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Wound Healing on Athymic Mice with Engineered Skin Substitutes Fabricated with Keratinocytes Harvested from an Automated Bioreactor

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Abstract

The Kerator is a computer controlled bioreactor for the automated culture and harvest of keratinocytes that can reduce labor and materials involved in the fabrication of engineered skin substitutes (ESS). Previous studies have shown that Kerator is comparable to tissue culture flasks by keratinocyte confluence during culture, clonogenic potential of harvested keratinocytes and microanatomy, cell viability and surface hydration of ESS fabricated with the harvested keratinocytes. In this study, the Kerator and tissue culture flasks were further compared by keratinocyte proliferation in vitro and wound healing after transplantation of ESS to athymic mice. The number of BrdU positive keratinocytes in ESS fabricated with keratinocytes harvested from Kerator after 2 weeks of in vitro maturation was 34 ± 3 per high power field (hpf) (mean ± SEM), which was not significantly different from that fabricated with keratinocytes harvested from flasks (34 ± 1.5 per hpf). Percentage original wound area 6 weeks after surgery of ESS fabricated with keratinocytes from the Kerator was 36 ± 3.3%, which was not significantly different from that of ESS fabricated with keratinocytes from flasks (30 ± 4.3%). In both cases, 78% (7 of 9) mice transplanted were positive for engraftment of human keratinocytes by direct immunofluoresence for HLA-ABC antigens. These results further confirm that the ESS fabricated with keratinocytes harvested from Kerator and flasks are equivalent in vitro and in vivo. Therefore, use of Kerator for large scale production of ESS can lead to increased availability at reduced cost while maintaining ESS quality for grafting.

Keywords

automation; bioreactor; keratinocytes; engineered skin; wound healing

Introduction

Skin is the largest organ in the body and provides a barrier against infections and fluid loss. Loss of this protective barrier contributes to the mortality and morbidity due to burns involving

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a significant amount of total body surface area (TBSA). Restoration of skin barrier is of definitive importance in the treatment of extensive burn injuries, and this has been accomplished traditionally by autograft or allograft. The limited availability of donor skin for autograft in large burns, and the risks of rejection and transmissible diseases associated with allograft, have led to the development of tissue engineered skin equivalents as alternatives in burn wound treatment. Various approaches have been taken towards the engineering of these therapeutic materials, including cultured epithelial autografts(1;2) (Epicel™); acellular dermal substitutes(3–6) (IntegraDRT™, AlloDerm™), cell populated dressings(7) (TransCyte™); cellular dermal substitutes(8) (Dermagraft™); and bilayered skin substitutes(9), (10) (Apligraf™, Orcel™).

Engineered Skin Substitutes (ESS) are composed of an epidermal substitute of autologous keratinocytes, attached to a dermal analog of bovine collagen-glycosaminoglycan sponge populated with autologous fibroblasts. In vitro prior to grafting, ESS demonstrate morphogenesis similar to native human skin and form a functional epidermal barrier(11;12). After transplantation, ESS have stable engraftment, undergo histogenesis and heal wounds with minimal scarring(13). ESS reduce the requirements for skin autograft in the treatment of massive burns and therefore demonstrate clinical efficacy as adjunctive therapy(14;15). Fabrication of ESS involves: 1) isolation of keratinocytes and fibroblasts from a skin biopsy; 2) primary culture of isolated cells; 3) cryopreservation of cells at the first passage; 4) recovery from cryopreservation and intermediate expansion of cells; 5) sequential inoculation of expanded fibroblasts and keratinocytes on collagen-GAG sponge; and, 6) maturation of ESS at the air-liquid interface prior to grafting(11). These processes are very intensive in terms of the labor and materials used which limits the amount of ESS that can be fabricated at a given time and also impacts the cost of the final product. As a means of increasing product availability and reducing costs we have evaluated the Kerator(16–18), a computer-controlled bioreactor, as an instrument for automation of keratinocyte expansion for fabrication of ESS(19).

