Original Research
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Phenotype changes of oral epithelial stem cells after *in vitro* culture

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Abstract: The aim of our study was to isolate populations of keratinocyte stem cells based on the expression of cell surface markers and to investigate whether the culture could affect their phenotype. keratinocytes from human oral mucosa were sorted based on the expression of the epithelial stem cell markers p75NTR and CD71. We also examined the co-expression of other epithelial stem markers such as integrins β1 and α6 and their stem cell-like proprieties in *in vitro* assays. Three passages after being sorted by MACS, more than 93% of the p75NTR^{+ve} cells lost the expression of p75NTR, while 5.46% of the p75NTR^{-ve} gained it. Within the small population of the p75NTR^{+ve} cells, 88% co-expressed other epithelial stem cell markers such as integrins β1 and α6, while only 28% of p75NTR^{-ve} cells co-expressed these markers. These results were confirmed by sorting cells by FACS. Additionally, when double staining was used for sorting cells, 99% of the p75NTR^{+ve}CD71^{-ve} and 33% of the p75NTR^{-ve}CD71^{+ve} cells expressed both integrins, but just one week after culture, only 1.74% of the p75NTR^{+ve}CD71^{-ve} cells still expressed p75NTR and only 0.32% still expressed CD71. Similar results were obtained when co-culturing p75NTR^{+ve} and p75NTR^{-ve} populations before analysis. Our results suggest that phenotype changes may be part of an intrinsic cellular mechanism to conserve levels of protein expression as they may found in the human body. In addition, *in vitro* culture may not offer ideal conditions for epithelial stem cell maintenance due to phenotype changes under standard culture conditions.

Keywords: Stem Cells; Mouth Mucosa.

Introduction

Isolation of progenitor epithelial cells has been proposed by using many different techniques such as the capacity of rapidly adherence to collagen IV,[1] the morphology of the colonies[2] or the ability to efflux Hoechst 33342 (side population).[3,4] Likewise, a variety of cell markers for isolating progenitor epithelial cells either by using Magnetic Activated Cell Sorting (MACS)[5] or by Fluorescence-Activated Cell Sorting (FACS) have been described.[6]

The p75NTR is member of the TNF receptor superfamily,[7] and it`s has been suggested as a potential epithelial stem cell marker in different tissues and organs including esophagus,[8] uterine cervix,[9] epithelial odontogenic

tumours[10] and oral mucosa.[5] The expression of integrins β1[11] and α6[12] and the absence of expression of CD71[13] have also been shown to be useful for identifying epithelial progenitor cells.

Here we isolated different populations of keratinocytes by MACS and FACS based on the expression of the epithelial stem cell markers P75NTR and CD71, respectively. We also analyzed the expression of these and other epithelial stem cells markers in the positive and negative populations. To assess the stem cell proprieties, we performed functional assays such as long-term growth potential, determination of the colony-forming efficiency and *in vitro* epithelial reconstruction.

Cell culture is a gold standard technique used to expand cells (including stem cells) prior to *in vitro* experiments or *in vivo* use. However, the literature is controversial in relation to whether culture conditions could affect cells phenotype[14,15] and there is no evidence that epithelial stem cells do not change after sorting.[5] Also, it's been recognized that cells double staining is more efficient than single staining to enrich a cell population with stem cells[13] but nothing is known about whether double staining would result in maintenance of stem cell phenotype under standard culture conditions.

Additionally, there is a lack of information whether cell sorting technics (FACS vs MACS) themselves or if the time that cells stay in culture could have any influence on either the phenotype maintenance or on their functional properties. Therefore, we have analyzed if the cell sorting technic and cell culture period could affect either the phenotype of our sorted populations or their functional properties.

