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ORIGINAL ARTICLE

Determining the origin of cells in tissue engineered skin substitutes: a pilot study employing in situ hybridization

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Abstract
Background Definitive and high-quality coverage of large and, in particular, massive skin defects remains a significant challenge in burn as well as plastic and reconstructive surgery because of donor site shortage. A novel and promising approach to overcome these problems is tissue engineering of skin. Clearly, before eventual clinical application, engineered skin substitutes of human origin must be grafted and then evaluated in animal models. For the various tests to be conducted it is indispensable to be able to identify human cells as such in culture and also to distinguish between graft and recipient tissue after transplantation. Here we describe a tool to identify human cells in vitro and in vivo.
Methods In situ hybridization allows for the detection and localization of specific DNA or RNA sequences in morphologically preserved cells in culture or tissue sections, respectively. We used digoxigenin-labeled DNA probes corresponding to human-specific Alu repeats in order to identify human keratinocytes grown in culture together with rat cells, and also to label split and full thickness skin grafts of human origin after transplantation on immuno-incompetent rats.
Results Digoxigenin-labeled DNA probing resulted in an intensive nuclear staining of human cells, both in culture and after transplantation onto recipient animals, while recipient animal cells (rat cells) did not stain.
Conclusion In situ hybridization using primate-specific Alu probes reliably allows distinguishing between cells of human and non-human origin both in culture as well as in histological sections. This method is an essential tool for those preclinical experiments (performed on non-primate animals) that must be conducted before novel tissue engineered skin substitutes might be introduced into clinical practice.

Keywords Tissue engineering · Skin substitute · In situ hybridization · Alu repeats

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Introduction

Large and especially massive skin defects resulting from burns, trauma, congenital giant nevi, and disease leading to skin necrosis, are significant clinical challenges. Clearly, such defects must be rapidly and definitively covered by autologous skin tissue. Surgeons are predominantly facing two problems: donor site shortage in the acute phase of therapy and scarring in the long run [1]. Especially in defects exceeding 50–60% of the total body surface area, donor site shortage is a dramatic and potentially life-threatening issue [2, 3]. Furthermore, transplanting split-thickness skin grafts (which is still the gold standard for coverage of large skin defects), is associated with disabling and disfiguring hypertrophic scarring or keloid formation, particularly in children [4, 5]. On the other side, full-thickness skin transplantation that causes no or only minimal scarring, can only be used to cover small defects (<1% body surface) [1].

Driven by the hypothesis that laboratory engineered skin substitutes with near normal anatomy might attenuate both donor site shortage and scarring [1], we have invested more than 10 years into tissue engineering of skin ("skingineering"). We have recently been able to grow dermo-epidermal skin substitutes of human origin in vitro [6–9]. Obviously, before their eventual clinical application, these substitutes must be evaluated with regard to various aspects in pre-clinical trials employing animal models. In this context, it is indispensable to be able to reliably identify human cells and tissues both in culture and after transplantation.

Human DNA contains considerable amounts of primate genome-specific Alu sequences [10, 11] that can be visualized by in situ hybridization [12]. Here we describe distinct steps and modifications of this technique to allow recognition of human cells in our experimental settings.

Materials and methods

Skin specimens

After approval by the Ethics Committee of the Canton of Zurich and after written informed consent by parents and/or patients, foreskins following circumcision were obtained. Samples were either left intact so as to be used as full thickness skin grafts, or they were cut using a dermatome in such a way as to obtain split thickness skin grafts.

Cell cultures

Isolation and culture of human as well as rat keratinocytes were performed according to standard protocols [7]. Skin biopsies were digested for 15–18 h at 4°C in 12 U ml^{-1} dispase in Hank's buffered salt solution containing 5 μg ml^{-1} gentamycin. Thereafter, epidermis and dermis were separated using forceps. The epidermis was further digested in 1% trypsin, 5 mM EDTA for maximally 3 min at 37°C. Epidermal cells were resuspended in serum-free keratinocyte medium containing 25 μg ml^{-1} bovine pituitary extract, 0.2 ng ml^{-1} EGF, and 5 μg ml^{-1} gentamycin. Culture medium was changed every 3 days. Rat keratinocytes were isolated and cultivated in the same way. All compounds were bought from Invitrogen (Basel, Switzerland).

