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Original Article

Xeno-free workflow exhibits comparable efficiency and quality of keratinocytes isolated from human skin biopsies

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ABSTRACT

Introduction: Regenerative solutions of the skin represent a hope for burn victims with extensive skin loss and chronic wound patients. The development of xeno-free workflow is crucial for clinical application in compliance with the directives of the European Medicines Agency. This study aimed at evaluating the outcome of the xeno-free isolation workflow of keratinocytes from human skin biopsy.
Methods: Skin biopsies were obtained from volunteers. The epidermis was digested with TrypLE™ Select, which was deactivated by dilution or with trypsin, deactivated by media with fetal bovine serum. Freshly isolated cells were compared for total cell number, viability, activity of caspase 3, gene expression and the presence of the keratinocyte surface markers cytokeratin 14. The cells were cultured in xeno-free conditions for one week and characterized regarding the number and viability as well as the metalloproteinase secretion.
Results: The number of obtained cells was similar in both workflows. The cell viability was less in the TrypLE group, with slight reduction of the cell surface marker cytokeratin 14. Caspase 3 activity was comparable as well as the gene expression of the apoptotic markers BAX, BCL2 and SLUG, as well as the keratinocyte markers cytokeratin 14, stratifin and filaggrin. Upon culture, the number of keratinocytes, their viability and secretion of matrix metalloproteinases 1 and 10 were equal in both groups.
Conclusion: This study reports the possibility of isolating functioning and viable keratinocytes through a xeno-free workflow for clinical application.

1. Introduction

The method of isolating and culturing human keratinocytes was originally developed by Rheinwald and Green in 1975 [1] and cultured keratinocytes were later used as a cell therapy for burn victims by applying *in vitro* grown cell sheets to the wound area [2]. In moderate and severe burn injuries, skin graft could be considered as the golden standard for management. Unfortunately, it is not possible to obtain healthy skin grafts in burns that involve large percentage of the total body surface area. Furthermore, obtaining the graft is associated with the development of a new wound at the donor site. Autologous cell therapy treatment of severe burn injuries using single-cell solutions sprayed on the wound, overcomes both these issues and can lead to improved scarring [3].

The standard procedure for isolating keratinocytes from human skin involves the use of several animal derived products. In the last few years, the European Medicines Agency (EMA) recommends that materials of animal origin should be avoided whenever possible, according to the Guideline on the use of bovine serum in the manufacture of human biological medicinal products (EMA/

Abbreviations: BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma-2; CK14, cytokeratin 14; EMA, European Medicines Agency; FBS, fetal bovine serum; GAPDH, Glyceraldehyde 3-phosphate Dehydrogenase; MMP, Matrix metalloproteinase; PBS, phosphate buffered saline.

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CHMP/BWP/457920/2012 rev 1). The agency stresses the importance of risk assessments, traceability throughout the process and safety testing verifying no detected bacteria, fungi, mycoplasma or virus in the final product. EMA claims however that a completely virus-safe serum does not exist. Bovine viral diarrhoea virus is a common infection of bovine animals and can only be avoided in serum from specifically controlled cattle donor herds. Additionally, bovine polyoma virus is commonly found in bovine serum.

Trypsin is the digestion enzyme that is widely used in cell culture routine as well as the dissociation of single cells from the epidermal layer [4]. Commercially available trypsin is a pancreatic serine protease, usually from porcine or bovine sources. Furthermore, the enzyme needs to be inactivated to prevent digestion of the cell membrane proteins. Media with fetal bovine serum (FBS) is usually used for this purpose, as FBS contains alpha 1 antitrypsin among other protease inhibitors. Thus, the use of a trypsin workflow is associated with the xeno-derived risk of the enzyme, as well as serum. TrypLE was introduced as a xeno-free enzyme, produced by recombinant technology. This enzyme is gentler on the cells and can be deactivated by simple washing with phosphate buffered saline (PBS) [5]. Manira et al. compared the effect of both enzymes on cell separation from tissue culture plastic [6]. At the meantime there is paucity in the studies investigated such effect on cell isolation from primary tissue, particularly the skin. Since the target in the Research and Development Unit for Skin and Cultured Cells, Linköping University Hospital, is to use *in vitro* expanded keratinocytes as an autologous cell treatment for severely burnt patients, we aim at establishing a xeno-free workflow for the isolation and expansion of human autologous keratinocytes. The aim of this study was to investigate and compare between the 'standard of practice' Trypsin–EDTA and the widely used xeno-free alternative TrypLE™ Select, for the isolation of human keratinocytes from skin biopsies.