Methodology

Tissues

Specimens were obtained in accordance with the Ethical Committee of the University of Sao Paulo, Brazil, and King`s College London, United Kingdom. All donors were informed about the procedure and provided consent for biopsy and cells harvest. Samples were obtained from healthy oral mucosa (gingiva and palate) from 22-35 years old patients undergoing to oral surgeries for clinical reasons. From those samples, two different keratinocyte stem cell lines named KSC-I and KSC-II were stablished. Both cell lines were used for functional assays (long-term growth potential, colony-forming efficiency and in vitro epithelial reconstruction). Only KSC-II was used for experiments involving cell sorting due to higher yield and thus resulting in a better cell density necessary for those experiments. All experiments were done in triplicates.

Primary culture of keratinocytes

Oral tissues were washed 3x in PBS and incubated in 2.5mg/ml of dispase (Gibco #17105-041) at 4°C for 12-16h. The epithelium was then mechanically separated from the underlying mesenchyme with tweezers and digested with TrypLETM (Gibco, #12563) at 37°C. Cells were immediately labelled with anti-p75NTR antibody and sorted ether by MACS or FACS. After sorting, cells were plated on the top of a feeder layer composed by irradiated 3T3-J2fibroblasts as previously described on the literature[16]. Keratinocytes were cultured in DMEM (Lonza, #12-604) with 30% of Ham's F-12 Nutrient (LifeTechnologies, #11765070), 10% of Fetal Bovine Serum (Hyclone, #SH30109.03), 1% of antibiotics (Gibco, #15240), 0.18mM of adenine (SIGMA, #A9795), 5µg/mL of insulin (SIGMA, #A9795), 0.4 µg/mL of hydrocortisone (SIGMA, #H0396), 0.1 nM of cholera toxin (SIGMA, #C8052), 20pM of triiodotironin (SIGMA, #058K8704), 10ng/mL of EGF and 1% of HEPES buffer (Gibco, #15630). When cells reached 80% of confluence, they were removed from the flask with TrypLETM and serially plated at a density of 6x10^{3} cell/cm^{2} on a freshly made monolayer of irradiated 3T3-J2 fibroblasts.

Magnetic cell sorting

Freshly dissociated human oral keratinocytes were passed thought a 40µm filter and labelled with 20 µL of p75NTR MicroBead Kit (Miltenyi Biotec #130-092-283) per 10^{7} total cells following manufacturer's instructions. Cells were sorted by MS Columns (Miltenyi Biotec #130-042-201) in the MiniMACS™ separator system (Miltenyi Biotec #130-042-102). After been sorted, the p75NTR^{+ve} and

p75NTR^{-ve} cells were plated at the same density and cultured separately under same conditions.

Cell sorting by flow cytometry

Once obtained single cells suspension by passing the cells thought a 40µm filter, they were re-suspended in 2% FBS in PBS and incubated at 4ºC for 15 minutes with PE-conjugated human anti-p75NTR antibody (Biolegend, #345105) using 10µl of antibody per 10^6 cells. After a PBS wash, cells were sorted using the BD Bioscience FACSAria.

Flow cytometry analysis

A single cells suspension was obtained as described above and then the cells were incubated with APC anti-human CD71 (eBioscience, #17-0719); PE-Cy7 anti-human Integrin α6 (eBioscience, #25-0495) and FITC anti-human Integrin β1 (eBioscience, #11-0299) using 10µl of antibody per 10^6 cells, for 15 min at 4ºC. Flow cytometry analysis was performed using the FACSCanto (BD Bioscience) and FlowJo 8.7 software (Tree Star Inc.; Ashland, OR). Dead cells and debris were identified using the gating strategy based on forward (FSC) and side scatter (SSC) (FSC-low and SSC-high). Single positive staining for each marker and unstained controls were used to establish compensation settings on the FACS. Each one of these setting samples provided a single parameter histogram from each it was possible to determine the ideal parameters of laser voltages and gating to identify both positive and negative populations for each staining.

Long-term growth potential

The long-term growth potential was assessed in both fractions of cells after been sorted either by MACS or by FACS. These populations were serially passaged at the density of $6x10^3$ cells/cm^2 until cells lost their proliferative capacity.