The Kerator bioreactor was originally designed for the production of an autologous wound dressing composed of subconfluent keratinocytes attached to a transparent, gas permeable fluorinated ethylene propylene (FEP) film(17;18). To fabricate ESS with keratinocytes cultured in the Kerator, a protocol for keratinocyte harvest was developed to maintain the clonogenic potential of harvested keratinocytes in the subsequent passage. Subconfluent keratinocytes in the Kerator were harvested by incubating in 0.02% EDTA for 8 – 10 minutes, followed by exposure to trypsin for 2 minutes. Keratinocytes harvested from the Kerator in this manner had colony forming efficiencies and growth rates similar to those harvested from flasks, both with and without pre-treatment with 0.02% EDTA. ESS fabricated with keratinocytes cultured in, and harvested from, the Kerator were comparable in vitro to control ESS fabricated with keratinocytes harvested from standard tissue culture flasks by histological organization, cellular viability and surface hydration(19). It is hypothesized based on these in vitro studies, that ESS fabricated with keratinocytes harvested form the Kerator will exhibit wound healing comparable to those fabricated with keratinocytes harvested from flasks, in vivo after transplantation to athymic mice. Demonstration of such comparability between Kerator and tissue culture flasks is an essential step towards the integration of the bioreactor in the process of ESS fabrication for clinical use, where the benefits of automation on cost and availability of the therapeutic product can be fully realized. In this study, engraftment of ESS prepared with normal human keratinocytes from the Kerator or flasks was compared.

Materials and Methods

Kerator Bioreactor

The Kerator has been described in detail in earlier publications(16–18). Briefly, it comprises of a modular polycarbonate growth chamber with fluorinated ethylene propylene (FEP) growth

surface, connected to reservoirs of fresh medium, cell suspension and waste by means of sterile silicone tubing. A bidirectional, multichannel peristaltic pump conveys medium and cells to and from the growth chamber. Fluid flow through the various tubes is regulated by means of pneumatic pinch valves (Figure 1). The growth chamber rests on a platform that can be tilted to one side by a pneumatic actuator (Figure 2). The peristaltic pump, pinch valves and pneumatic actuator are controlled by a custom Virtual Instrument (VI) designed in LabVIEW 6.1, which provides automated fluid handling during medium changes and keratinocyte harvest. A mass flow controller mixes air and 100% CO_2 to provide 5% CO_2 that is filtered and humidified before entering the growth chamber. The growth chamber is located in an insulated box in which the temperature is maintained at 37°C by means of a hot plate and thermostat. The K erator is also equipped with a camera system that allows real time visualization and monitoring of keratinocytes in culture.

Keratinocyte culture and harvest from Kerator

Normal human keratinocytes were inoculated in the Kerator at a density of $4\times10^3/cm^2$ of growth area. A custom VI was programmed to perform fully automated medium changes in the Kerator once every 48 hours. The growth of keratinocytes was monitored by the online image acquisition and analysis system. When the keratinocytes were considered to be 75 – 90% confluent by image analysis (usually on the seventh day of culture), the cells were harvested by treatment with 0.02% ethylenediamine tetra-acetic acid (EDTA) for 8 –10 minutes followed by brief (1–2 minutes) exposure to trypsin. The resultant cell suspension was collected in a reservoir of 10% fetal bovine serum, following which the growth chamber was rinsed twice with HBS to collect the remaining cells. The cell suspension was centrifuged and the pellet was resuspended in modified MCDB 153. The cells were counted under a microscope using a hemacytometer and used for inoculation of ESS.

Tissue culture flasks were inoculated with keratinocytes at the same density and medium was changed manually every 2 days. When keratinocytes reached 75 – 90% confluence, they were harvested by a 15-second rinse with HBS followed by exposure to trypsin for 5 minutes. The cell suspension was collected in 10% FBS and processed for ESS inoculation as described above.