2. Methods

2.1. Isolation of human keratinocytes and fibroblasts from skin biopsies

Skin biopsies were obtained from four healthy donors during abdominoplasty and/or breast reduction procedures, under the ethical approval no. 2015/177-31 by the Swedish Ethical Review Authority. The biopsies were cut into 1–2 mm^2 and incubated in Dispase II solution (Life Technologies, Japan) overnight at 4 °C. The epidermis was then gently peeled off from the dermis and incubated with one of the dissociation enzymes on a tube rotator at 37 °C for 30 min. The dermis was used later for fibroblast isolation. Two dissociation protocols were followed; the classical protocol, in which epidermis was exposed to Trypsin–EDTA (Sigma–Aldrich, USA) and deactivated using DMEM (Life Technologies, UK) with 10% FBS (Life Technologies, Brazil). Alternatively, a xeno-free protocol was followed with TrypLE™ Select™ CTS™ (Life Technologies, Denmark) and deactivated by PBS (Life Technologies, UK). The isolated keratinocyte cell suspensions were then centrifuged at 2900×*g* for 4 min, supernatant discarded, and the cell pellets were washed twice with PBS. The cells were cultured in Gibco™ EpiLife™ Media with 60 μM calcium supplemented with S7 supplement, classified as animal origin-free (Life Technologies, USA) and maintained in a humidified chamber with 5% CO_2 at 37 °C for one week.

The dermis was incubated with digestion solution of 0.1% collagenase I (Life Technologies, USA) in DMEM, at 37 °C for 90 min. The digestion was stopped when the tissue structure had been lost by adding DMEM with 10% FBS.

2.2. Determining human keratinocyte yield and viability

Freshly isolated and cultured keratinocytes were stained with 0.4% trypan blue (1:1) and counted using a TC20 automated cell counter (Bio-Rad Inc., Singapore). Viability percentage was recorded as well as total cell count, based on the following equation:

$$\text{Viable cells } (\%) = \frac{\text{Total number of viable cells per 1 mL of suspension}}{\text{Total number of cell per 1 mL of suspension}} * 100\%$$

2.3. Characterization of isolated human keratinocytes using flowcytometry analysis

Flow cytometry analysis was used in order to determine the purity of the isolated cell population. To detect human keratinocytes, cells were stained with the primary antibody anti-cytokeratin 14 (CK14) (Abcam, United Kingdom) coupled with the secondary antibody Goat anti-Mouse IgG Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Life Technologies, Netherlands). To detect fibroblast contamination, cells were instead stained with the negative staining control anti-CD90 (R&D Systems, USA), coupled with the secondary antibody Donkey anti-Sheep IgG Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (Life Technologies, Netherlands). Human fibroblasts were used as a negative control for CK14 and positive control for CD90. Cells were analysed on a Gallios Flow Cytometer (Beckman Coulter, USA) equipped with three lasers. Alexa Flour 647 was detected with red laser (633 nm). Stained and unstained cells were analysed by acquiring minimum of 10,000 events per sample. Forward and side light scatter gates were set to exclude clumps and cell debris. All the flow cytometric acquired data were analysed using Kaluza Analysis Software (Beckman Coulter, USA).

