SUPPLEMENTARY INFORMATION

Ms. Title: “Keratin intracellular concentration revisited: Implications for keratin function in surface epithelia”

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Content: - Materials and Methods (and relevant references)
- Supplementary Tables S1-S3

MATERIALS AND METHODS

Isolation and FACS analysis of primary basal keratinocytes

All studies involving mice were reviewed and approved by the Johns Hopkins’ Institutional Animal Care and Use Committee. Keratinocytes from P2 pup skin of C57Bl/6 mice were isolated as described (Bernot et al., 2004). Freshly isolated keratinocytes were filtered using 70μm cell strainer and processed for flow cytometric cell sorting as follows. A single cell suspension was prepared in BSA-containing FACS buffer (R&D Systems, Minneapolis, MN), blocked with anti-mouse CD16/CD32 Fc-receptor block (BD Biosciences, San Diego, CA) for 5min at 4°C, and labeled with anti-mouse Integrin β-1/phycoerythrin (R&D Systems) for 30 min at room temperature. Stained cells were washed in FACS buffer twice and sorted by a MoFlo
MLS high-speed cell sorter (Beckman Coulter, Miami, FL). Dead and apoptotic cells were excluded on the basis of propidium iodide positive staining. Two cell populations (integrin β-1-dim, integrin β-1-bright) were sorted based on the fluorescence intensity of the surface antigen: cells with the intermediate fluorescent intensity were sorted as integrin β-1-dim, and cells with the highest intensity as integrin β-1-bright.

**Transmission electron microscopy**

Sorted basal cells or tissue specimens from back skin of C57Bl/6 P0 mice were fixed in 4% formaldehyde/1% glutaraldehyde/0.1M cacodylate buffer (pH 7.4) overnight at 4°C, post-fixed in 1% osmium tetroxide/0.1M cacodylate buffer for 30 min, en bloc stained with 2% uranyl acetate for 1 hour at room temperature, dehydrated in a graded series of ethanol (50%, 70%, 95% and 100% ethanol for 15 min each), infiltrated in a graded series of plastic/propylene oxide (0/100 for 30 min, 50/50 for 1 hour, 70/30 for overnight, and 100/0 for 1 hour), and polymerized overnight at 60°C. The cell or tissue pellets were re-embedded in “OO” beem capsules and polymerized for 24 hours at 60°C. Thin (50-70 nm) sections were counterstained with uranyl acetate and lead citrate, and visualized using a Hitachi HU-12A electron microscope (Hitachi, Tokyo, Japan) operated at 75kV. For skin tissue specimens, keratinocytes located in the basal layer were selected for study.

**Measurements of the size and volume of basal epidermal keratinocytes**

Fifty electron micrographs of sorted and in situ basal cells were taken at nominal magnifications of 6000x and 5000x respectively, and individually printed on 8x10 inch Kodak photographic paper (enlargement factor: 2.9x). Diameters (D), and heights/widths (h/d) were measured for whole cells and nuclei of sorted and in situ basal cells respectively. Different mathematical equations were used to calculate the volume of the entire cell, its nucleus and cytoplasm,
assuming that a sorted basal cell is a sphere (volume = \(\frac{4}{3} \pi (D/2)^3\)) while a basal cell *in situ* is a cylinder (volume = \(\pi (d/2)^2 h\)). The cytoplasmic volume was obtained by subtracting the nuclear volume from the entire cell volume.

**Immunofluorescence staining of P2 mouse skin**

Back skin tissue was isolated from C57Bl/6 P2 mice and freshly embedded in Sakura Tissue-Tek OCT. (VWR, Radnor, PA) and stored at -20°C. Samples were cut in 5μm sections using HM 550 cryostat (Thermo Scientific, Kalamazoo, MI) and fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS). Frozen sections were blocked in 5% normal goat serum/0.1% Triton X-100/PBS for 1 hour at room temperature, incubated in primary antibody solution for 1 hour at room temperature, washed in PBS, incubated in secondary antibody solution for 1 hour, counterstained in DAPI (Life Technologies, Grand Island, NY), and mounted in Fluoro-gel mounting medium (Electron Microscopy Sciences, Hatfield, PA). Antibodies used include anti-K15 (chicken, 1:500, Covance, Gaithersburg, MD), anti-K17 (rabbit, 1:1000, McGowan and Coulombe, 1998), anti-chicken and anti-rabbit IgG conjugated to Alexa Fluor 594 and 488 respectively (1:1000, Life Technologies). Pictures were acquired using an inverted Zeiss fluorescence microscope with ApoTome attachment (Carl Zeiss, Thornwood, NY).