Transplantation

Anaesthesia for all procedures in rats was performed using isoflurane (Abbott AG, Baar, Switzerland) according to defined standards [6].

Full thickness skin defects were created surgically on the back of 10-week-old female athymic Nu/Nu rats. Then, polypropylene rings, 27 mm in diameter (modified Fusenig chamber [13, 14]) were sutured to the wound edges in order to shelter the wound bed from surrounding skin. Full thickness or, alternatively, split thickness skin grafts were then transplanted on the wound bed within the chamber and covered with Fucidin-Gaze (LEO), Garamycin ointment (Essex), and N-A Ultra Dressing (Johnson & Johnson). After 10 days, healed grafts were completely excised and processed for in situ hybridization.

DNA extraction and probe labeling with digoxigenin (DIG)

PCR was performed as described by Just [10]. A mix of 175 ng/ml genomic DNA, $MgCl_2$ (25 mM), PCR buffer, dNTP (10 mM), and Alu-sense and Alu-antisense primer (each 0.4 μM) was used.

After pre-denaturation at 95°C for 5 min, Taq polymerase (2.6 U/μl) was added ("Hot Start"). Denaturation (95°C for 30 s), hybridization (58°C for 45 s), and elongation (72°C for 45 s) were carried out for 25 cycles, followed by a final elongation for 10 min at 72°C [15]. PCR resulted in a 224 bp long fragment of the most conserved areas of human Alu sequences. The PCR product was electrophoresed on an agarose gel (2% agarose in 1× TAE, 1.5 μg/ml ethidium bromide) together with a molecular mass marker (PhiX174 DNA-Hae III). Thereafter, the DNA band of 224 bp was eluted using the High pure PCR product purification kit (Roche). To determine the concentration, 5 μl of the PCR product were electrophoresed on a 2.5% agarose gel in TBE with 1.5 μg/ml ethidium bromide.

To produce DIG-labeled DNA probes, the same PCR protocol as described above was used and DIG-labeled nucleotides (PCR DIG probe synthesis kit (Roche)) were added. The resulting PCR product was prepared by agarose gel electrophoresis and the labeled probe was purified according to the protocol that came with the PCR DIG probe synthesis kit.

In situ hybridization of cultured cells

Human keratinocytes alone, rat keratinocytes alone, and both cell types in combination were cultured in a density of 3.4×10^4 cells/chamber on chamber slides (Falcon). Thereafter, cells were put in a 2 mM calcium-containing solution for 2 h and then rinsed with PBS.

The cells were fixed in methanol/acetone at −20°C for 5 min, air-dried and acetylated twice using TEA buffer for 5 min.

After three washes for 5 min with PBS, the cells were permeabilized using PBS containing 0.3% Triton X-100, and thereafter treated with proteinase K (2 μl/ml) in TE buffer for 30 min at 37°C. The proteinase K digestion of

membrane and cytoskeleton proteins allowed the probe to enter the nucleus.

After the slides were rinsed again three times for 5 min in PBS, they were acetylated using acetic anhydride containing TEA buffer to reduce nonspecific binding of the probe.

The slides were then pre-hybridized in hybridization buffer for 150 min at 85°C in a moist chamber (50% formamide, 25% H_2O, 25% 20× SSC). During the next 2 h, slides were incubated at the same temperature with a fresh hybridization buffer containing 50 ng/ml denatured DIG-labeled DNA probe. Thereafter, slides were immediately transferred into ice for 10 min, to prevent the denaturized DNS strands from rehybridizing. After overnight incubation at 42°C, slides were briefly rinsed in 2× SSC at room temperature and three times in 0.1× SSC for 15 min at 42°C to remove unspecifically bound probe. Detection of the DIG-labeled DNA probe was performed according to the protocol of the DIG nucleic acid detection kit (Roche). Unspecific antibody binding was blocked for 30 min with blocking buffer (1% blocking reagent (Roche) in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) and then incubated with alkaline phosphatase-conjugated antibody solution (1:2,000 in blocking buffer containing 0.1% Triton X-100) for 1 h.

After rinsing four times with maleic acid buffer for 15 min, slides were rinsed for 5 min in TBS and exposed to a substrate solution for 2–6 h to allow the color reaction to occur. To terminate the enzymatic reaction, stop buffer was added and slides were rinsed three times with PBS, air dried, and mounted using mounting-medium (Dako).