2.4. Caspase-3 colorimetric assay

Freshly isolated keratinocytes were lysed and the cell lysate was assayed for caspase-3 activity using a colorimetric kit (R&D Systems, USA). The cell lysate was incubated with the reaction buffer, prepared according to the manufacturer's instructions, and incubated at 37 °C for 2 h. The developed color was read at 405 nm using SpectraMax Plus 384 Microplate Reader (Molecular Devices, USA). The absorbance was corrected to the total protein content of each sample.

2.5. Gene expression analysis

Quantitative real-time PCR was performed to detect the expression of keratinocytes markers along with apoptotic markers. Total cellular RNA was extracted using RNA/DNA/Protein purification plus kit (Norgen Biotek, Canada). Next, reverse transcription was performed using QuantiTect reverse transcription kit (Qiagen, Germany) as directed by the manufacturer, and the expression of

these genes were determined by the powerup SYBR green mater mix (Applied biosystem, USA). The primers sequences of the targeted genes are listed in Table 1. Real-time quantitative PCR was carried out using Applied Biosystems™ 7500 Real-Time PCR System (Applied biosystem, USA) and amplification was monitored and analyzed by 7500 Software v2.x software (Applied biosystem, USA). The relative fold change in mRNA expression was calculated using delta delta Ct (ΔΔCt) method and normalized to GAPDH.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Supernatant samples were collected after one week of culturing the isolated keratinocytes. Commercially available kits for human Matrix metalloproteinase −1 (MMP-1; Abcam, United Kingdom) and MMP-10 (Abcam, United Kingdom) were used according to the manufacturer's protocol. In brief, the samples and standards were incubated at room temperature for 2.5 h, while the biotinylated antibody was incubated for 1 h with MMP-1 or MMP-10. The developed color was read at 450 nm using SpectraMax Plus 384 Microplate Reader (Molecular Devices, USA).

2.7. Statistical analysis

The statistics was analyzed by Microsoft Excel version 16.34 for Microsoft Office 365 with the add-on 'Data Analysis ToolPak'. Student's t-test was used to evaluate the statistical significance of difference. The figures showed the mean of three to four replicates and the standard error of mean. *p*-value was considered as significant when less than 0.05.

3. Results

3.1. Characterization of freshly isolated keratinocytes using both workflows

Cell count and viability measurements are the most important features to improve the cellular therapy products. Keratinocytes freshly isolated with the classical protocol had no difference for total cell count per cm^2 skin; total cell count per mL skin; total cell count per mL epidermis when comparing with the xeno-free protocol (Fig. 1A–C). Furthermore, cell viability was higher in cells isolated with trypsin by 152% (Fig. 1D). Caspase-3 (Fig. 1E) showed a trend of higher activity, by 150% in the cells isolated with TrypLE in comparison to keratinocytes isolated by trypsin (*p*-value = 0.09). On average, 86.3% of the cells isolated using Trypsin–EDTA were positive for CK14, in comparison to 70.3% of those isolated with TrypLE Select ($p < 0.05$). The cells isolated by both enzymes showed similar low level of expression of the fibroblastic cell marker CD90 (Fig. 2). To investigate the effect of TrypLE select isolation method at the molecular level. BAX, BCL2 and SLUG apoptotic genes were screened along with keratinocytes markers p63, CK14, Stratifin and Filaggrin. Our results showed no significant difference in the genes expression levels in comparison with the classical workflow (Fig. 3).

3.2. Characterization of cultured cells following the isolation by both workflows

The total cell number of cultured keratinocytes showed no significant difference between the standard or xeno-free conditions (Fig. 3B). The viability of cultured cells was similar for both

Table 1
Primers sequences of the genes of interest.

