Boosting and Rescuing Epidermal Superior Population from Fresh Keratinocyte Cultures

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Epidermal stem cells (EpSCs) hold great expectations in a regenerative medicine context, but innovative methods that permit to obtain a significant yield of EpSCs or stem-like epidermal cells are still required. We propose a two-step strategy to obtain a superior epidermal stem-like cell fraction among primary keratinocytes (KCs) isolated from adult human skin. The approach is based on the combination of rapid adherence to collagen IV with the rock-associated kinase inhibitor (ROCKi) treatment, and the subsequent immunomagnetic separation of the $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ cell subset. The combined collagen IV and ROCKi treatment showed not only to enhance cells clonogenic capacity, but also to induce an early epidermal phenotypic signature, along with the diminished expression of late differentiation-associated markers. More importantly, collagen IV and the ROCKi efficiently promoted a synergized effect over $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ expression, boosting the number of highly proliferative KCs stem-like cells as demonstrated by the expression of ki67. This cell fraction showed a superior ability to generate a 3D stratified epithelium formed by cells with successive differentiation phenotypes. Overall, this strategy indulged the possibility to uncover, among adult KCs, a superior epidermal cell population with stem-like proliferation capacity and early differentiation degree to be used in numerous skin regeneration approaches.

Introduction

SKIN KERATINOCYTES (KCs) are accessible cells that, when applied as epidermal sheets or as a part of tissue engineering constructs, have proved useful in the healing of burns, chronic wounds, and ulcers. Nonetheless, primary KCs have limited lifespan in culture, which reduces their proliferative potential and success in clinics [1]. As suggested by Watt et al. [2], better approaches for establishing primary KCs cultures or for increasing epidermal stem cells (EpSCs) numbers in those cultures are required to improve cell-based therapies and to shorten the time to treat skin-wounded patients.

The interfollicular epidermis, located between the orifices of hair follicles, regenerates throughout adult life and is known to ensure the continuous replacement of the outermost layer of terminally differentiated KCs that are continuously shed from skin surface [3,4]. This seems to indicate that, under normal homeostatic conditions, bulge stem cells do not contribute to the regeneration of interfollicular epidermis [5], which relies on its resident stem cell population within the basal layer, the EpSCs.

The need for specific and exclusive EpSCs markers has been, and still remains, a shortcoming of the field. Many works have been addressing this issue and nonexclusive

markers, including $\alpha 6$ -integrin [6,7], $\beta 1$ -integrin [8], Keratin 15, as well as Keratin 19 [9] related to EpSCs. Moreover, elevated expression of E-cadherin in adherens junctions and reduced levels of desmosomes [10] have also been associated with EpSCs, stressing the importance of both extracellular and intercellular cues, but confirming the lack of specificity of these markers. EpSCs are distinguished from other basal cells by the high expression of the adhesion molecule $\alpha 6$ and low levels of CD71; thus, cells with this combined phenotype were elected to represent the stem cell subpopulation within basal cells [6]. However, the fraction of isolated skin KCs capable as behaving as EpSCs, that is, to undergo continuous cell-renewal and expansion on demand and lead regeneration, is limited in in vitro cultures. In fact, the estimated keratinocytes stem cells (KSCs) frequency in human epidermis ranges from 1% to 10% [6], and its success is highly dependent on the isolation and culture methodologies [11].

The attempts to overcome this problem started by proposing rapidly adhering epidermal cells to skin basement membrane proteins, such as collagen IV, as EpSCs due to their very high proliferative capacity [12]. Nonetheless, further data demonstrated that this method was not exclusive, as both EpSCs and transit amplifying (TA) cells adhered rapidly to this matrix, both of which exhibited high $\beta 1$ -

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integrin levels [13]. Thus, these data demonstrated that a complementary approach is needed to select EpSCs. ROCK kinase, central mediator of the signals from Ras homolog gene family, member A (RhoA), appears to play a critical role in the regulation of the balance between human KCs (hKCs) proliferation and differentiation [14]. It has been reported that blocking ROCK signaling in the initial 6 days of primary culture of epithelial cells from different sources, with the pharmacological inhibitor Y-27632, leads to an increased number of KCs with higher clonogenic capacity, in a concentration-dependent manner [15].

