RESEARCH REPORTS
Biological

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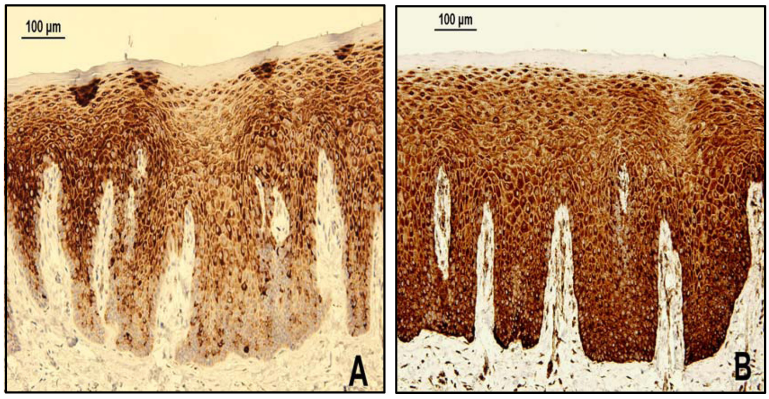
Pharmacological Retention of Oral Mucosa Progenitor/Stem Cells

APPENDICES

Appendix 1

Immunohistochemistry

Non-cultured palatal tissue was fixed in 10% neutral formalin. Paraffin-embedded specimens were cut into 5-µm sections and de-paraffinized. 3% hydrogen peroxide in methanol was used to quench endogenous peroxidase activity. For antigen retrieval, sections were boiled in Tris-EDTA solution (10 mM Tris and 1 mM EDTA; Sigma, St. Louis, MO, USA), pH 9.2, for 10 min. After the sections were incubated with 2% bovine serum albumin (Fisher Scientific, Pittsburgh, PA, USA) for 1 hr, they were incubated in a humidified chamber with rabbit monoclonal antibodies against phosphorylated-ribosomal S6 protein, and ribosomal S6 protein, dilution 1:200 (Cell Signaling Technology, Beverly, MA, USA) overnight at 4°C. For control sections, the primary antibody for the respective specimen was omitted. The sections were incubated with the peroxidase-labeled polymer conjugated to goat anti-rabbit immunoglobulins (Dako USA, Carpinteria, CA, USA) for 1 hr, and the immune-complex was visualized by incubation with diaminobenzidine substrate solution (Dako USA) for 2 min at room temperature. Sections were lightly counterstained with hematoxylin.



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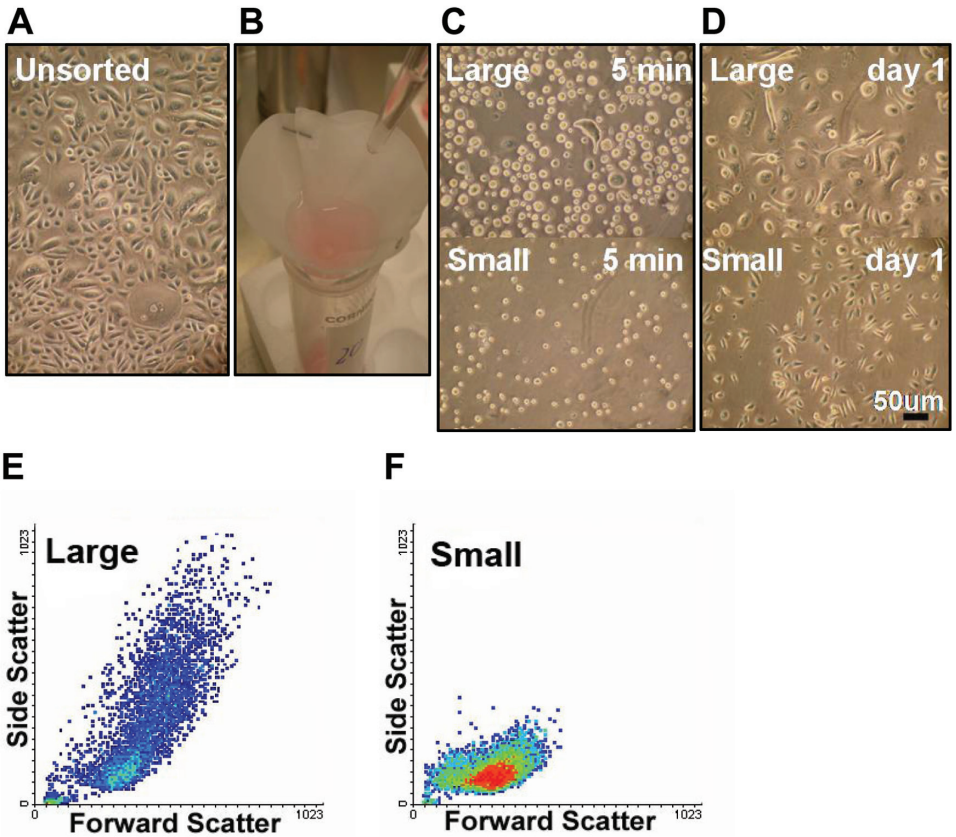


