
Skin fragments (5 to 8 cm2) undergo a sterilization process and microbiological control testing to guarantee that they are pure and free from viral or bacterial infections.

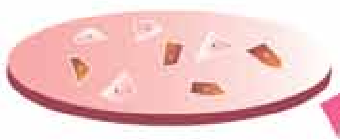


3 CELL ISOLATION
The main skin cells are isolated next: fibroblasts, which are responsible for producing the proteins that keep the skin firm; keratinocytes, which protect the skin; and melanocytes, which provide pigmentation.

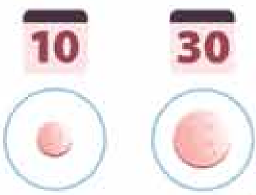
Artificial skin affords greater test accuracy since it is made from a pool of cells obtained from a number of individuals



4 CONSTRUCTION OF THE DERMIS
The first step in reconstituting full-thickness skin (dermis + epidermis) is to synthesize the dermis. Scientists induce the growth of human fibroblasts in a collagen matrix.



5 RECONSTITUTION OF THE EPIDERMIS
Keratinocytes and melanocytes are cultured onto the reconstructed dermis. Culturing can also be done over a polycarbonate filter membrane.



6 GROWTH TIME
It takes 10 days to grow smaller pieces of skin (1.5 cm in diameter). Larger fragments (3 cm) take 30 days. Reconstituted skin lasts up to 7 days.

ADVANTAGES

Reduces or eliminates the need for animal testing in the cosmetic and pharmaceutical industries

Increases test reliability, since reconstructed skin is more like human tissue than the skin of laboratory animals

Enables the testing of a number of formulations to identify which are the safest and most effective

Makes it possible to reduce human trials ing a protective barrier, and melanocytes, which produce the melanin pigment that colors the skin. The dermis is reconstituted by culturing human fibroblasts, which are grown in a collagen gel. Fibroblasts are responsible for producing the proteins that can synthesize collagen and elastic fibers.

The growth characteristics of all of these lab-grown cell structures are quite similar to those of human skin, and this enhances test uniformity and reproducibility. They also have much more in common with human skin than the mouse skin that is generally used in testing new products. The full-thickness skin, formed by a dermis and an epidermis, is ideal for studying diseases and assessing new drugs, while the structure formed by the epidermis alone suffices for the corrosion and irritation tests that are performed by the cosmetic industry.

3D SKIN

According to Márcio Lorencini, Grupo Boticário began developing its technology, which allows a number of tests to be performed on the same piece of reconstituted skin, in 2009. "3D skin affords greater breadth and accuracy in testing since it is made from a set of cells that come from a number of people [this is common practice among all groups researching and producing artificial skin with current techniques]. Using a pool of cells reduces individual variability. If we used cells from a single person, we might get responses that vary from individual to individual, and this isn't ideal in evaluating toxicity and efficacy parameters for cosmetic products and raw materials," states Lorencini. In addition to testing for toxicity and skin corrosion and irritation, the company employs artificial skin to assess the efficacy of melanin production; to analyze the gene and protein expression of various skin markers, such as collagen, elastin, and keratin; and to study cytokines, which are biomarkers of inflammation.

Grupo Boticário self-funded the development of the model and received no assistance from any academic partners. Its team, however, included biologist Carla Abdo Brohem, who trained at the USP Laboratory of Skin Biology under a FAPESP doctoral fellowship. In 2010 and 2011, with FAPESP support, Brohem did postdoctoral work as an intern at the laboratory of Australian researcher Pritinder Kaur, a member of the Peter MacCallum Cancer Centre, a medical institution in Melbourne specialized in cancer research and treatment. Considered a top expert in the study of epithelial stem cells, Kaur collaborates with Professor Maria-Engler's group. Brohem now coordinates the Safety and Efficacy Evaluation Group at the company's R&D Center.


1

Testing liquid makeup on artificial skin at USP. Each patch can be used only once

In São Paulo, Maria-Engler, coordinator of the USP Laboratory of Skin Biology, finished her first model of human skin, which was reconstructed *in vitro*, in 2006. Of special note among the more recent studies are the development of an aged skin for use in anti-aging cosmetics tests, an epidermis that is similar to commercial models, and a 3D skin meant for skin cancer studies. This line of research by Maria-Engler's group has already yielded 45 published papers.

"It's essential that Brazil master the technology for making reconstructed human skin and gain autonomy in this field of research," says the scientist. "The full-thickness skin and epidermis models that we have developed are identical to those produced abroad. We are transferring this knowledge to society through the USP Pharmaceutical Research Institute Foundation, FipFarma. We have already been contacted by a number of cosmetic manufacturers who are interested in being trained to make these tissues in the laboratory," she says.