Determination of the colony-forming efficiency

After sorting, cells were expanded up to the third passage and plated in triplicate, in low density (1.5 cells/cm^2), to evaluate their colony-forming efficiency. On the 6th day of culture the colonies were fixed with methanol at -20ºC for 10 min, stained with toluidine blue and counted. All colonies with 50 or more cells were scored and an average number of colonies were obtained. The number of colonies on the p75NTR^{+ve} and p75NTR^{-ve} populations were analyzed independently using the t-Test on the SPSS v.25 software. Values were considered significant when p-value < 0.05

In vitro epithelial reconstruction

At the third passage, cells from both fractions of P75NTR (positives and negatives) were plated in triplicates, in six-well plates, and allowed to grow for 3 days more after they reached confluence. The epitheliums were removed from the wells using dispase diluted in DMEM (2.5 mg/ml), for 1h at 37ºC. After 1h fixation in 4% formaldehyde, tissues were histologically processed for paraffin embedding and sectioned on microtome. The samples were stained with haematoxylin & eosin and analyzed under optical microscope.

Results

Characterization of keratinocytes sorted by MACS

Three passages after cell sorting by MACS both p75NTR^{+ve} and p75NTR^{-ve} populations were analyzed by FACS. At this moment it was possible to observe that just 6.93% of the population p75NTR^{+ve} retained the expression of p75NTR (Figure 1A) while a similar percentage of cells (5.46%) gained the expression of p75NTR in the negative population (Figure 1B).

When the co-expression of other epithelial stem cells markers (Integrin β1 and α6) was studied on the same passages of the p75NTR^{-ve} and p75NTR^{-ve} populations, it was possible to observe that 88% of the p75NTR^{+ve} cells were co-expressing both integrins in contrast with just 28% of p75NTR^{-ve} cells. Moreover, 6% of the cells were p75NTR^{+ve}CD71^{-ve} and from this population 99% were expressing both integrins β1 and α6. Meanwhile, 33% of the cells were p75NTR^{-ve}CD71^{+ve} and of this population just 56% expressed both integrins.

Characterization of keratinocytes sorted by FACS

Based on the results described above and considering that the double staining could be

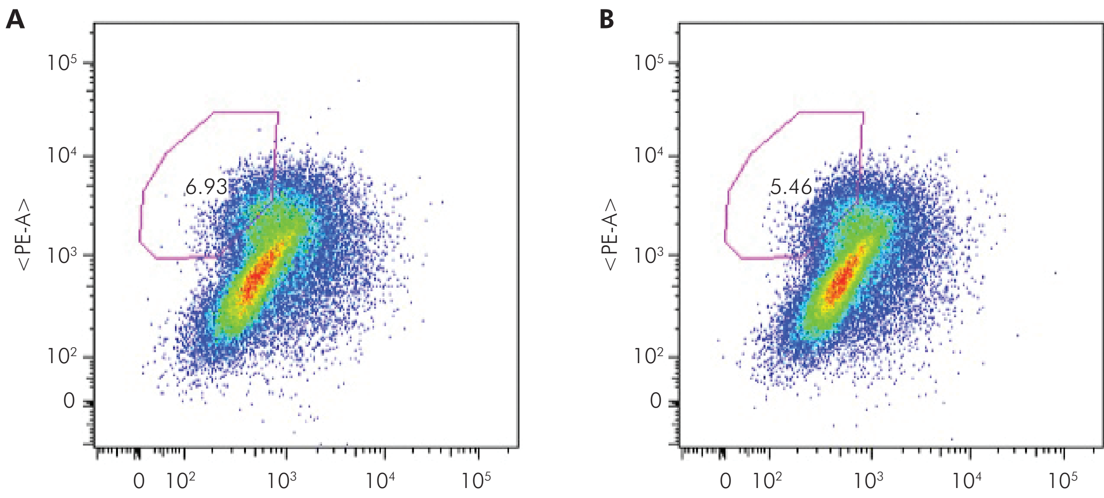

Figure 1. Characterization of keratinocytes sorted by MACS three passages after sorting. (A) Amount of cells that remain p75NTR^{+ve} on a population originally p75NTR^{+ve} .(B) Amount of cells that become p75NTR^{+ve} on a population originally p75NTR^{-ve}.