Appendix 2 Figure. Phase contrast and FACS analysis of pre- and post-GACS cell size. (**A**) Unsorted normal human oral keratinocytes prior to GACS. (**B**) Filtering of cell suspension. (**C**) Phase contrast microscopic features 5 min after GACS. Large cells showed a variety of cell sizes (top). In contrast, the small-cell population was relatively small and uniform in size (bottom). (**D**) Phase contrast microscopic features of large and small cells one day after GACS. (**E**) Light-scatter dot plots of large-cell populations after GACS, analyzed by fluorescence-activated cell-sorting (FACS). This revealed that GACS allowed cross-contamination by small cells in a large-cell population. (**F**) Light-scatter dot plots of small-cell populations after GACS, analyzed by FACS. Small-sized cells were enriched in a small-cell population.

Appendix 2

Cell Sorting by the Technique of Gravity-assisted Cell Sorting (GACS)

Nylon net filters (90 mm in diameter, pore sizes of 30 m and 20 m; Millipore, Billerica, MA, USA) were overlapped and stapled to shape a funnel, then soaked in 95% ethanol overnight. After the ethanol was removed from the filter with PBS, cell suspension of 3 x 10^6 cells in 5 mL was poured into a 30- m filter funnel, at a rate of 2-3 drops *per* second. The cell suspension was allowed to pass passively through the 30- m filter into a 50-mL conical tube. To collect large cells, we removed the stapled portion of the 30-µm filter using scissors, and the remaining cells, not filtered and trapped on the 30- m filter, were vigorously washed away with forceps when the filter was shaken into 10 mL of culture medium in a 50-mL conical tube. The first filtrate was passed again through the 20- m filter, and the second filtrate represented the small cells.

Characteristics of Cells Sorted by GACS

As previously reported, the oral keratinocytes progenitor/stem cells are enriched in a small-sized cell population *in vitro* (Izumi *et al.,* 2007). In addition, we tested the GACS technique to determine if this novel serial filtration system can efficiently and consistently sort out small-sized cultured oral mucosa cell populations, and concluded that oral mucosa keratinocyte progenitor/stem cells appear to be enriched based on a functional test (CFE and LLTP) and the regenerative capability of an oral mucosa epithelium, another functional "test" (Miin *et al.*, 2007). Thus, GACS is likely to be more cost-effective to sort small-sized oral keratinocytes efficiently and consistently, and post-GACS "small cells" are thought to be a progenitor/stem cell population.

Nylon net filters are commercially available in pore sizes of 11, 20, 30, 40, and larger. We used a combination of 30- m and 20- m filters. The 30- m filter removed larger-sized, differentiated cells, while the 20- m filter allowed the "putative" progenitor/stem cells to pass through, since their size was thought to be less than 20 m (Barrandon and Green, 1987). However, post-GACS "large cells" also contained "small cells", referred to as "cross-contamination". The small-sized cells trapped in the 30-µm filter stayed in the "large cell" group, which occasionally showed regenerative capability equal to that of the "small cell" group. Cells that did not pass through the 40-µm filter no longer proliferated. In contrast, few viable cells were present in filtrate of the 11-µm filter.