Gene name	forward primer	reverse primer_	Reference
GAPDH	CCTGCACCACCAACTGCTTA	GGCCATCCACAGTCTTCTGAG	[7]
BAX	CATGTTTTCTGACGGCAACTTC	AGGGCCTTGAGCACCAGTTT	[8]
BCL2	GGTGGTGGAGGAGCTCTTCA	TGACGCTCTCCACACACATGA	[9]
SLUG	TGTTGCAGTGAGGGCAAGAA	GACCCTGGTTGCTTCAAGGA	[10]
p63	GAAAACAATGCCCAGACTCAA	TGCGCGTGGTCTGTGTTA	[11]
CK14	CCTCCTCCAGCCGCCAAATCC	TTGGTGCGAAGGACCTGCTCG	[12]
Stratifin	ACTTTTCCGTCTTCCACTACGA	ACAGTGTCAGGTTGTCTCGC	[12]
Filaggrin	TGAAGCCTATGACACCACTGA	TCCCCTACGCTTTCTTGTCCT	[12]

Abbreviations: GAPDH (Glyceraldehyde 3-phosphate Dehydrogenase), BAX (BCL-2-associated X protein), BCL-2 (B-cell lymphoma-2), CK14 (Cytokeratin 14). (12).

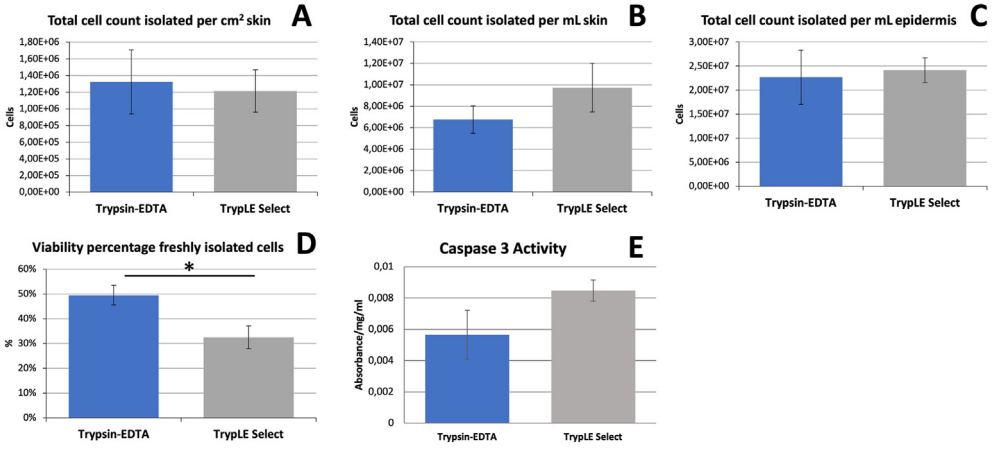

Fig. 1. Keratinocytes isolated from human epidermis with trypsin and TrypLE (A–D). No significant difference in the cell count in relation to the skin biopsy size (A), volume (B) or the epidermis volume (C). On the contrary, the cell viability was significantly different ($p = 0.032$) between the two groups (D), while the activity of caspase-3 activity (E) showed a trend of increase with TrypLE.