**Determination of keratin concentration in average basal keratinocyte**

Recombinant keratin proteins, including human K5, human K14, and mouse K15 were expressed in bacteria and purified in multiple steps, including preparation of inclusion bodies, urea-based solubilization of keratin proteins, and anion-exchange chromatography using HiTrapQ and MonoQ columns (GE Healthcare, Pittsburgh, PA) as described previously (Lee and Coulombe, 2009). Sorted basal cells were lysed in urea lysis buffer (pH 7.5, 6.5M urea, 1X PBS, 1mM ethylenediaminetetraacetic acid, 2mM dithiothreitol, proteinase inhibitor cocktail (Roche,
Indianapolis, IN), 1mM phenylmethylsulfonyl fluoride). Known amounts of purified keratin standards and cell lysates were run side by side on a 4-20% gradient sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose. Quantitative infrared Western blotting analysis (Li-Cor Biosciences, Lincoln, NE) were performed using anti-K5 (rabbit, 1:3000, Covance), rabbit anti-K14 (rabbit, 1:5000), or anti-K15 antibody (chicken, 1:1000), followed by a goat anti-rabbit or goat anti-chicken IgG antibody conjugated to infrared fluorescent dye IRDye 800 (1:20000, Li-Cor Biosciences). To determine each keratin fraction within total cell protein in the sorted basal cells, a concentration calibration curve was derived from the known value of the purified keratin standard and Western signal intensity using densitometry on the Li-Cor and ImageJ software programs. Data is presented as average ± standard deviation (s.d.) from eight (for K5) or six (for K14, K15) independent experiments.

**Average length of individual keratin filaments in basal keratinocytes of epidermis**

For our calculations we assumed an average length of 4 micrometers for individual K5/K14 filaments in basal keratinocytes of the epidermis in situ. This estimate is based on a) the dimensions of basal keratinocytes (this study); b) the orientation of keratin filaments in such basal keratinocytes; and range of lengths observed for individual K5/K14 filaments reconstituted from purified proteins in vitro (e.g., Coulombe and Fuchs, 1990; Lee and Coulombe, 2009). We note that confocal microscopy does not offer the resolution needed to make a determination about this quantity, while thin-section TEM analysis (see Figure 1) does not allow us to trace the outline of single 10 nm filaments, even at high magnifications, owing in part to the bundled organization of most filaments and the high electron density of the cytoplasm.
References

Bernt KM, Coulombe PA, Wong P (2004) Skin: an ideal model system to study keratin genes and proteins. *Methods Cell Biol* 78: 453-87

Coulombe PA (1993) The cellular and molecular biology of keratins: beginning a new era. *Curr Opin Cell Biol* 5: 17-29

Coulombe PA, Fuchs E (1990) Elucidating the early stages of keratin filament assembly. *J Cell Biol* 111: 153-69.

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**Supplementary Table S1. Assumptions (keratin molar ratio, average filament length in basal keratinocytes)**

| Parameters                                      | Value         | Source                                      |
|-------------------------------------------------|---------------|---------------------------------------------|
| Type I : Type II keratin molar ratio            | 1:1           | Fuchs et al., Ann N Y Acad Sci 1985         |
| Polymerized keratin fraction                    | 98%           | Bernot et al., J Cell Biol 2005             |
| No. monomer in IF polymer                       | 700/μm        | Herrmann and Aebl, Subcell Biochem 1998     |
| Length of individual filaments                  | 2 μm and/or 4 μm | This study                                 |

**Supplementary Table S2. Relevant dimensions for epidermal basal keratinocytes**

| Sample                                    | Parameters                  | Value                        |
|-------------------------------------------|-----------------------------|------------------------------|
| Basal keratinocyte (sorted)               | Diameter of cell            | 8.31 ± 0.50 μm               |
|                                           | Volume of cell              | 0.30 ± 0.05 pL               |
|                                           | Volume of cytoplasm         | 0.21 ± 0.04 pL               |
| Basal keratinocyte (in situ)              | Diameter of cell            | Height = 11.82±1.84 μm; Width = 6.85±1.12 μm |
|                                           | Volume of cell              | 0.45 ± 0.17 pL               |
|                                           | Volume of cytoplasm         | 0.26 ± 0.14 pL               |

**Supplementary Table S3. Keratin amount and concentration in sorted epidermal basal keratinocytes**

| Parameter                                                | Value                                   |
|----------------------------------------------------------|-----------------------------------------|
| Total cellular protein amount                            | 181.2 ± 1.32 μg/μL                      |
| Total cellular protein content – average basal keratinocyte | 36.81 ± 4.34 pg/cell                   |
| Keratin amount within total cellular proteins            |                                        |
| K5: 13.35 ± 1.94 %                                         |                                        |
| K14: 5.92 ± 3.01 %                                        |                                        |
| K15: 2.82 ± 0.16 %                                        |                                        |
| Total keratins: 22.09 ± 5.11 %                            |                                        |
| Total concentration of keratin protein                   | 40.03±9.55 μg/μL; 523.7 ± 59.4 μM       |
| Total concentration of soluble keratin                   | 0.80±0.19 μg/μL; 10.5 ± 1.2 μM          |
| Total concentration of polymerized keratin               | 39.23±9.36 μg/μL; 513.2 ± 58.2 μM       |
| Keratin protein content – average basal keratinocyte     |                                        |
| K5: 4.91 ± 1.07 pg/cell                                  |                                        |
| K14: 2.18 ± 0.80 pg/cell                                 |                                        |
| K15: 0.98 ± 0.01 pg/cell                                 |                                        |
| Total keratin concentration – average basal keratinocyte | 8.07 ± 1.88 pg/cell                    |