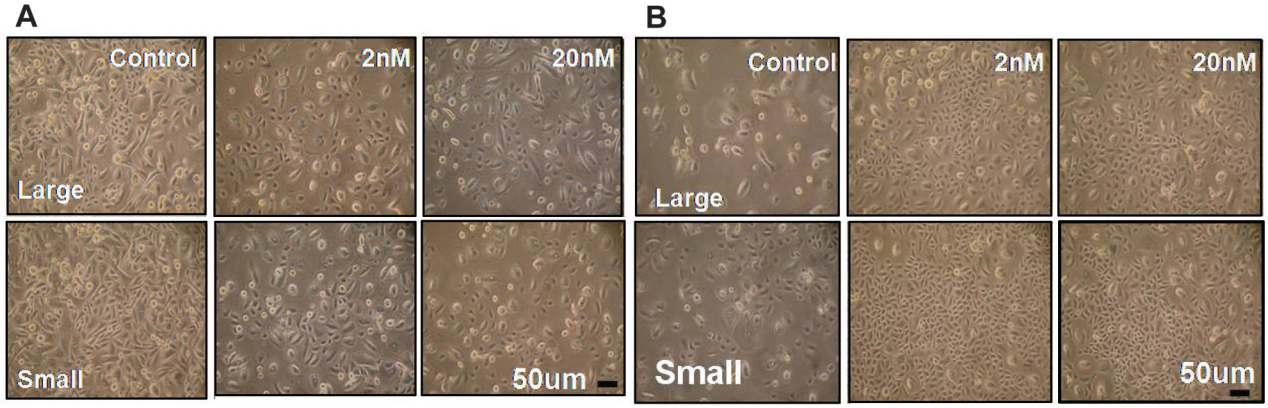
APPENDIX REFERENCES

Barrandon Y, Green H (1987). Three clonal types of keratinocyte with different capacities for multiplication. *Proc Natl Acad Sci USA* 84:2302-2306.

Izumi K, Tobita T, Feinberg SE (2007). Isolation of human oral keratinocyte progenitor/stem cells. *J Dent Res* 86:341-346.

Miin JC, Izumi K, Feinberg SE (2007). Physical isolation of progenitor/stem cells from oral mucosa (abstract). *J Dent Res* 86(Spec Iss):1835 (http://iadr.confex.com/iadr/2007orleans/techprogram/abstract_89211.htm).

Appendix 3



Appendix 3 Figure. Representative phase-contrast microscopic images of large and small cells without and with rapamycin treatment (2 nM and 20 nM) prior to the first passage, after 6 days in culture (A), and prior to the third passage, at 17 days in culture (B). (**A**) Large and small control cells continued to proliferate after GACS. A variety of cells was present, of which the major proliferative subpopulation was small-sized cells. However, the proliferation of cells treated with rapamycin was slowed for the first few passages compared with control cells. (**B**) Proliferation of control cells plunged after several passages and was unable to catch up with that of rapamycin-treated cells. In contrast, proliferation of rapamycin-treated cells recovered during the next few passages, and then accelerated, dominated by uniform, small-sized cells.

Appendix Table 1. "Maximum Cumulative Population-doubling" and "Days in Culture" of Control and Rapamycin-treated (2 and 20 nM) Cells after Gravity-assisted Cell Sorting (GACS) during the Serial Passages

	Large-cell Rapamycin Treatment			Small-cell Rapamycin Treatment		
	Control	2 nM	20 nM	Control	2 nM	20 nM
Maximum CPD	3.7 ± 3.5	15.7 ± 5.7	14.5 ± 4.0	5.0 ± 3.0	17.0 ± 5.7	17.0 ± 5.0
Days in Culture	31.1 ± 16.2	174.9 ± 72.6	166.7 ± 64.3	34.9 ± 3.0	178.9 ± 69.5	166.7 ± 64.3

Maximum cumulative population-doublings (CPD) and days in culture were significantly larger in 2-nM and 20-nM rapamycin-treated cells than in control cells ($p < 0.001$) in both large and small cells. There was no difference in maximum CPD as well as days in culture between 2-nM and 20-nM rapamycin-treated cells in each cell size. In control cells, small cells had larger maximum CPD than large cells. Numbers indicate means ± Standard Deviation.

Appendix Table 2. The Percentages of Cells in Each Cycle Phase of Control and Rapamycin-treated (2 nM and 20 nM) Cells

	Large-cell Rapamycin Treatment			Small-cell Rapamycin Treatment		
Phase	Control	2 nM	20 nM	Control	2 nM	20 nM
G_0/G_1%	64.6 ± 4.9	72.0 ± 6.2	74.2 ± 5.6	66.2 ± 6.9	73.8 ± 6.2	76.7 ± 6.3
S%	13.3 ± 5.0	11.4 ± 4.3	11.0 ± 3.0	11.0 ± 3.8	12.0 ± 4.9	10.8 ± 2.4
G_2/M%	22.0 ± 3.6	16.6 ± 6.0	14.8 ± 5.7	22.7 ± 6.0	14.2 ± 4.5	12.5 ± 5.4

Numbers indicate means ± Standard Deviation. There was statistical significance between control cells and both 2-nM and 20-nM rapamycin-treated cells in each cell size group on cells in the G_0/G_1 and G_2/M phases ($p < 0.001$), while there was no statistical significance between 2-nM and 20-nM rapamycin-treated cells in each cell size group. In cells in the S phase, no significant difference was noted.