In situ hybridization of crysections

The excised tissue was embedded in OCT compound (Sakura Finetek/Digitana AG, Horgen, Switzerland) and frozen at −20°C before being cut into 12 µm sections using a cryotome. Pre-hybridization and hybridization were performed as described above. Before mounting, some cryosections (treated with or without proteinase K) were stained with Hoechst reagent (1 µg/ml) for 15 min.

In situ hybridization of paraffin sections

Excised tissue specimens were cut into 5 µm thick paraffin sections using a rotation microtome. Sections were floated on a 40°C water bath containing distilled water before being transferred onto silane coated glass slides. Tissue slides were dried at 37°C overnight, deparaffinized, and rehydrated. Subsequently, slides were processed for pre-hybridization and hybridization as described above (including proteinase K treatment to permeabilize cell membranes).

Results

DNA extraction and probe labeling with DIG

Alu-specific DNA fragments were amplified from human genomic DNA by PCR. As expected, a band of 245 bp was detected by electrophoresis (Fig. 1). The oligomers were isolated, eluted and labeled using a PCR DIG probe synthesis kit (Roche).

In situ hybridization of cultured cells

A first series of in situ hybridization with Alu probes was successfully carried out on cultured human keratinocytes, rat keratinocytes, and on co-cultures of both cell types (Fig. 2). While all nuclei of the cultured human keratinocytes exhibited an intensive purple staining (Fig. 2a), rat keratinocytes did not stain at all (Fig. 2b). Before in situ hybridization, it was not possible to morphologically

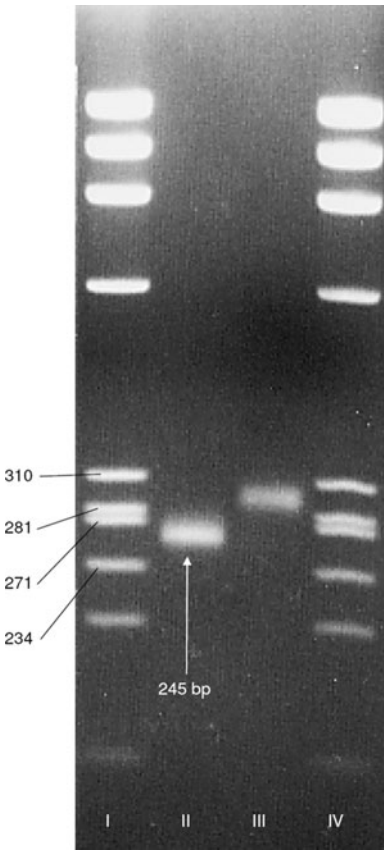

Fig. 1 Agarose gel analysis of PCR products. PCR products were electrophoretically separated electrophoresed on a 2.5% agarose gel and visualised by ethidium bromide staining. *Lane I* "DNA molecular weight marker" (PhiX174 DNA-Hae III, BioLabs). *Lane II* Alu DNA fragment (245 bp, unmarked) *Lane III* Alu DNA fragment (DIG-marked). *Lane IV* "DNA molecular weight marker" (PhiX174 DNA-Hae III, BioLabs)

distinguish between human and rat keratinocytes in co-cultures. Thereafter, distinction was easy and clear cut. Clusters of human cells exhibiting stained nuclei were detectable next to unstained, hence Alu-negative, nuclei of rat keratinocytes (Fig. 2c).

In situ hybridization of cryo- and paraffin tissue sections

Figure 3 shows a cryo- (Fig. 3a) and a paraffin (Fig. 3b) section with densely packed nuclei of the living fraction of keratinocytes. The pictures also reveal that fibroblasts of human origin are still present in the dermal part of the grafts. As proteinase K was not used on these sections, the epidermal and dermal architecture remained largely intact. However, only the nuclei damaged by sectioning were accessible for the probe and therefore stained positive. In contrast, intact nuclei remain unstained, as we demonstrate using Hoechst staining and microscopical inspection (Fig. 3c).

In situ hybridization on paraffin sections resulted in an excellent preservation of the histological structure, even after proteinase K treatment (Fig. 4). Hematoxylin/eosin stained histological sections, excised 10 days after transplantation of human split thickness skin, did not allow to distinguish between human and rat tissue (Fig. 4a). In contrast, using DIG-labeled DNA probes corresponding to human specific Alu repeats, clearly revealed the human cells by their nuclear staining, whereas the cells of the underlying recipient tissue (of rat origin) did not stain (Fig. 4b).