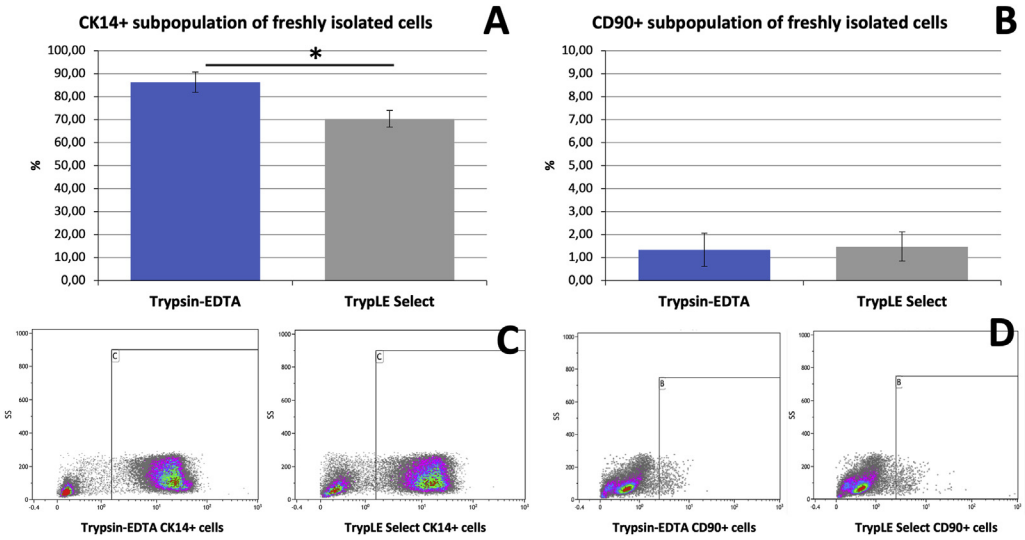

Fig. 2. Keratinocytes isolated with trypsin had a significantly higher expression of the cell surface marker cytokeratin 14 (CK14; A, C) than those isolated with TrypLE. The $CD90^{+}$ subpopulations (B, D) were similar in the two study groups.

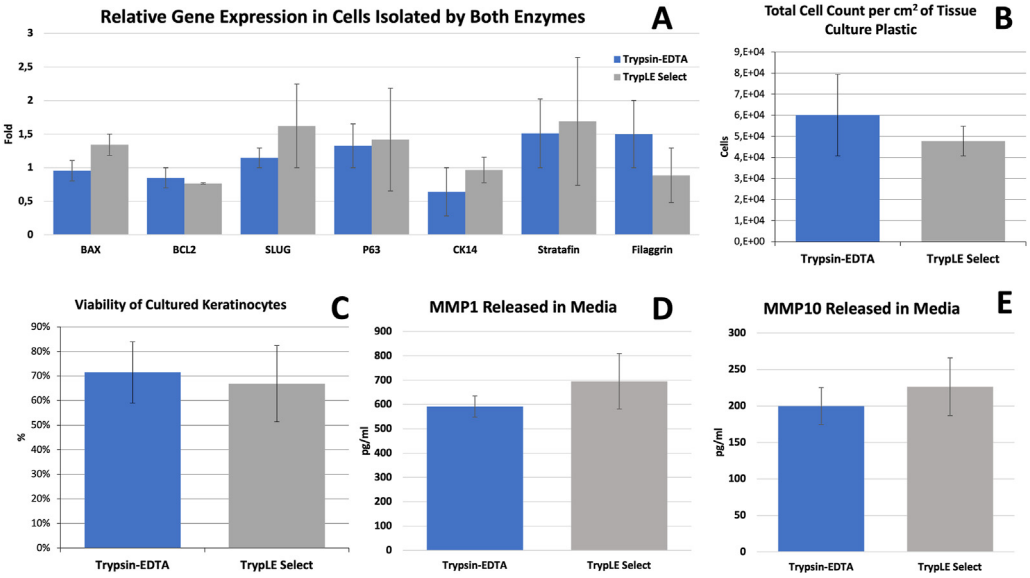

Fig. 3. The gene expression of freshly isolated cells showed no significant difference for either the apoptosis or keratinocytes markers by either workflow (A). There was no difference between the cell number (B) or viability (C) of cultured keratinocytes isolated by either enzyme from tissue culture plastic (TCP). The amount of MMP-1 (D) or MMP-10 (E) released in media was similar in cells isolated by TrypLE or trypsin.

conditions and showed higher values in comparison to the freshly isolated cells (Fig. 3C). Functionally, the secretion of both MMP-1 and MMP-10 (Fig. 3D, E) was comparable in keratinocytes isolated using both workflows.