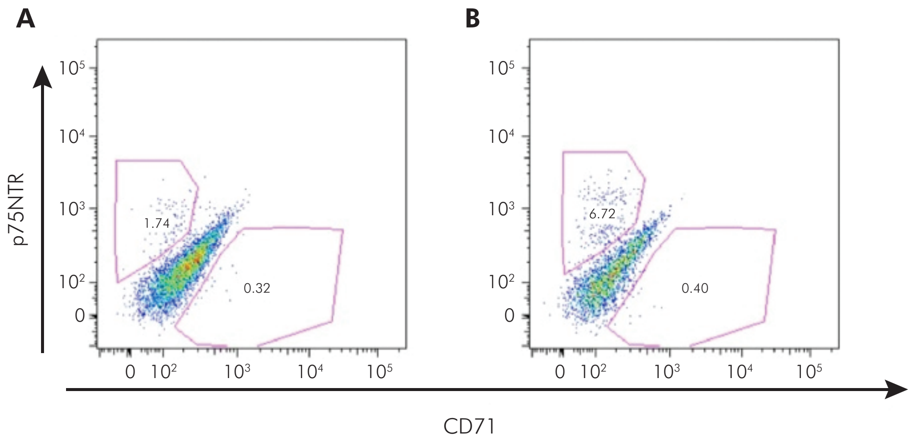

Figure 2. Characterization of keratinocytes sorted by FACS after only 1 week in culture. (A) Percentage of cells expressing p75NTR and CD71 among the population of cells that were originally p75NTR^{+ve}CD71^{-ve}. (B) Percentage of cells expressing p75NTR and CD71 in the population of cells that were originally p75NTR^{-ve}CD71^{+ve}.

useful for selecting a population expressing other useful stem cells markers, oral keratinocytes were harvested, sorted by FACS and plated separately at the same density, as population of p75NTR^{+ve} and p75NTR^{-ve} (group I), and populations of p75NTR^{+ve}CD71^{-ve} and p75NTR^{-ve}CD71^{+ve} (group II). After 1 week in culture, cells reached semi-confluence and then were reanalyzed by FACS. Among the p75NTR^{+ve} cells, just 7.48% remained p75NTR^{+ve} while 11.9% of the p75NTR^{-ve} became positive. Under the same conditions of analysis, just 1.74% of the cells were still expressing p75NTR on the population of p75NTR^{+ve}CD71^{-ve} and 0.32% were expressing CD71 (Figure 2A). Once again, a significant percentage of the cells of the p75NTR^{-ve}CD71^{+ve} population (6.72 %) gained the expression of p75NTRT while just 0.4% were still expressing CD71 (Figure 2B).

The expression of p75NTR was also assessed in freshly dissociated oral keratinocytes, without