The first company to take the USP training course was OneSkin Technologies, a biotech startup based in San Francisco that specializes in tissue engineering and was founded by three Brazilian researchers. "With the training we re-

PHOTOS 1 AND 2 LÉO RAMOS CHAVES 3 AND 4 ENRIQUE BOCCARDO / USP

2

The first sample of reconstructed human skin at USP was completed in 2006

ceived at USP, we were successful in building our *in vitro* human epidermis model. We're now working to develop full-thickness skin," reports biochemist Carolina Reis de Oliveira, a founding partner of OneSkin. Working since March 2016 with IndieBio, one of the largest biotech accelerators in the United States, OneSkin wants to master the technology for making 3D skin so that it can enter the anti-aging cosmetics market. "Our next challenge is to develop a type of aged skin that allows us to study mechanisms to prevent aging," says Oliveira. When this goal is reached, OneSkin will focus on the search for molecules with anti-aging potential. "Our idea is to license relevant molecules or produce new cosmetics with them." OneSkin was invited to receive funding and mentorship from IndieBio after taking part in an event for startups in Brazil and catching the attention of foreign investors.

At the USP Laboratory of Skin Biology, biologist Paula Comune Pennacchi is working on a line of research similar to OneSkin's. She devised a human skin model that simulates physiological cutaneous aging and the skin changes observed in diabetics. The study formed the basis of her doctoral dissertation, which she defended in February 2016. "We recreated a model that can respond to the action of cosmetics and drugs that work on skin aging. Our reconstructed skin has also contributed to understanding phenomena associated with inadequate scar formation and greater skin inflammation in diabetics," the researcher says.

AWAITING REGULATIONS

Grupo Boticário will not share the 3D skin constructed in its laboratories until validation is available for this type of product in Brazil. The French company L'Oréal, which decided to invest in the area in Brazil, is also waiting for the product to be regulated before placing tissues from its EpiSkin line on the market, as it now does in Europe and Asia. "Until there are clear regulations governing the distribution of tissues, we are in Brazil for research purposes only. We make roughly 150,000 units of reconstructed skin in France per year, while we manufacture another 30,000 units of pigmented skin tissue in China," says Rodrigo De Vecchi, manager of Advanced Research for L'Oréal in Brazil. The company will begin by marketing just its model of reconstructed human epidermis, or RHE, which is made from human keratinocytes, the main type of epithelial cell in this tissue. RHE tissue has been approved by the European Union Reference Lab for Alternatives to Animal Testing (EURL ECVAM) to replace animals in cosmetic safety tests. "When the RHE model is available in Brazil, we will have at our disposal a tool for use with cosmetics and in research areas like biomedicine, regenerative medicine, and toxicity assessment," says De Vecchi.

L'Oréal recently established a partnership with the D'Or Institute for Research and Education (IDOR) in Rio de Janeiro with the aim of refining its reconstructed epidermis model. "Our proposal is to reinnervate the reconstructed human skin model with neurons produced by us, making it even more like natural human skin," reports neuroscientist Stevens Rehen, research coordinator at IDOR. This research holds tremendous biotechnological potential, according to Rehen, who is also a professor at the Institute of Biomedical Sciences of the Federal University

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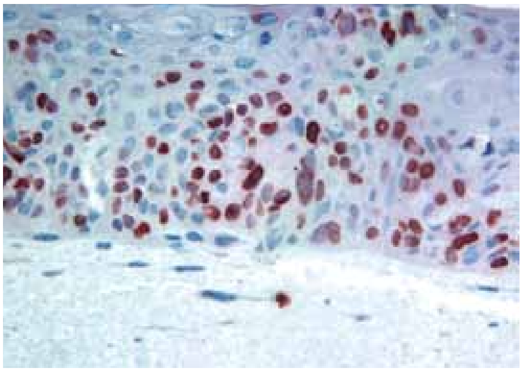