Discussion

Here we describe a successfully applied, optimized technique of in situ hybridization to distinguish between cells of human and non-primate origin in vitro as well as in vivo. Generally speaking, our data show that in situ hybridization using primate-specific Alu probes is a reliable tool to selectively recognize human cells in culture and also after transplanting different types of human skin grafts onto rats. Although, for the sake of labour and cost effectiveness, we transplanted split and full thickness skin grafts and not yet expensive laboratory engineered skin substitutes, there is little doubt that the method applied here will work with the same reliability when cultured skin is used for transplantation.

The described technique has indubitably great value for a number of crucial preclinical experiments to test laboratory engineered skin substitutes in our well-established animal model (human cell-derived skin substitutes transplanted on athymic rats). For instance, we must study how these human skin substitutes take on a rat wound bed during the first days after transplantation (e.g. vascularization, survival of the epidermis), how they develop and

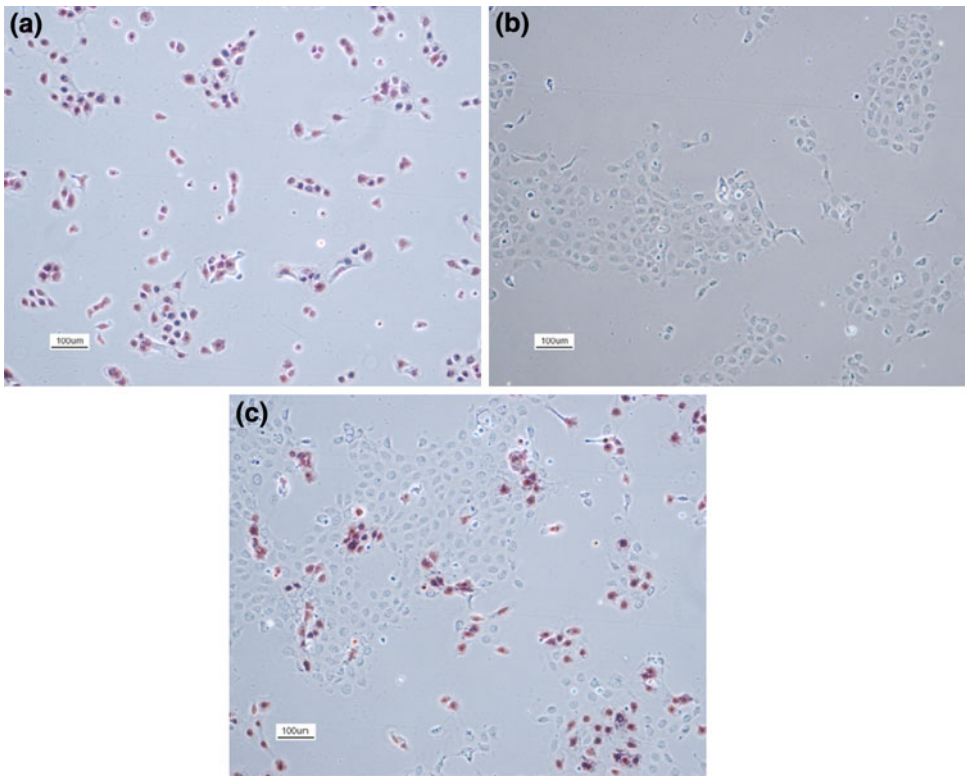

Fig. 2 Human cells are selectively recognized using an Alu probe. **a** All nuclei of cultured human keratinocytes are intensely stained using a DIG-marked Alu probe. **b** There are no Alu sequences in rat DNA, hence nuclei remain unstained. **c** In co-cultures of human and rat keratinocytes, clusters of human cells exhibited stained nuclei, whereas the nuclei of rat cells remained unstained