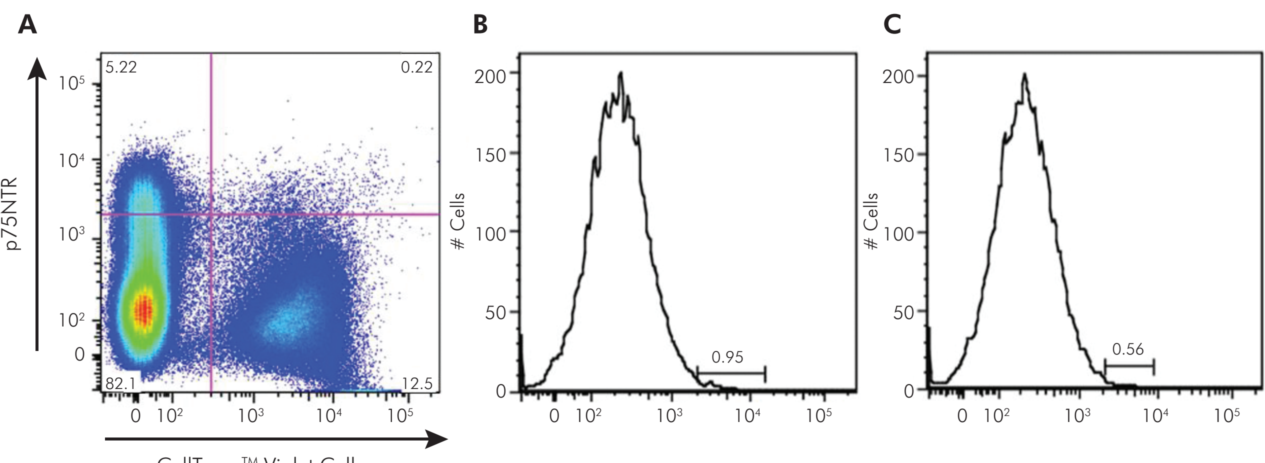

Figure 3. Expression of p75NTR in co-culture of p75NTR^{+ve} and p75NTR^{-ve} cells (marked with Cell Trace™). (A) Most of the original positive population became negative and some cells of the original negative population turned out expressing p75NTR after 4 days in co-culture. (B) Histogram reveling that 0.95% of the cells originally p75NTR^{-ves} become p75NTR^{-ves} even after been cultivated separately from the p75NTR^{+ve} population. (C) Similarly, a percentage of cells (0.56%) originally expressing p75NTR lost the expression of this marker after the same period of time.

prior culture and an average of 1.4% of the cells were expressing p75NTR.

In order to consider whether co-culturing p75NTR^{+ve} and p75NTR^{-ve} could maintain a higher number of p75NTR^{+ve} cells, fresh oral keratinocytes were sorted by FACS based on the expression of p75NTR and the negative cells were stained with Cell Trace™ Violet Cell Proliferation Kit (Molecular Probes, #C34557 and seeded in triplicate onto 6-well plates as (I) p75NTR^{+ves}, (II) p75NTR^{-ves} and (III) 50% p75NTR^{+ves} and 50% p75NTR^{-ves}, respecting the same densities and culture conditions used before. Four days later, the cells were removed from the plate and re-analyzed by FACS. As a result, we noticed that even co-culturing p75NTR^{+ves} and p75NTR^{-ves} (group III) for a short period of time, more than 90% of the p75NTR^{+ves} cells lost the expression of the stem cell marker (Figure 3A). For the groups that were cultured separately (groups I and II), the loss of expression was even higher. Less than 0.6% of the p75NTR^{+ves} retained the expression of p75NTR (Figure 3B). On the other hand, almost 1% of the cells gained the expression of p75NTR on the p75NTR^{-ves} population (Figure 3C). Unstained and stained compensation controls were used as parameters.

Functional assays

Long-term growth potential

Long-term growth potential was evaluated in both p75NTR^{+ve} and p75NTR^{-ve} cells sorted by MACS and FACS and on the p75NTR^{+ve}CD71^{-ve} and p75NTR^{-ve}CD71^{+ve} sorted by FACS, in both studied cell lineages (KSC-I and KSC-II). No significant difference in the proliferative capacity was observed between positive and negative populations. Both p75TR^{+ve} and p75NTR^{-ve} counterparts were completely senescent on the 13th and 14th passages, on the KSC-I and KSC-II cell lineages, respectively. Furthermore, when cells were sorted by FACS using double staining (p75NTR^{+ve}CD71^{-ve} and p75NTR^{-ve}CD71^{+ve}), they were senescent just 2 or 3 passages after sorting.