ESS fabrication

ESS was fabricated as described in earlier publications(12;15), with syngeneic normal human fibroblasts and keratinocytes, and collagen – glycosaminoglycan sponge. Fibroblasts were inoculated on the sponge at a density of $0.5\times10^6/cm^2$. The day following fibroblast inoculation, keratinocytes harvested from the Kerator or flasks were inoculated on the sponge at a density of $1\times10^6/cm^2$. The ESS was incubated the air-liquid interface for 3 weeks following keratinocyte inoculation. UCMC 160 medium(20) was used for the first three days of ESS culture and UCMC 161 medium was used for the remaining incubation period, with daily medium changes.

BrdU Immunofluorescence

The number of actively proliferating keratinocytes in the basal layers of ESS fabricated with keratinocytes harvested from Kerator or flasks was quantified by bromo-deoxyuridine (BrdU) immunostaining of CSS in vitro. After 2 weeks of maturation, test and control ESS (n=3) were incubated in 65μM BrdU for 22 h at 37°C and 5% CO_2. Following incubation, ESS were fixed in formalin, embedded in paraffin and sectioned. The sections were processed for BrdU immunostaining by baking at 60°C for 2 h followed by deparaffinizatio n with xylene and rehydration with graded alcohols. The sections were then co-labeled with 1:10 anti-BrdU fluorescein isocyanate and 1:50 primary anti-pancytokeratin by a procedure described previously. After labeling, the slides were washed with PBS and Milli-Q water and

coverslipped with Vectashield hard mount media containing 4′, 6′-diamidino-2-phenylindole (DAPI). The slides were examined under the microscope and 10 unique fields per ESS were analyzed for the number of BrdU positive cells (total 30 fields per condition). Data are expressed as mean ± SEM of BrdU positive cells per field.

ESS transplantation to athymic mice

All animal care and handling in this study was performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Cincinnati. ESS fabricated with keratinocytes harvested from Kerator or flasks were allowed to mature for two weeks in vitro prior to transplantation on athymic mice (nu/nu, Harlan; $n = 9$ animals per condition). Briefly, a full thickness skin wound measuring 2cm × 2cm was prepared on the dorsolateral aspect of each mouse, sparing the panniculus carnosus. ESS along with an overlying piece of nonadherent dressing was placed orthotopically on the wound and was secured to the wound margin with sutures. The grafted ESS was dressed with a piece of sterile gauze coated with antibiotic ointment (containing Bactroban, Nystatin and Neomycin in equal parts) and the gauze was held in place by tying the sutures over it. The grafted site was covered with an occlusive dressing (OpSite) and the mice were wrapped in Coban. Mice were left undisturbed until two weeks after the surgery when the dressings and sutures were removed. Thereafter, the mice were maintained without dressings until six weeks post-operative, at which time they were euthanized.

Wound areas on athymic mice

Mice were photographed at biweekly intervals from 2 to 6 weeks after surgery. The wound perimeters were traced at the time of surgery and at weekly intervals from 2 to 6 weeks post-operatively (n = 9 per condition at each time point). Wound area at each time point was determined from these tracings using computer planimetry. Percent original wound area was defined as the wound area at serial time points divided by the wound area at the time of surgery × 100%. Data for each time point were expressed as percent original wound area (mean ± SEM).

ESS engraftment on athymic mice

Six weeks after surgery, the mice were euthanized and two biopsies of the graft along with adjoining mouse skin were collected. One biopsy was processed by paraffin embedding and the other for cryomicrotomy. The paraffin embedded specimen was stained by H&E and viewed under the microscope to analyze the histological organization of healed tissue. The frozen sections were stained immunohistochemically for HLA-ABC antigens by a procedure previously described(11;12), to confirm the engraftment of human keratinocytes on the mice. ESS engraftment was expressed as the percentage of animals staining positive for HLA – ABC ($n = 9$ each for Kerator or flasks).

Statistical Analysis

Data for wound area and ESS engraftment were analyzed for by one-way repeated measures analysis of variance (RM-ANOVA) followed by Student-Neuman-Keul's test for pair wise comparisons. Data for BrdU incorporation were compared by Student's t-test. Statistical significance was accepted at the 95% confidence level ($p<0.05$).