4. Discussion

The new directives of the European Directorate for the Quality of Medicines & Health Care recommend against the use of animal derived products during cell manufacturing. The mission of the Research and Development Unit for Skin and Cultured Cells in Linköping University Hospital is to provide a “simple and safe skin regenerative solution for burn victims and chronic wound patients”. Autologous keratinocytes can be considered as an attractive target for this purpose. The extraction process of these cells from skin biopsies involves multiple steps, including digestion of epidermis with trypsin and deactivation of the enzyme by media with FBS. Both trypsin and FBS should not be considered for advanced medicinal products, such as cultured keratinocytes. Previously published data by Tuschong et al. highlighted that FBS antibodies could be found in patients who underwent cell transfer using *in vitro* cultured lymphocytes. They further reported elevated levels of anti-FCS IgG antibodies in a patient more than 8 years after having received T cell gene therapy, despite extensive washing of the cells prior to infusion. They suggested that FCS antigens were integrated into the cell membranes *in vitro*, and later entering the patient's circulation [13]. Selvaggi et al. presented in a previous study that antibodies against FCS were detected in 67% of HIV patients treated with extensively washed lymphocyte transfers, prepared from their identical twin and cultured *in vitro* supplemented

with 5% FCS. Following the transfusion, 92% of the patients presented clinical side effects such as prolonged fever, myalgias and chest tightness. Fifty percent of the patients presented specific so-called arthus-like symptoms [14]. These findings stress the importance of aborting the use of FBS during cell culture prior to human cell therapy, and instead exploring xeno-free alternatives. Furthermore, variations can be found in the composition of FBS. As cells are sensitive to even the slightest changes in their microenvironment, using FBS has the potential risk of influencing the cells and affecting their performance characteristics and attributes [15,16]. Thus, in order to get a more reproducible outcome, it is most likely better to avoid the involvement of FBS. TrypLE is a recombinant enzyme that is inactivated solely by dilution, hence FBS is not needed. Moreover, by excluding products of animal origin during cell isolation and expansion, the risk of introducing xenotropic-viruses and immunogenic agents to the patient is eliminated.

The use of different dissociating enzymes could have an effect on the cell quantity and quality [17]. In a previous study, Tsuji et al. examined different cell-detaching reagents, such as trypsin and TrypLE. They found that both trypsin and TrypLE dissociated adherent cells within a few minutes, however trypsin reduced the expression of several cell surface antigens whilst TrypLE did not affect the antigen expression [18]. Vrtačnik et al. found profound RNA degradation as a result of using trypsin during dissociation of adherent cells prior to RNA isolation. No signs of RNA degradation were found when instead using alternative procedures, such as TrypLE, during RNA extraction. They suggested that RNA degradation with trypsin is a result of extracellular RNases contaminating the enzyme, which originates from animal pancreas [19].

In this study, the enzymatic efficiency was investigated during *in vitro* cell isolation comparing the standard workflow, using Trypsin–EDTA and inactivation with FBS, with xeno-free conditions, using TrypLE™ Select and inactivation with PBS. Our data did not show significant differences of the cell number isolated from skin biopsies, with the xeno-free agent TrypLE™ Select in comparison to Trypsin–EDTA. The cell number was corrected to the volume of skin and detached epidermis as well as the surface area of the skin, with similar efficiency of isolation between the two workflows. The epidermal thickness and the number of keratinocytes can vary between individuals and even topologically within the same individual, being richer at the sun exposed areas, which makes the correlation between the cell number to the epidermal thickness more logical than the skin surface area or whole skin thickness [20]. Although, the cell viability seems to be more preserved with the classical trypsin workflow. This pattern was absent for cell separation from tissue culture plastic, which is in agreement with Manira et al. [6]. This decline of cell viability with TrypLE could not be explained by the activity level of caspase-3, which is a pro-apoptotic enzyme that is not involved with keratinocyte differentiation but with cytoskeletal rearrangement during apoptosis [21,22], as the enzyme was similarly expressed in both groups. Similarly, the expression of apoptosis related genes, BAX, BCL2 and SLUG was very comparable. BCL2 can protect the cells from going through apoptosis by the regulation of BAX. The overexpression of one of these two genes antagonizes the other's effect [23,24]. SLUG is considered as a potent inducer of cell survival and movement; apoptosis can be induced by suppression of this gene [25]. This data can exclude a potentially noxious risk of any of the two enzymes, but it cannot provide a reason for the difference in viability. On the other hand, the effect of both enzymes on stratum corneum may provide an explanation for this result. The uppermost layer of epidermis consists mainly of dead enucleated cells, which has a very important function as a protective barrier. Trypsin has a tendency to digest and dissolve this layer and digest up to 75% of epidermal proteins [26]. TrypLE is known to be gentler on primary cells, including keratinocytes, in comparison to trypsin [27]. Thus, the corneocytes could have be released with TrypLE and counted as dead cells, while digested with trypsin. Unfortunately, no studies can be found to describe the effect of TrypLE on stratum corneum.