Considering the need for a superior epidermal cell fraction with enhanced proliferation capacity at an early maturation stage for further use in skin regeneration purposes, we propose a two-step approach that reunites the use of collagen IV for the selection of rapid adherent cells in combination with Rock-associated kinase inhibitor (ROCKi) treatment, and the subsequent separation of $\alpha 6^{\text{high}}/\text{CD71}^{\text{dim}}$ cell subset. We hypothesized that the joined effect of collagen IV and ROCKi would boost the number of cells with KC stem-like cell behavior among freshly isolated adult human skin KCs, which when selected would allow obtaining a fraction that would exhibit higher proliferation capacity, early differentiation marker expression, lack of late KCs associated markers, and ideally holding the capacity to form a 3D stratified epithelia in vitro. Evidence that the proposed approach permitted to obtain a high number of cells with superior characteristics to explore in the skin regenerative medicine context is demonstrated here.

Materials and Methods

KCs isolation from human skin

Healthy skin was obtained from consenting adult female patients who underwent abdominoplasty surgical procedures in Hospital da Prelada, Porto, Portugal, under a collaboration protocol with the 3B's Research Group approved by the ethics committees of the involved institutions. Briefly, skin specimens (ranging between 20 and 75 cm²) were incubated in Dispase (2.4 U/mL) (BD Biosciences), epidermis was peeled off, and dermis was used to isolate human dermal fibroblasts (hDFb) (see Supplementary Materials and Methods; Supplementary Data are available online at www.liebertpub.com/scd) for the organotypic culture. Epidermis was then enzymatically digested by Trypsin-EDTA (0.05%) (Invitrogen) and resuspended in KC serum-free medium (KSFm) (Invitrogen) with 1% Antibiotic/Antimycotic (Invitrogen).

Cell culture

Freshly isolated hKCs were cultured in KSFm at a density of 2×10^4 cells/cm² in polystyrene culture plates (BD Biosciences) at 37°C and 5% CO₂ until cultures reached 80% confluence. The ROCKi cultures were obtained by adding Y-27632 (Sigma) to KSFm at a final concentration of 10 μM . Cell culture flasks (BD Biosciences), coated with collagen IV from human placenta (5 mg/cm²) (Sigma) for 1 h at 37°C, were used to obtain the rapidly adherent cells from freshly isolated adult skin KCs suspensions by removing nonadherent cells 30 min after plating. The combined effect of collagen IV and ROCKi was obtained by adding KSFm with Y-27632 at a

final concentration of 10 μM to RACs. Media were changed every 3 days in control and collagen IV conditions as well as in the ROCKi-containing cultures to maintain its activity (Fig. 1).

Immunomagnetic selection

The separation of $\text{CD71}^-/\alpha 6^+$ fraction was carried out using immunomagnetic Dynal M-450 Epoxy beads (Dyna-beads) (Invitrogen), according to the manufacturer's instructions. Supernatant containing the unbound cells (CD71^- fraction) was plated in KSFm, and cells were expanded until reaching 80% confluence (P2). Protocol was repeated for the selection of CD49f^+ cells from the CD71^- fraction, and the obtained population was plated for further analysis (P3). A fraction of the original cell population, without the immunomagnetic selection, was kept as a control.

Colony-forming unit assay

To determine the clonogenic capacity of cells obtained after collagen IV, ROCKi and combined treatment in comparison to the untreated cells, an in vitro colony-forming units (CFUs) assay originally described by Rheinwald and Green [16] was performed. Cells were plated at densities ranging from 52 cells/cm² (5×10^2 cells) to 520 cells/cm² (5×10^3 cells) in six-well plates (Falcon, BD Biosciences). After 5 days of culture, plates were fixed with 3.7% formalin (Sigma) and stained with crystal violet (Sigma). The number and diameter of CFUs when 5×10^2 cells were plated were determined by analyzing the results for four independent samples with Image J software (Wayne, Rasband, NIH).