Results

BrdU Immunostaining

Representative images of BrdU-positive keratinocytes in ESS are shown in Figure 3. In both conditions, the BrdU positive keratinocytes were located in the basal layers of the epidermal

portion of the ESS. Results of the number of keratinocytes in ESS which were positive for BrdU uptake day 14 of in vitro maturation is summarized in Figure 4. There were no significant differences between the Kerator (34 ± 3 per hpf) and flasks (34 ± 1.5 per hpf).

Wound closure on athymic mice

Figure 5 shows representative animals grafted with ESS fabricated with keratinocytes harvested from the Kerator and flasks, at 2 and 6 weeks after surgery. In both conditions, the ESS attached to the wound and to the surrounding margins of native mouse skin. The surfaces of the grafted ESS were dry and well keratinized. ESS from both conditions displayed stable engraftment over the six week study period.

Wound areas on athymic mice

Results of wound area measurements are illustrated in Figure 6. The percent original wound area in mice grafted with ESS fabricated with keratinocytes harvested from the Kerator was 83 ± 6, 60 ± 10, 36 ± 3, 35 ± 3.2 and 36 ± 3.3 at 2, 3, 4, 5 and 6 weeks after surgery, respectively. At parallel time points, the percent wound area in mice grafted with ESS fabricated with keratinocytes harvested from flasks was 82 ± 5.3, 59 ± 10.2, 40 ± 4.1, 33 ± 4.3 and 30 ± 4.3. In both conditions, the wounds areas decreased significantly between 2 and 4 weeks ($p < 0.05$) but there was no change in wound area between 4 and 6 weeks post-op. There were no significant differences in wound areas between the two conditions at parallel time points.

ESS engraftment on athymic mice

Representative H&E stained paraffin sections of healed ESS from test and control conditions are shown in Figure 7A and 7C, respectively. Confirmation of engraftment of human keratinocytes was performed by direct immunofluoresence for HLA-ABC antigens. Positive staining for HLA-ABC antigens in the epidermis appears net-like, corresponding to the distribution of those antigens on the surface of keratinocytes. The staining is absent in the adjacent edge of normal mouse skin (Figure 7B and 7D, arrow). Results of scoring for HLA-ABC immunostaining showed that in both conditions, 78% of animals (7 of 9) had keratinocytes staining positive for HLA-ABC.

Discussion

The Kerator bioreactor provides automation of keratinocyte culture(16–18) and harvest, thereby reducing the labor and materials involved in the cell expansion phase of ESS fabrication. Data presented in this study demonstrate comparability of ESS with keratinocytes from Kerator and flasks by BrdU incorporation in vitro, and wound healing after transplantation to athymic mice.

Previous studies have shown that ESS fabricated with keratinocytes harvested from Kerator and flasks are comparable in vitro by microanatomy, cell viability and surface hydration(19). In this study, ESS from the two conditions were compared in vitro by BrdU incorporation in the epidermis as a measure of keratinocyte proliferation. No significant differences were found, validating the findings from earlier studies (Figure 2). Also, it is known that the formation of a functional, barrier-forming epithelium both in vitro and in vivo depends on a source of proliferative keratinocytes that give rise to the mature corneocytes of stratum corneum. Because the BrdU studies on ESS were done at the time of their transplantation, the results of these studies served as an indicator of their ability to heal wounds effectively.

The athymic mouse has been the preferred model to study the wound healing capability of human skin substitutes(21–33), because of its immunologic tolerance of xenograft and the limited scarring that occurs during healing. ESS grafts fabricated with keratinocytes harvested

from the Kerator had well keratinized and desquamating surfaces after two weeks following surgery (Figure 3). ESS have been shown to form a functional epidermal barrier approaching that of native human skin after transplantation to athymic mice(34;35). Although surface hydration was not measured in vivo in the present study, the dry appearance of these grafts indicated well-formed epithelium. Evaluation of wound areas demonstrated that ESS fabricated with keratinocytes harvested from the Kerator heal wounds in a manner comparable to those fabricated with keratinocytes harvested from standard tissue culture flasks (Figure 4).