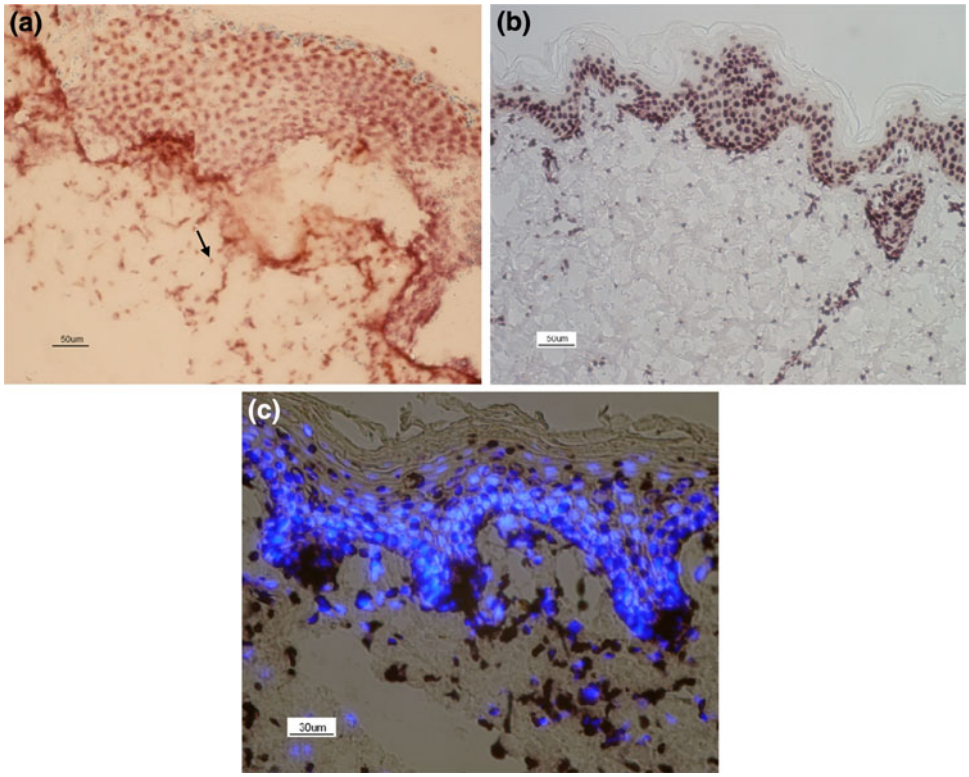

Fig. 3 The result of the hybridization depends on the type of section: 12 μm thick cryosections (**a**) versus 5 μm thick paraffin sections (**b**). **a** Cryosections reveal densely packed purple colored nuclei of the living fraction of epidermal keratinocytes. It is also obvious that fibroblasts of human origin are present in the dermal part of the graft (*arrow*). Note the partial separation of dermis from epidermis. **b** Paraffin sections show basically the same result, except that the epidermal and dermal architecture remained largely intact. **c** As proteinase K was not used on these sections, epidermal and dermal structures remained largely intact. However, only nuclei damaged by sectioning were accessible for the probe and therefore stained positively. Intact nuclei remained unstained. Both nuclear Hoechst staining and nuclear Alu-signals were superposed (Section 12 μm)

mature over the ensuing weeks (e.g. tracking the progeny of human stem cells, maturation of the dermo-epidermal junction), and what the long-term result after 1 year or more is going to be. In all these experiments the distinction between human and rat tissue is definitely essential. The opportunity to study the biological behavior of laboratory engineered human skin on a rat host at any given time desired with the intriguing prospect to always reliably discern human from host tissue is a promising and needed prerequisite on the way to clinical application.

With a broader look on the growing field of tissue engineering, testing of any sort of engineered tissue substitutes of human origin in immuno-incompetent animal models will become an indispensable preclinical routine for analogous reasons as mentioned from our setting. We therefore anticipate that the approach outlined here might find widespread application in future tissue engineering research.

There are a few more aspects calling for a detailed consideration: basically, there are several methods to distinguish human and non-primate animal cells including human specific antibodies, cell detection by the expression of green fluorescent protein (GFP), labeling of cells with fluorescence dyes (cell tracking), and, finally in situ hybridization employing primate-specific Alu sequences [16–18] as used in our experiments.

Obviously, each technique has particular features that may be advantageous or disadvantageous depending on the actual experimental design: for example, antibodies may cause misleading cross reactions between human and animal tissue and also yield considerable unspecific background staining [19]. GFP labeling allows for the monitoring of living cells (provided the GFP gene is stably integrated into the cell's genome), however, the highly expressed reporter system might alter cell physiology [19].