Flow cytometry

For surface marker analysis, cells were incubated with CD71-PE (0.4 $\mu\text{g}/\mu\text{L}$; BD Biosciences) and CD49f-APC (0.4 $\mu\text{g}/\mu\text{L}$; eBioscience) antibodies. For intracellular staining, Fix and Perm Cell Fixation & Cell Permeabilization Kit (Invitrogen) was used, and manufacturer's indications were followed. Cells were first fixed and permeabilized with reagent B before incubation with Keratin 14-FITC (1:50; AbD Serotec) or Cytokeratin 19 AF488 (1:20; ExBio) antibodies. For keratin 5 (1:1,000; Covance), intracellular indirect staining was performed with Alexa Fluor 488 donkey anti-Rabbit secondary antibody (1:500; Invitrogen). Samples were analyzed with an FACSCalibur flow cytometer (BD Biosciences) and Cell Quest Pro version 4.0.2 (BD Biosciences) software.

Organotypic culture

Organotypic cultures were prepared as previously described [17], with minor modifications. Briefly, 5×10^5 hDFb were incorporated in a 4 mg/mL rat tail collagen I (Invitrogen) matrix that was cast into polycarbonate transwells (BD Biosciences). The different cell populations, treated and untreated whole and $\text{CD71}^-/\alpha 6^+$ fractions (1×10^6 cells/gel), were then seeded on the top of collagen I gel and cultured in KSFm. After 7 days, constructs rose to air-liquid interface in 3D prime medium CnT-02-3DP1 (CELLnTEC). Cultures were allowed to stratify for 14 days and were then fixed in 3.7% formalin overnight. Samples were paraffin embedded and sectioned for further analysis.

FIG. 1. Schematic representation of the overall workflow. (A) Illustration of the different cell types present in the adult human skin stratified epidermis. (B) Keratinocytes (KCs) freshly isolated from human skin were cultured for approximately 80% confluence in standard (nontreated) and collagen IV-coated (Col. IV) tissue culture plates, and, respectively, in the presence of rock-associated kinase inhibitor (ROCKi) and Col. IV + ROCKi, as KC stem-like cells enrichment strategies. (C) After culture under the different conditions, the CD71⁻ cell fraction was separated by negative selection using CD71 antibody-coated magnetic beads. (D) The CD71⁻ / α6⁺ subpopulation was then obtained from the CD71⁻ population by positive selection with α6 integrin antibody-coated magnetic beads, and cultured for approximately 80% confluence for (E) expansion under the initial defined culture conditions.

Immunocytochemical staining

The different cell populations and samples from the organotypic culture were characterized by fluorescent immunocytochemistry. Primary antibodies were used as follows: keratin 5 (1:1,000), Keratin 10 (1:1,000; Covance), p63 (1:100; Biolegend), β1-integrin (1:30; BD Biosciences), involucrin (1:100; Sigma), and ki67 (1:50; Abcam). AlexaFluor 488/594 (Invitrogen) secondary antibodies (1:500) were used. All fluorescence microscopy images were captured in AXIOIMAGER Z1M (Zeiss), using the Axiovision software (Zeiss). Image J software was used to determine the total number of cells (number of nuclei stained with DAPI) and the percentage of positive cells for involucrin, p63, and ki67. Three random images were analyzed for each one of the four independent samples.

Statistical analysis

Human skin samples from four different donors (n=4) were used in each group for a specific time point. Standard deviation is reported as a measure of sample deviation. Statistical analysis of the flow cytometry data of non-treated and treated keratinocytes was performed using two-way

ANOVA with Bonferroni post-tests. CFUs data and p63, ki67 and involucrin expression results were evaluated by one-way ANOVA with Tukey's post-tests (GraphPad Prism 4.02). Significance levels between groups, determined using post-tests, were set for $p \leq 0.05$.

Results

Treated KCs exhibit typical epidermal basal cells morphology and higher clonogenic ability

The cells obtained from the fresh KCs isolated from human adult skin after culture under the distinct treatment conditions were characterized, in relation to nontreated cells, with regard to morphology and proliferative capacity. The KCs selected by rapid adherence to collagen IV, cultured in the presence of ROCKi and in both collagen IV and ROCKi (P0), were actively dividing, reaching confluence at 6 days after isolation (Supplementary Fig. S1A–C). In contrast, nontreated cultures, at the same culture time, exhibited a few small cells colonies (Supplementary Fig. S1D) and only reached confluence between day 10 and 15. Furthermore, the morphology of the cells treated with collagen IV

(Supplementary Fig. S1A), ROCKi (Supplementary Fig. S1B), or Col IV+ROCKi (Supplementary Fig. S1C) was more homogenous, exhibiting a small and cuboidal appearance, characteristic of epidermal basal cells.