Engineered skin fabricated with keratinocytes from flasks were shown to persist for as long as 1 year on athymic mice(36). Tumorigenicity tests done by subcutaneous implantation of such cells in athymic mice ruled out neoplastic transformation after 26-week observation(37). ESS fabricated with keratinocytes harvested form the Kerator showed stable integration with host skin for the six-week period of the present study. However long-term animal studies with these cells and ESS fabricated with them will be necessary to test the stability of ESS. Although FEP has been used as culture surface for growing cell types such as nerve(38) and corneal epithelial cells(39), use of the polymer for clinical will require demonstration of safety and efficacy by such studies.

Pigmented skin was seen within the graft region in 3 out of 9 animals in both conditions, beginning from the fourth week post-op (Fig. 5B and 5D). This gave an early indication of the persistence of human-derived cells because the skin of host animal is devoid of any pigment. This observation also suggests that the FEP culture surface may support the attachment and proliferation of melanocytes, which may be applicable for large scale culture of these cells in the Kerator.

Demonstration of comparability between the Kerator and flasks, both by in vitro and in vivo studies, suggests that the bioreactor can be used for automation of keratinocyte culture for large scale fabrication of engineered skin. Apart from automation, the hands-free operation of cell culture facilitated by the Kerator limits the chances of contamination(40). By integrating online monitoring sensors in the Kerator it can be possible to regulate medium changes according to preset thresholds of biochemical parameters. Such dynamic bioprocess optimization has been shown to enhance cell yield of hybridoma cells(41). Adaptation of this technique to keratinocyte culture can prospectively lead to earlier achievement of an adequate cell yield for ESS fabrication, thereby reducing the lead time between biopsy sourcing and clinical application. Metabolic regulation of keratinocyte culture in the Kerator could also lead to improved cell physiology, thereby resulting in improved ESS quality that may potentially translate into better wound healing in vivo. Although analysis of cost involved in operating the bioreactor has not been performed, automation has historically resulted in greater product availability at lower cost, higher consistency and greater availability. As more tissue engineered products become available commercially, bioreactors such as the Kerator will be valuable tools in making them more available by the manufacturer, and more affordable to the patient

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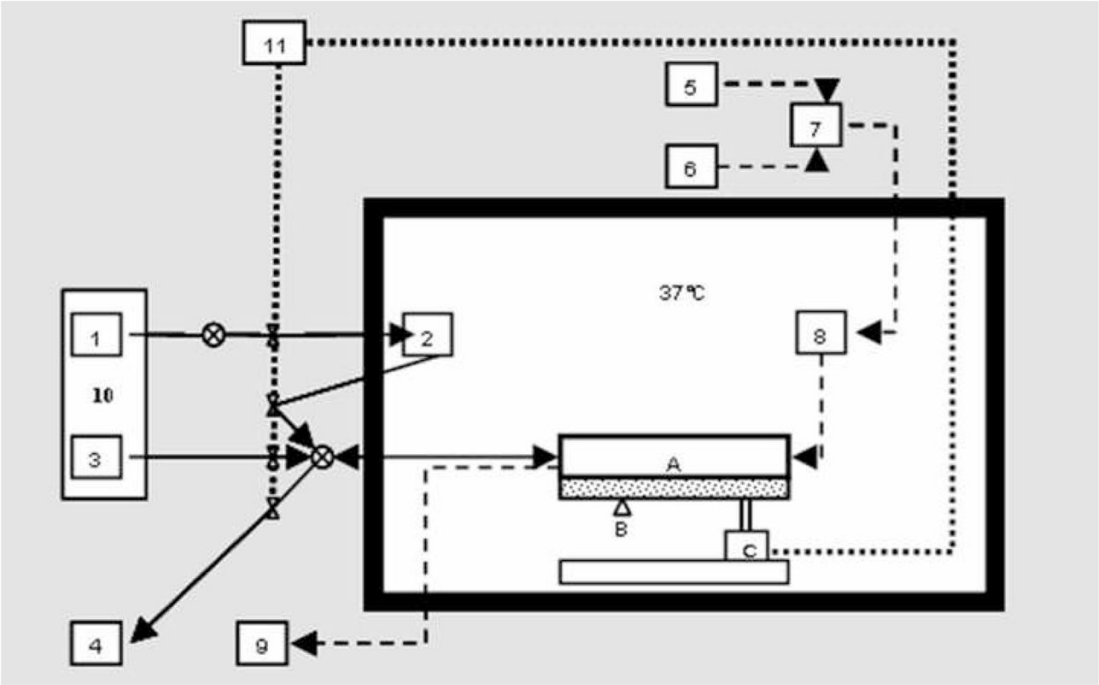