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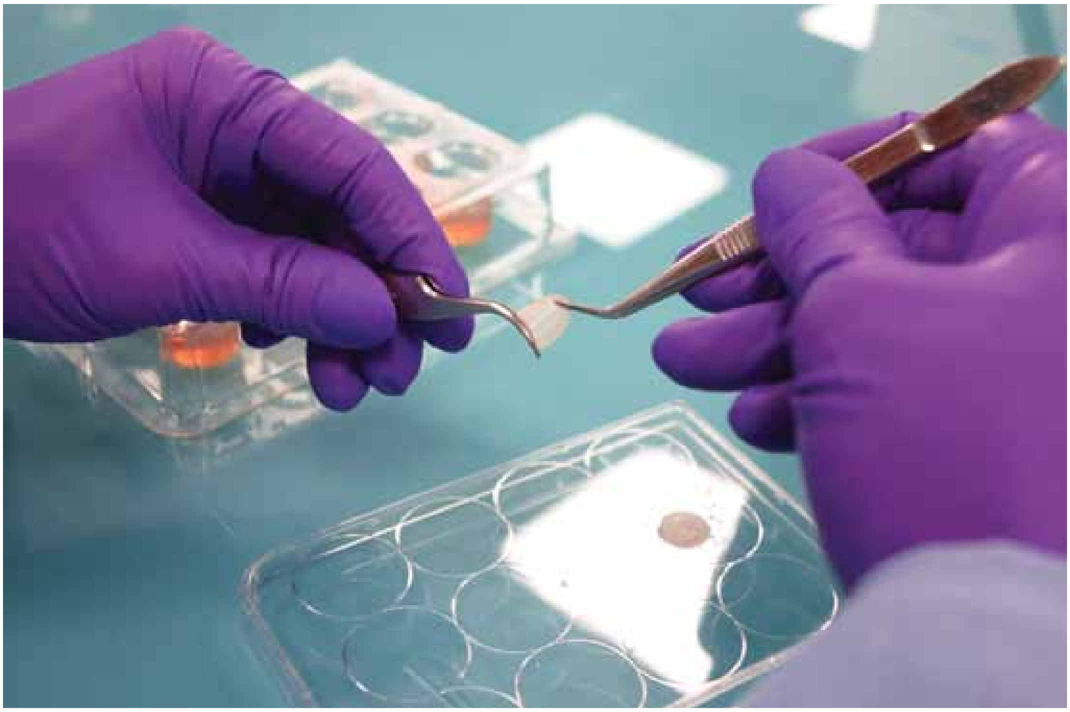
Microscopic view of infection caused by human papillomavirus (HPV), left, and normal artificial skin in experiments at the Institute of Biomedical Sciences (ICB)



Reconstructed skin by L'Oréal: research moves toward introducing neurons into the product

of Rio de Janeiro (ICB-UFRJ). The researcher's area of interest is the biology of reprogrammed stem cells. "We've had a partnership with L'Oréal since 2014, involving the use of stem cells to create human cell models in the laboratory," he says. "We believe that by innervating reconstructed human epidermis with neurons, we will boost the model's predictive ability."

In addition to growing skin as a platform for testing makeup and personal hygiene products, the skin can also serve as a tool for validating new drugs and studying diseases, such as human papillomavirus (HPV) and melanoma. At USP, studies of this nature are being conducted in Professor Maria-Engler's laboratory and at the Institute of Biomedical Sciences. At the Institute, Professor Enrique Boccardo developed a human skin model *in vitro* to investigate the cell transformation mechanisms associated with HPV to further advance research on cervical cancer, which is caused by the virus. "With the support of FAPESP, I brought this technology from the United States in 2001, when I was working at the Ludwig Institute for Cancer Research (ILPC) in São Paulo," says Boccardo.

"In order to study the biology of the virus more thoroughly, we introduced an *in vitro* cell culture system in Brazil that makes it possible to repro-

Alternatives to animal experimentation

The use of reconstructed skin in Brazil depends on an expensive validation process

Brazil is the world's fourth-largest market for beauty products, outranked only by the United States, China, and Japan. According to the Brazilian Personal Hygiene, Perfumery, and Cosmetics Industry Association (ABIHPEC), the approximately 2,500 companies in the industry recorded sales of R$42.6 billion in 2015. As of 2019, any new beauty product must undergo dermatological testing on reconstructed human skin, in Brazil or abroad. "There are no studies indicating the size of the skin equivalents market in Brazil, but, in theory, it must be substantial, since many cosmetics are introduced to the market every year," states USP Professor Maria-Engler, who sits on ABIHPEC's Science Council.

Two years ago, the National Council for the Control of Animal Experimentation (CONCEA)—the agency of Brazil's Ministry of Science, Technology, Innovation and Communication (MCTIC) that is responsible for regulating animal experimentation in Brazil—stipulated that animal testing must be replaced with tests on skin equivalent models. The agency recognized 17 alternatives to the use of animals in research, two of which call for a human epidermis equivalent to be used in validating cosmetics. "One is meant for ascertaining the irritation potential of new products and the other serves in corrosion assessments of the tested substances," explains former CONCEA coordinator José Mauro Granjeiro. The two alternative methods

PHOTOS 1 L'ORÉAL 2 GUILHERME PUPO

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Skin production at Boticário: testing for toxicity and for skin corrosion and irritation

duce the environment where the microorganism goes through its cycle. Similar to skin, this tissue is composed of human keratinocytes, collagen, and fibroblasts," Boccardo explains. "We've used the model to analyze the molecular mechanisms employed by the virus to evade the body's immune response and understand how HPV manipulates cells in order to synthesize its genetic material and reproduce new viral particles."