The influence of the different treatments in the clonogenic capacity of the respective cells was analyzed for approxi-

mately passage 3 in order to confirm the suitability of the culture conditions along the passages (Fig. 2i). The number of colonies generated by the treated and nontreated hKCs was determined for the plating density of 500 cells/well (Fig. 2ii), as for the other densities the edges were difficult to define due to the overlap with neighboring colonies.

FIG. 2. (i) Representative photographs of colony-forming units (CFUs) from passage 3 keratinocytes, nontreated and previously treated with collagen IV, ROCKi, and with both collagen IV and ROCKi, seeded at 500, 1,000, and 5,000 cells/well and after 5 days in culture in keratinocyte serum-free medium. (ii) Quantification of the CFUs number and diameter when cells were plated at a density of 500 cells/well. Results are presented as mean $\pm$ standard deviation. In the CFUs quantification, the indicated statistical difference is in relation to the no-treatment condition. * $P < 0.05$; ** $P < 0.01$, $n = 4$.

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CFUs Number	
No Treatment	82 $\pm$ 5.7
Collagen IV	37 $\pm$ 4.2*
ROCKi	38 $\pm$ 11.3*
Collagen IV + ROCKi	31 $\pm$ 4.3**

FIG. 3. Phenotypic profile of nontreated and treated KCs, at passage 3, assessed by (i) flow cytometry and (ii, iii) immunocytochemistry. (i) Representative flow cytometry histograms and data regarding the percentage of cells positive for K5, K14, and K19 confirming the high expression of early differentiation associated markers K5, K14 by all of the groups, and a (*) significantly higher expression of K19 after treatment in comparison to the nontreated cells. Filled areas in the flow cytometry histograms represent the negative population for the respective marker. (ii) (A–P) Immunofluorescence micrographs illustrating the higher expression of early differentiation-associated markers K5 (E, I, M), p63 (F, J, N), and β 1-integrin (G, K, O) in treated KCs, when compared with nontreated ones (A–C). Conversely, late differentiation-associated marker involucrin was clearly expressed in nontreated cells (D) and collagen IV (H) and ROCKi independently treated KCs (L), but almost absent in the collagen IV and ROCKi combined treated KCs (P). A few k10 positive cells (arrowheads) were observed in collagen IV (E) and ROCKi (I) independently treated KCs and none in the nontreated cells (A) and in the collagen IV and ROCKi combined treated KCs (M). Scale bar corresponds to 50 μ m. DAPI was used as nuclear staining. (iii) Graphical representations of p63 and involucrin positive cells quantified after immunocytochemistry. * $P < 0.05$, ** $P < 0.01$, $n = 4$.

(Continued →)

FIG. 3. (Continued).

Nontreated hKCs formed a significantly ($P < 0.05$) higher number of colonies than the treated ones. However, treated hKCs led to significantly larger ($P < 0.05$) colonies than untreated ones. Moreover, ROCKi+Col IV-treated cells proliferated at higher rates than those treated with ROCKi, giving rise to significantly higher ($P < 0.05$) diameter colonies (Fig. 2ii).

KCs simultaneously treated with collagen IV and ROCKi display an early-associated phenotypic profile and diminished expression of terminal differentiation markers

In addition to the distinct clonogenic properties, the phenotype of the cultures obtained after different treatments is of major significance with regard to cells' ultimate performance. The profile of early (K5, K14, p63, β 1-integrin, and K19) and late (K10, involucrin)-associated epidermal differentiation/maturation markers was, therefore, analyzed (Fig. 3). The expression of early markers, in general, was higher in the Col IV + ROCKi-treated cultures, although K14/K5 ex-

pression, in particular, did not vary with the treatments, being expressed by the majority of the cells in all the conditions tested (Fig. 3i, ii). A significantly higher ($P < 0.05$) percentage of cells expressing p63 (Fig. 3ii, iii), in comparison to no treatment, was detected for the collagen IV and ROCKi+Col IV conditions. Similarly, the combined Col IV + ROCKi treatment also resulted in a significantly higher ($P < 0.05$) percentage of cells expressing K19 in comparison to untreated cells (Fig. 3i). In what concerns the expression β 1-integrin, a higher percentage of cells expressing this marker was identified after the treatments but at the levels observed for the untreated cells. (Fig. 3ii)