Figure 1.
Schematic representation of the Kerator bioreactor, showing A) Growth chamber, B) Camera, C) Tilting actuator, 1) Cold medium reservoir, 2) Warm medium reservoir, 3) Cell seeding and collection reservoir, 4) Waste, 5) CO_2 supply, 6) Air supply, 7) Mass flow controller, 8) Humidifier bottle, 9) CO_2 detector, 10) Peltier cooler, 11) Compressed air supply.

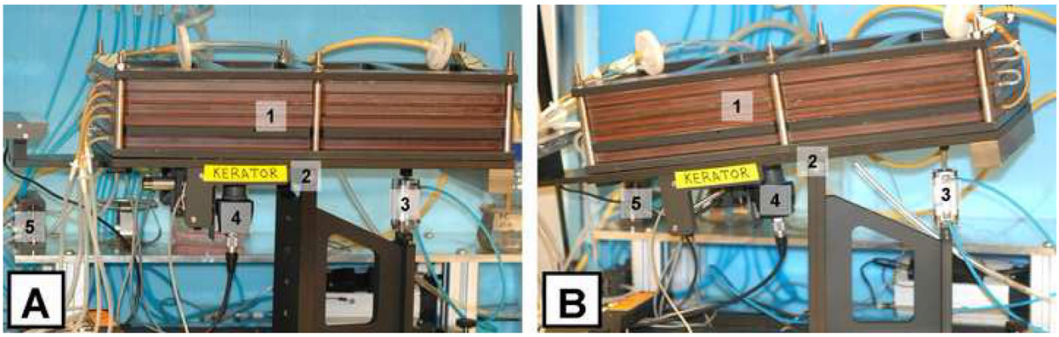

Figure 2.
Frontal view of the Kerator growth chamber in the horizontal (A) and tilted (B) positions. 1) Five-layered growth chamber, 2) Rocking platform, 3) Pneumatic actuator, 4) Camera, and 5) Pneumatic pinch valves. Tilting the resting platform by activating the pneumatic actuator (3) allows medium and cells to be drained from the growth chamber.

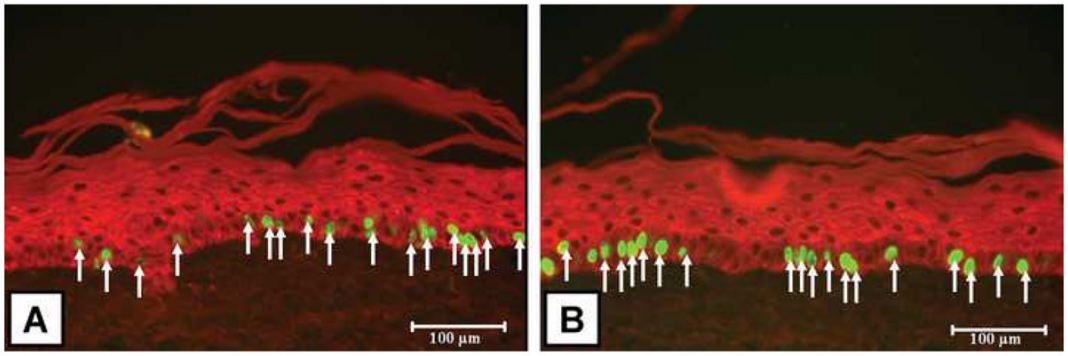

Figure 3.
Representative images of ESS sections (viewed at 20X magnification) stained for BrdU positive keratinocytes. (A) Kerator; (B) Flasks. BrdU positive nuclei stain fluorescent green and are located predominantly in the basal layers of the epidermis. Keratinocytes in the epidermis are stained red by anti-pancytokeratin (see methods). Scale bar = 100μm.