At the USP School of Pharmaceutical Sciences, postdoctoral researcher Fernanda Faião Flores relies on the artificial tissue developed by Professor Maria-Engler's group to study mechanisms for resisting melanoma, the deadliest form of skin cancer. "We use cell lineages, patient samples, and a human skin model reconstructed *in vitro* that mimics the invasion and spreading of melanoma cells," she says. "In this way, we test compounds and can characterize the phenomenon of resistance to a drug called vemurafenib, which inhibits tumor proliferation." Reconstructed skin with melanoma was used to evaluate the compound as a possible chemotherapy agent.

TREATING BURNS

In the medical field, another option is to use the reconstituted human skin and cell therapy based on transplanted skin cells to treat skin ulcers and burn victims. In Campinas, dermatologist Maria Beatriz Puzzi, coordinator of the Skin Cell Culture Laboratory at the School of Medical Sciences of the University of Campinas (FCM-Unicamp), studies the laboratory reconstruction of cell tissue for grafting purposes using cells that have been isolated from the victim, which means that the two tissues—the natural and the reconstituted skin—display very similar structures. This method allows for transplantation of the patient's own tissue (autologous transplantation), which lowers the risk of rejection. "The problem with this methodology is that it takes about 45 to 60 days to reconstitute the skin in the laboratory, and burn victims need to be treated immediately," says Puzzi.

To circumvent this problem, the group decided to perform cell therapy using skin cells instead of grafting the reconstructed skin. "We remove a small bit of skin from the patient, isolate the keratinocytes and fibroblasts, and culture these cells in the laboratory. After 15 days, they are mixed with a gel and applied to the patient. In a short while, they spread over the wounds and reconstruct the skin," she says. "We've had really positive results with this method, which speeds up healing, shortens hospital stays, and lowers patient morbidity." ■

have been approved by the Organization for Economic Cooperation and Development (OECD), the agency that endorsed the methods used in Europe, on which the Brazilian standards are based.

"The 3D skin that we developed at USP was created for scientific studies, but it can be used commercially, as long as it undergoes a validation process," says Maria-Engler. In this process, samples of skin grown in vitro must be put through an extensive battery of tests at an estimated cost of R$1 million. One to three independent laboratories usually take part in the validation, which is coordinated by the Brazilian Center for the Validation of Alternative Methods (BRACVAM), with the support of the National Network for Alternative Methods (RENAMA), which was established in 2012 by the federal government. "Because of the high cost, it is only feasible with the support of private businesses and laboratories," notes pharmacist and biochemist Silvia Berlanga Barros, a professor at the USP School of Pharmaceutical Sciences and a member of the Maria-Engler-led group that created the artificial skin.

Projects

1. Development of artificial skin containing glycated dermal equivalent in the assessment of efficacy and toxicity of compound anti-glycation (No. 2011/14327-6); **Grant Mechanism:** Regular Research Grant; **Principal Investigator:** Silvya Stuchi Maria-Engler (USP); **Investment:** R$85,925.35.
2. Generation of human artificial skins and invasive melanomas as a platform for pharmacological testing (No. 2008/58817-4); **Grant Mechanism:** Regular Research Grant; **Principal Investigator:** Silvya Stuchi Maria-Engler (USP); **Investment:** R$165,075.55.
3. Impact of RECK expression on the control of melanoma invasion: Study of monolayers and artificial skin (No. 2010/50157-5); **Grant Mechanism:** Fellowships in Brazil – Postdoctoral; **Principal Investigator:** Silvya Stuchi Maria-Engler (USP); **Grant Recipient:** Carla Abdo Brohem (USP); **Investment:** R$32,690.51.
4. Study of the possible implication of p53 on the effects of tumor necrosis factor-alpha (TNF-alpha) on cells immortalized by the human papillomavirus (HPV) (No. 1998/07087-2); **Grant Mechanism:** Fellowships in Brazil – Regular; **Principal Investigator:** Luisa Lina Villa (USP); **Grant Recipient:** Enrique Mario Boccardo Pierulivo (USP); **Investment:** R$104,861.71.
5. Analysis of polarity protein expression in neoplastic processes associated with human papillomavirus using organotypic cultures (FAPESP-CONICET) (No. 2012/51017-8); **Grant Mechanism:** Regular Research Grant; **Principal Investigator:** Enrique Mario Boccardo Pierulivo (USP); **Investment:** R$22,988.33.