In what concerns late differentiation-associated markers expression, a few k10 positive cells were observed in collagen IV (Fig. 3ii, E) and ROCKi (Fig. 3ii, I) independently treated KCs. Moreover, K10 expression was not detected in the nontreated cells (Fig. 3ii, A) and in the Col IV + ROCKi-treated KCs (Fig. 3ii, M). In opposition, a significantly higher ($P < 0.05$) number of cells expressing involucrin, in comparison to the collagen IV and Col IV + ROCKi

treatments, were identified in the untreated condition (Fig. 3ii, iii).

The reduced number of cells, observed between the different culture conditions in the immunocytochemistry analysis (Fig. 3ii, A–P), and in particular in the no treatment condition (Fig. 3ii, A–D), might be related with the distinct type of colonies formed (Fig. 2).

Collagen IV and ROCKi synergize to boost the expression of $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ subset, which is enriched in early associated markers

Considering that the balance between the expression of the adhesion molecule $\alpha 6$ and CD71 permits to distinguish a stem-like cell subpopulation within basal cells, the influence of collagen IV, ROCKi, and the combined treatment over $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ subpopulation incidence was analyzed by flow cytometry (Fig. 4). The occurrence of this $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ subset was affected by the proposed treatments (Fig. 4ii). An average of 2.04% of the nontreated KCs represented the referred subpopulation. In the collagen IV and ROCKi-treated cultures, the percentage of $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ cells, respectively, increased to an average of 4.01% and 4.82%. While these values were not statistically different, the percentage of $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ cells (9.32%) identified in the Col IV + ROCKi-treated cultures was significantly higher ($P < 0.05$) than the 2.04% observed in the untreated cells.

The selected $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ subpopulation was then analyzed regarding the expression of the early keratin 14 and the proliferative-associated marker ki67 (Fig. 5). Keratin 14 was expressed by the majority of the population, as before the separation (Fig. 5i). However, the percentage of cells expressing Ki67 was significantly higher ($P < 0.05$), when compared with the subset isolated from the untreated cells,

in the $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ fraction obtained from the Col IV + ROCKi treatment (Fig. 5ii).

Selected $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ subpopulations have the ability to form 3D stratified epithelia

The retention of the differentiation/maturation capacity of treated and nontreated KCs, and of KC stem-like cells obtained by immunomagnetic selection of the respective $\alpha 6^+$ /CD71[−] subsets, was assessed based on their capacity to generate a stratified epithelium. Independently of the condition, all the populations were able to generate a uniform stratified epithelium (Fig. 6). The whole KCs fraction, independently of the culture conditions, formed poorly organized epithelia (Fig. 6ii) A–D). In contrast, the $\alpha 6^+$ /CD71[−] subsets selected from the treated populations gave rise to more defined structures (Fig. 6ii, F–H). These organized 3D epithelia with K14 positive cells in the basal, and the differentiated K10 positive cells in the suprabasal layers, were more evident for the $\alpha 6^+$ /CD71[−] subsets obtained from the collagen IV (Fig. 6ii, F) and Col IV + ROCKi-treated populations (Fig. 6ii, H). Interestingly, dynabeads were distinguished in the outermost layers of the stratified epithelia generated by the nontreated and treated cells KC stem-like cells, the $\alpha 6^+$ /CD71[−] subsets (Fig. 6i, B, D, F, H). This confirmed not only the differentiation/maturation of the selected cells but also the distinct effect of the test conditions denoted by the observed diversity of patterns.

Discussion

Purified EpSCs offer the opportunity to develop next-generation products with enhanced features, overcoming issues such as typical terminal differentiation of epidermal cells caused by extensive culture that results in poor re-epithelialization on transplantation [18]. However, to

FIG. 4. (i) Representative flow cytometry dot plots of nontreated and treated keratinocytes, from four independent samples, highlighting the distinct potential of the treatments on the enrichment of $71^{\text{dim}}\alpha 6^{\text{high}}$ subset. **(ii)** The $71^{\text{dim}}\alpha 6^{\text{high}}$ subpopulation of interest (upper left region in the graphs) represents around 2% of the nontreated culture, respectively, increasing to 4% and 5% in the collagen IV and in the ROCKi independently treated cultures and to 9% in the combined collagen IV and ROCKi-treated cells. *represents statistical difference ($P < 0.05$) to no treatment condition. Data are presented as mean $\pm$ standard deviation, $n = 4$.