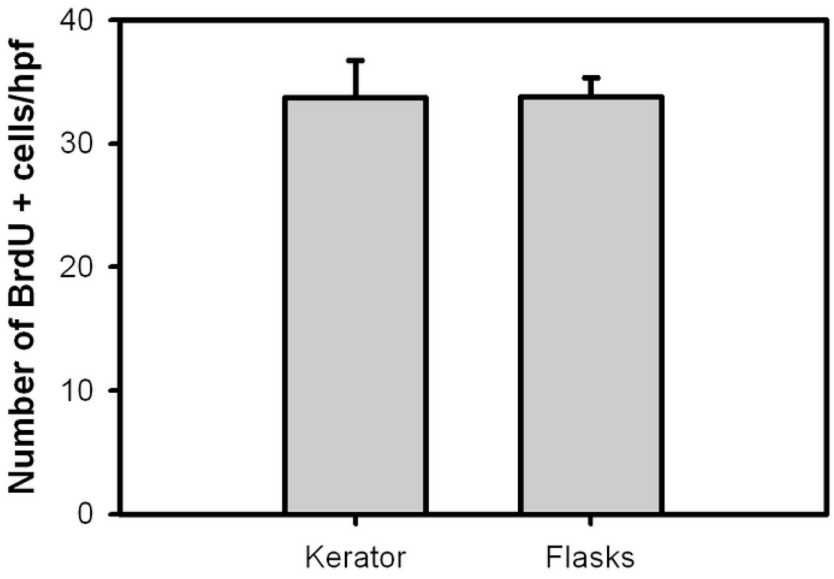

Figure 4.
Number of BrdU positive keratinocytes per high power field in ESS fabricated with keratinocytes harvested from Kerator and flasks, after 2 weeks of maturation in vitro. There were no significant differences between the conditions.

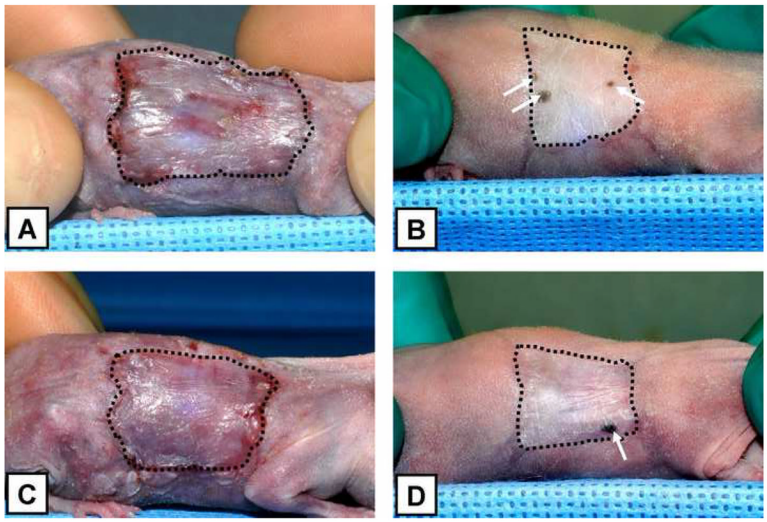

Figure 5.
Representative images, at 2 and 6 weeks after surgery, of athymic mice grafted with ESS fabricated with keratinocytes harvested from Kerator (A and B) and flasks (C and D). The perimeter of grafted area has been delineated with dashed lines. Note the extent of wound contraction in both conditions. Also remarkable is the presence of pigmented spots within the grafted area at 6 weeks in both conditions (arrows), suggesting the presence of human derived cells in those regions.