Colony-forming efficiency and in vitro epithelial reconstruction

After being sorted as p75NTR^{+ve} and p75NTR^{-ve}, cells were expanded up to the third passage and plated at low density, fixed and stained as previously described (Figure 4A and B). The number of colonies from the p75NTR^{+ve} population was 1.6 and 5.97-fold higher than from p75NTR^{-ve}, on both the KSC-I and KSC-II lineages (Figure 4C) and statically

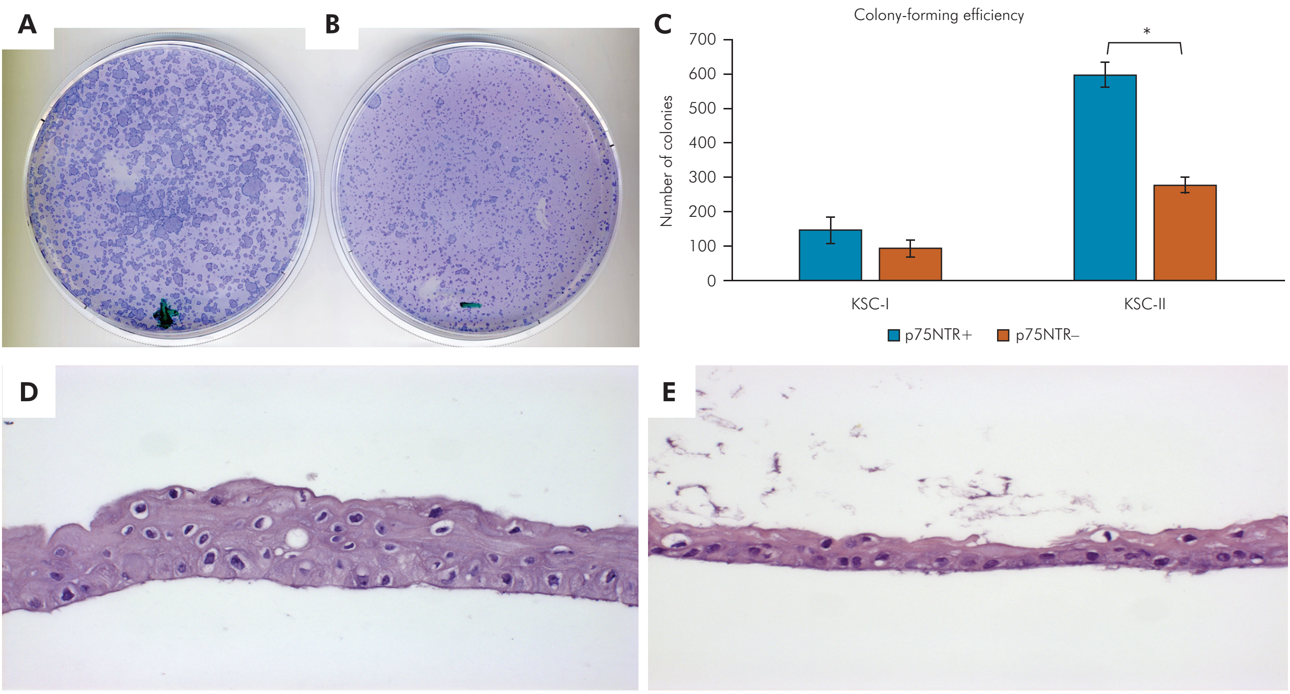

Figure 4. Colony-forming efficiency and *in vitro* epithelial reconstruction. (A) Morphology of the colonies of the p75NTR^{+ve} cells and (B) p75NTR^{-ve} cells. (C) Average number of colonies formed by the p75NTR^{+ve} compared with the number of colonies formed by the p75NTR^{-ve} in two different cell lineages (KSC-I and KSC-II). (*) Represents statically significant difference (D) *In vitro*-formed stratified epithelium by p75NTR^{+ve} cells. (E) *In vitro*-formed stratified epithelium by p75NTR^{-ve} cells.

significant on the KSC-II (p=0.001). The p75NTR^{+ve} cells were found to be capable of forming more organized and thicker stratified epithelium *in vitro* (Figure 4D and E).

It was not possible to perform the same experiments with the p75NTR^{+ve}CD71^{-ve} or p75NTR^{+ve}CD71^{-ve} because, as mentioned before, the cells did not survive more than 2 or 3 passages after been sorted which means that we did not have sufficient cells to perform the experiment.