FIG. 5. (i) Immunohistochemical analysis of the expression of (A–D) keratin 14 and (E–H) proliferation-associated marker ki67 in $\alpha 6^{+}/71^{-}$ cell fractions obtained from (A, E) nontreated, (B, F) collagen IV, (C, G) ROCKi and (D, H) combined collagen IV and ROCKi-treated keratinocytes, respectively, showing the early keratin profile expression and the proliferative nature of this $71^{-}\alpha 6^{+}$ subset. Scale bar represents 50 μ m. DAPI was used as nuclear staining. (ii) Graphical representation of the number of cells expressing Ki67 confirming the higher proliferative character of the combined collagen IV and ROCKi treated cells. * $P < 0.05$, $n = 4$.

propose innovative methodologies to obtain purified stem-like epidermal cells for further use in skin regeneration purposes has been a continuous challenge and a few works [7,12,15] have addressed this need. The current work envisioned to achieve a superior purified epidermal cell fraction by boosting KC stem-like cells among freshly isolated human skin KCs having as rationale the available knowledge regarding the EpSCs interfollicular basal niche. The combination of rapid adherence to collagen IV with the effect of the

pharmacological inhibitor of rho-associated protein kinases, ROCKi, followed by the immunomagnetic separation of $\alpha 6^{+}/CD71^{-}$ cell subset was hypothesized as a reliable method to enable the establishment of purified hKC stem-like cell cultures.

In accordance to previous works [12,13,19] which demonstrated that both EpSCs and TA cells comprise rapidly adhering cells to collagen IV, we were able to obtain cells with typical basal cell morphology, independently of using collagen IV and ROCKi as individual or combined treatments. It is also known that KCs expressing high levels of $\beta 1$ -integrin, collagen IV ligand, give rise to holoclones, whereas KCs with low levels of $\beta 1$ -integrin produce small colonies [12]. In fact, larger holoclone-like colonies formed by more proliferative cells were generated by the collagen IV and Col IV + ROCKi-treated cells, despite the higher number of CFUs formed by the nontreated cells. Moreover, cells treated only with ROCKi also gave rise to significantly larger colonies than in the control, although smaller than those formed in the ROCKi + Col IV treatment. ROCKi has a safety history in preclinical studies [1], and several reports [15,20,21] have described the involvement of ROCK signaling in KCs differentiation process and in prolonging KC lifespan. Thus, it seems that the combination of collagen IV and ROCKi is effective in the selection of epidermal basal cells. In fact, the phenotypic analysis of the obtained populations showed that Col IV + ROCKi-treated KCs not only maintain the expression of early associated epidermal markers, such as K14 and K5, but also depicted increased expression of K19 and p63, which is usually observed in interfollicular adult epidermis [22,23]. In addition, the proposed Col IV + ROCKi treatment diminished the expression of involucrin, a late-stage epidermal marker [24], in a more effective way than the independent ROCKi treatment. This reinforces the effectiveness of the ROCKi role in inhibiting epidermal differentiation when the initial culture contains more KCs stem-like cells selected by rapid adherence. This assumption was, in fact, confirmed based on the premise that the $\alpha 6^{\text{high}}/CD71^{\text{dim}}$ subset represents the stem-like cells within the treated KCs populations, significantly enhanced by the Col IV + ROCKi treatment. Overall, and considering that estimated KSCs frequency ranges from 1% to 10% [6] and only 0.1%–1% of adult freshly isolated epidermal cells can act as KSCs [16,25,26], we were able to significantly increase those numbers probably by enhancing the frequency of the symmetric division of stem-like cells resultant from the highest clonogenic nature of the Col IV + ROCKi treatment. The expected expression of the early epidermal differentiation-associated marker keratin 14 was demonstrated and displayed by hKC stem-like cells obtained from every treatment, and also from nontreated hKC, as shown before the separation. Nonetheless, further epidermal basal cells features were demonstrated after selection and subsequent culture, by the expression profile of the ki67 marker, typically expressed in a small percentage of epidermal cells in the basal layer and in the hair follicles. This marker was differently expressed in the KCs stem-like cells obtained from distinctively treated cells and in a significantly higher percentage by the cells of the $\alpha 6^{\text{high}}/CD71^{\text{dim}}$ fraction obtained from the Col IV + ROCKi treatment.