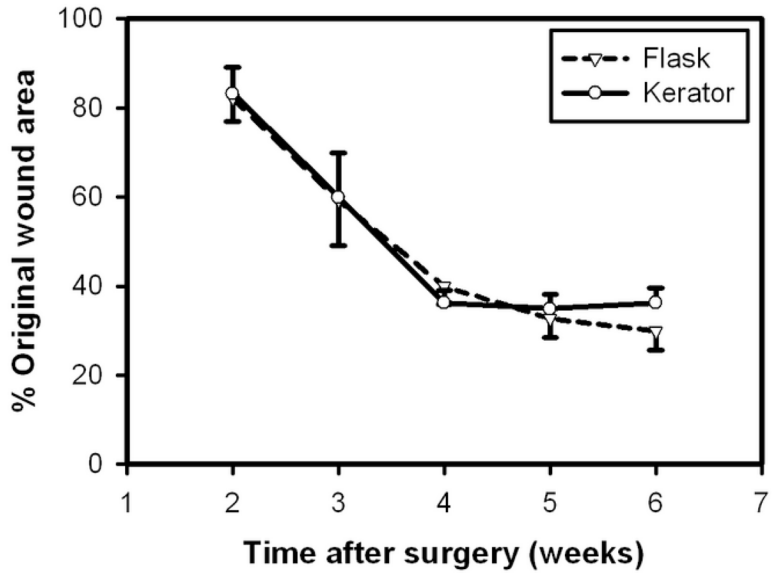

Figure 6.
Percentage original wound area, of ESS fabricated with keratinocytes harvested from Kerator and flasks transplanted on athymic mice. There were no significant differences between the two conditions at parallel time points.

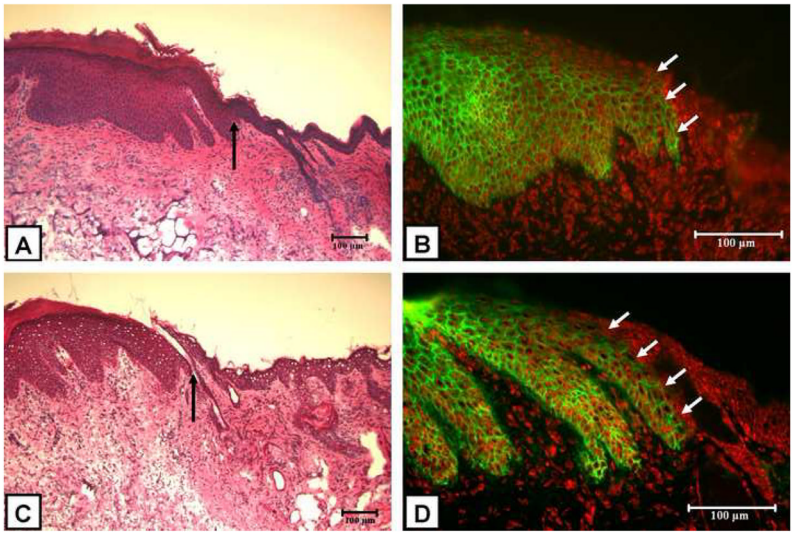

Figure 7.

Representative H&E stained sections and HLA-ABC immunofluorescence of grafted ESS fabricated with keratinocytes harvested from Kerator (A and B, respectively) and flasks (C and D, respectively). In both conditions, H&E sections (A and C) show normal mouse epidermis (to the right of arrow) which is thin and lacks rete ridges. The thicker epithelium with prominent rete ridges (to the left of arrow) resembles human skin and is derived from the grafted ESS. Direct immunofluoresence for HLA-ABC antigens (B and D) shows net-like distribution of HLA-ABC (green) on human derived keratinocytes in the epidermis. Adjacent mouse epidermis (arrow) stains negatively for HLA-ABC. Nuclei have been stained red by propidium iodide. Scale bar = 100μm.