Discussion

Keratinocytes from oral mucosa have been successfully used for many proposes, including reconstruction of the oral epithelium after tumor resection[17] and oral implants,[18] for the prevention of stenosis after endoscopic mucosal resection,[19] for re-epithelialization of the corneal surfaces in patients with total limbal stem-cell deficiency,[20] reconstruction of urethra,[21] regeneration of skin,[22] dental tissue engineering[23] and creation of induced pluripotent stem cells (iPS).[24]

Numerous studies have reported the isolation of stem/progenitor cells from keratinocytes. In most cases, the cell sorting is based on the expression of cell surface markers[5,8,11,25,26] and the stem cell-like proprieties are assessed by *in vitro* assays. What is not common is the analyses of expression of these cell markers at different time points, following sorting and culture.

We observed that despite of the p75NTR^{+ves} cells have higher colony-forming efficiency and better *in vitro* stratified epithelium reconstruction capacity as reported by Nakamura *et al.* 2007, they didn't survive for longer periods than the negative ones. This could mean that these cells were not capable of self-renewal and proliferate for longer periods as expected from a stem cell population or that the p75NTR population might not be maintained in culture conditions. With this in mind, we proceeded with analysis of the sorted populations after culturing.

The p75NTR^{+ve} population had a higher number of cells co-expressing other epithelial stem cells markers, such as integrins β1[25] and α6[13] than the negative one (88% against 28%). Furthermore, when we used a second marker (CD71), we could obtain a purer population (99% of the p75NTR^{+ve}CD71^{-ve} where both integrins β1 and α6 positive). However, this population had the worst performance in the *in vitro* assays when compared with the single stained ones (p75NTR^{+ve} only). The explanation could lie in the culture procedure as this purer population could face difficulties to maintain their numbers due to low densities after sorting.

The p75NTR^{+ve}CD71^{-ve} cells were 99% positive for stem cells markers described in the literature, but only survived for 2 or 3 passages after sorting, implying that a sorted keratinocyte stem cell phenotype does not guarantee a stem cell behavior *in vitro*.

We further observed that independently of the cell sorting technic used (MACS or FACS) and for how long the cells have been cultured (3 passages, one week or only 4 days) they changed their phenotype dramatically: the positive population lost the expression of their markers and the negative one, gained them. These lead us to assume that positive and the negative populations may need each other and possibly there is an intrinsic mechanism that controls and balances the protein expressions such as the one seen in the human body. This suggests that the use of freshly sorted cells (without further culture) or even unsorted cells could offer better results. This hypothesis needs further investigation.

Our findings showing the numbers of positive and negative cells and that the p75NTR^{+ve} population showed better functional properties when compared with negative cells in fresh oral keratinocytes are consistent with previously published results[5,9]. Nakamura *et al* (2007) also analyzed the expression of p75NTR in human cells from fresh oral mucosa and described that 7.35 ± 3.41% of the total keratinocytes expressed p75NTR[5]. In a similar way, Kunimura et al.[9] described that 1.5% of their population was p75NTR^{+ve} and had very little telomerase activity. Although these authors also described a gain of function in terms of functional assays, none of them checked the cell's phenotype after sorting and culturing.

Finally, considering that the p75NTR positive population may need the negative population, we investigated whether co-culturing p75NTR^{-ve} cells with positive cells would help both populations maintain their phenotype. However, this approach proved to be inefficient as after only four days in culture, almost 95% of the positive cells lost the expression of p75NTR, while 0.22% of the negative gained it.

Our findings suggest that *in vitro* culture may not offer ideal conditions for epithelial stem cell phenotype maintenance as it results in changes in the sorted populations in an attempt to establish or maintain a balance between different populations. The phenotypic changes may be part of an intrinsic cellular mechanism in an effort to re-stabilize the levels of protein expression in the same proportions found in the human body.

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Conflict of interest

The authors declare no potential conflict of interest.

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