In order to determine the characteristics of freshly isolated keratinocytes, using either standard or xeno-free conditions, the cells were analysed with flow cytometry for the keratinocyte specific marker CK14 and a fibroblast specific surface antigen CD90 as fibroblasts are the most common contaminant of keratinocyte isolation process. Our data showed slight significant reduction, by 16%, in the percentage of the cell population isolated using the xeno-free conditions in CK14. These findings were not in agreement with Tsuji et al. findings with synovial mesenchymal stem cells, as mentioned earlier, but with agreement with the findings for isolating $CD133^{+}$ from glioma cells, as well as by previous work of our research team comparing accutase to trypsin [18,28]. CK14 is one of the most commonly used markers for keratinocytes. It is mainly expressed in the basal layers, which is the young cells that will differentiate to the upper layers of the skin. The expression of this marker decreases as the cells migrate to the top of the epidermis and is absent in stratum corneum, which supports our assumption about the gentle effect of TrypLE on corneocytes and their consideration during the flow analysis characterization. At the molecular level, no significant difference could be found in the studied genetic markers. p63 is a master regulator of epidermal development, it controls the expression of the genes that have a role in the cell adhesion, signalling, and lineage-specific components of the cytoskeleton, such as keratins [29]. The latter are important markers for the differentiation stage as well as the location of human keratinocytes. Keratinocytes in skin basal layer express CK14, CK15 and CK5 [30]. The release of Stratifin from keratinocytes at the dermal–epidermal junction has a paracrine signalling effect on fibroblasts and strongly stimulate MMPs activity, which is important in wound repair [31]. Filaggrin is a filament-associated protein which binds to keratin in epithelial cells at the stratum corneum [32]. The absence of difference of gene expression may enforce the role of corneocytes in the differences noticed in the viability and CK14 expression as a surface marker, as this subpopulation of keratinocytes are metabolically inactive and cannot influence the gene expression pattern [33]. Nevertheless, the statistical reduction of CK14 positive population should not affect the biological validity of the xeno-free protocols, especially in consideration with the similarities in the cell number, viability and functionality when the cells from both workflows were cultured.

The ultimate goal of the isolation process is to have viable and functional keratinocytes that can be expanded in xeno-free culture conditions and can be transplanted in autologous fashion. Our data showed that the cells isolated by both workflows showed similar cell count and viability after one week. Furthermore, the production and secretion of MMP-1 and MMP-10 was similar. The expression of these MMPs is crucial to allow keratinocyte cell migration and wound repair. This enzyme is usually produced by the cells at the wound edge *in vivo* and released in the media when keratinocytes are cultured on collagen *in vitro,* through the activation of ERK signalling pathway [34–36]. As skin topology varies between different sites in the body, our data may need further confirmation for biopsies obtained from other sites of the body than the abdomen or breast.

5. Conclusion

This study validated the use of a xeno-free workflow for the isolation of keratinocytes from epidermis against the classical system that includes trypsin and FBS. Both systems showed

comparable efficiency in isolating the cells, which would recommend the use of TrypLE for keratinocyte isolation from skin biopsies in clinical settings.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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