After careful evaluation of the above options, we have chosen in situ hybridization for the following reasons: first, it can easily, reliably, and without any (potentially harmful) preceding cell manipulation be applied to all human cells since the Alu-repeats are present in all nucleated human cells. Second, even degenerated or otherwise abnormal human cells (pathologic protein synthesis/

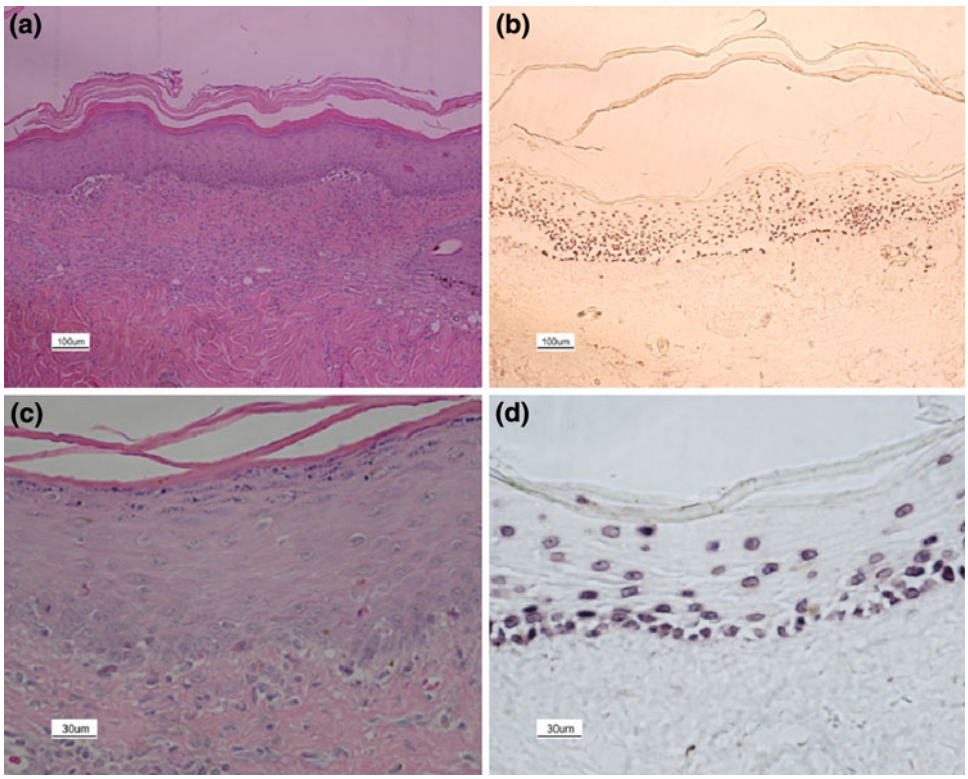

Fig. 4 Human split thickness skin engrafted on an immuno-incompetent rat. **a** and **c** HE stained paraffin sections do not allow to distinguish between human and rat cells (paraffin section, 5 μm). **b** and **d** Grafted human cells can be clearly distinguished from the unstained rat tissue after the in situ hybridization with Alu sequences (paraffin section, 5 μm)

surface abnormality) can be detected due to their high content of Alu sequences [20]. Third, labeled Alu probes can easily and cost-effectively be generated by PCR. Once the specific probe is produced, it can be used as a template for further amplification without the need for new genomic DNA isolation. Since these probes detect highly repetitive sequences that are more or less statistically distributed over the genome and found in excess of a million copies, the resulting signals are much more intense compared to probes detecting single genes or single chromosomes [20, 21].

The last comment regards the use of proteinase K and tissue sectioning. Proteinase K digests cell membranes and thus acts as a door opener for the probe to enter the nucleus. But, at the same time, it also gnaws on tissue integrity. Therefore, we recommend doing paraffin rather than cryosections, as the former yields more solid tissue slices (although production of paraffin sections is more laborious, time-consuming, and also more expensive than the production of cryosections).

In conclusion, in situ hybridization using primate-specific Alu probes consistently permits distinction between human and non-human cells, both in culture as well as histologically. This technique is an instrumental tool for the many preclinical experiments that must be accomplished, testing human cell-derived skin substitutes on non-primate animals before tissue engineered skin can eventually be applied clinically.

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Conflict of interest The authors declare that none of them has any conflict of interest.

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