Based on the properties of the treated KCs cultures before and after $\alpha 6^{\text{high}}/CD71^{\text{dim}}$ subset selection, the question of

FIG. 6. (i) H&E stained histological transversal sections of the 21 days 3D organotypic co-cultures of human dermal fibroblasts with the (A, C, E, G) whole fraction and (B, D, F, H) the respective $\alpha 6^{+}/71^{-}$ subset of nontreated (A, B) and (C, D) collagen IV, (E, F) ROCKi and (G, H) combined collagen IV and ROCKi treated keratinocytes, at passage 3. Dynabeads (arrowheads) are visible in the mature part of the generated epithelia. * represents stratified areas and ** collagen I gels. (ii) Immunohistochemical analysis of the 3D stratified epithelia generated by (A–D) whole keratinocytes fraction and by (E–H) stem-like cells ($\alpha 6^{+}/71^{-}$ subset) (A, E) nontreated, and subjected to (B, F) collagen IV, (C, G) ROCKi and (D, H) combined collagen IV, and ROCKi treatments, revealing an organized structure with K14-positive cells in the basal, and the differentiated K10-positive cells in the suprabasal differentiated layers. DAPI was used as nuclear staining. Scale bar corresponds to 50 μ m.

whether the obtained cells were capable of entering the KCs differentiation program in which stratification occurs was raised [27]. Cells from the whole fraction generated poorly organized 3D-stratified epithelia in organotypic cultures. In contrast, selected hKC stem-like cells, the $\alpha 6^{\text{high}}/\text{CD}71^{\text{dim}}$ subsets, formed more organized 3D stratified structures. In fact, the magnetic beads used to select the $\alpha 6^{+}$ cells were found in the outermost cell layers of the generated epithelium, thus confirming that the purified cells were able to give rise to daughter cells, cells without magnetic beads in basal layer, and to go into the normal differentiation program reaching the epithelium surface layer. Nonetheless, it seems that epithelia generated by the $\alpha 6^{+}/\text{CD}71^{-}$ subsets obtained from the collagen IV and ROCKi + Col IV-treated cells presented a pattern of successively higher differentiation degree than those from the ROCKi-treated condition. Despite the known rock signaling effect in epidermal stratification [14], it appears that rapid adherence to collagen IV affect the capacity of the $\alpha 6^{+}/\text{CD}71^{-}$ cells to differentiate under the defined conditions.

Hence, the proposed methodology allowed rescuing the cells of interest, actively proliferating, exhibiting an early differentiation phenotype, and capable to generate a fully

differentiated and stratified epithelium. A two-step methodology for the boost and isolation of a superior epidermal stem-like cell subset, from freshly isolated adult human skin KCs, is successfully anticipated here. Rapid adhesion to collagen IV and ROCKi administration led to increased KCs stem-like numbers, which demonstrated a superior functional behavior by generating a fully stratified epithelium.

Acknowledgments

The authors would like to thank Hospital da Prelada (Porto), in particular Paulo Costa for human skin collection. Financial support by Skingineering (PTDC/SAU-OSM/099422/2008), Portuguese Foundation for Science and Technology (FCT) funded project, is also acknowledged.

Part of this work has been presented at XXXVIII Congress of the European Society for Artificial Organs (ESAO 2011) and IV Biennial Congress of the International Federation on Artificial Organs (IFAO 2011) held in Porto, Portugal, October 2011, and 7th International Meeting of the Portuguese Society for Stem Cells and Cell Therapies (SPCE-TC), held in Porto Portugal, May 2012, as oral communications.

Author Disclosure Statement

No competing financial interests exist.

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Received for publication January 16, 2013

Accepted after revision August 21, 2013

Prepublished on Liebert Instant Online August 22, 2013