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Selective Matrix (Hyaluronan) Interaction with CD44 and RhoGTPase Signaling Promotes Keratinocyte Functions and Overcomes Age-related Epidermal Dysfunction

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Abstract

Background—Mouse epidermal chronologic aging is closely associated with aberrant matrix (hyaluronan, HA) -size distribution/production and impaired keratinocyte proliferation/differentiation, leading to a marked thinning of the epidermis with functional consequence that causes a slower recovery of permeability barrier function.

Objective—The goal of this study is to demonstrate mechanism-based, corrective therapeutic strategies using topical applications of small HA (HA_S) and/or large HA (HA_L) [or a sequential small HA (HA_S) and large HA (HA_L) (HA_S→HA_L) treatment] as well as RhoGTPase signaling perturbation agents to regulate HA/CD44-mediated signaling, thereby restoring normal epidermal function, and permeability barrier homeostasis in aged mouse skin.

Methods—A number of biochemical, cell biological/molecular, pharmacological and physiological approaches were used to investigate matrix HA-CD44-mediated RhoGTPase signaling in regulating epidermal functions and skin aging.

Results—In this study we demonstrated that topical application of small HA (HA_S) promotes keratinocyte proliferation and increases skin thickness, while it fails to upregulate keratinocyte differentiation or permeability barrier repair in aged mouse skin. In contrast, large HA (HA_L) induces only minimal changes in keratinocyte proliferation and skin thickness, but restores keratinocyte differentiation and improves permeability barrier function in aged epidermis. Since neither HA_S nor HA_L corrects these epidermal defects in aged CD44 knock-out mice, CD44 likely mediates HA-associated epidermal functions in aged mouse skin. Finally, blockade of Rho-kinase activity with Y27632 or protein kinase-N activity with Ro31-8220 significantly decreased the HA (HA_S or HA_L)-mediated changes in epidermal function in aged mouse skin.

Conclusion—The results of our study show first that HA application of different sizes regulates epidermal proliferation, differentiation and barrier function in aged mouse skin. Second, manipulation of matrix (HA) interaction with CD44 and RhoGTPase signaling could provide further novel therapeutic approaches that could be targeted for the treatment of various aging-related skin disorders.

Keywords

Matrix hyaluronan; CD44; RhoGTPase signaling; Keratinocyte functions; Skin aging

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INTRODUCTION

Chronological skin aging is a universal and inevitable process characterized by physiological alterations in keratinocyte activities and epidermal function, as well as dermal changes independent of photo-induced alterations [1]. Epidermal dysfunction in aged skin can contribute to important clinical consequences, such as epidermal thinning (atrophy), permeability barrier dysfunction, xerosis/xerotic eczema, delayed wound healing, altered drug permeability, as well as increased susceptibility to ulceration and irritant contact dermatitis [1–3]. Yet, the cellular and molecular mechanisms that cause epidermal dysfunction during skin aging are not well understood, creating a strong rationale to elucidate alteration in the epidermal biology that underlies aged skin-related diseases.

In the epidermis, extracellular matrix (ECM) components, such as hyaluronan (HA), form an integral part of hemidesmosomes, and mediate keratinocyte attachment to the its underlying basement membrane [4–6]. The dynamic nature of HA, particularly with regard to cellular interactions, is just beginning to be appreciated [4–6]. A general concept that has emerged from several studies is that HA fragments [Small Molecular Weight HA (HA_S)], and their larger precursor molecules [i.e. Large Molecular Weight HA (HA_L)] display distinct biological activities [7–10]. The degradation of HA_L to HA_S often leads to the generation of biologically active ECM fragment (HA_S, M.W. $\sim 1 \times 10^5 - 1 \times 10^4$ Da) from the intact/large size ECM (HA_L, M.W. $\sim 7 \times 10^5 - 1 \times 10^6$ Da), during periods of epidermal proliferation, differentiation and development, as well as following epidermal injury [7].

Age-related changes in the sizes of HA have also been reported [11]. Specifically, while HA_S appears to predominate in young (4-week-old) mouse skin, the amount of HA_L increases in older (52+ week-old) mouse skin [11]. Age-related declines in total HA production have also been documented in both rodent [11] and human aged skin [12]. In addition, alterations in HA metabolism are associated with reduced skin growth [13] and impaired wound healing [14], and/or delayed resolution of a variety of skin diseases [1–3]. All of these observations support the idea that both low levels of HA deposition and HA size modifications could underlie age-associated changes that occur during skin disease progression.

Interaction of HA with CD44 (a HA receptor) often induces unique downstream functions in many different cell types [15]. Our previous study showed that a CD44 deficiency is accompanied by a reduction in HA staining as well as marked alterations in keratinocyte proliferation, differentiation and altered barrier function in CD44 knock-out (k/o) mouse skin [16]. Downregulation of CD44 in cultured keratinocytes (using CD44siRNA) also significantly inhibits HA-mediated keratinocyte differentiation and lipid synthesis [16]. Together, these observations suggest the importance of both CD44 and HA for several key epidermal/keratinocyte functions. However, the mechanisms by which HA (HA_S vs. HA_L) and its receptor (CD44) contribute to the regulation of distinct keratinocyte functions (e.g., proliferation, differentiation, and permeability barrier recovery) in aged epidermis have not yet been determined.

A number of studies indicate that HA binding to CD44 promotes RhoGTPase signaling in a variety of cell types including keratinocytes [15, 17–19]. RhoGTPases (e.g., RhoA and Rac1) belong to members of the Rho subclass of the Ras superfamily [20] that are known to cycle between an active GTP-bound state and an inactive GDP-bound state which transmit diverse signals from the cell surface to intracellular targets. They function as molecular switches that, in response to external stimuli, regulate key signaling pathways that in turn control a variety of downstream metabolic activities [21]. RhoA signaling is known to be important in keratinocyte functions [22–24]. Several enzymes have been identified as

possible downstream targets for RhoA in cellular functions. One such enzyme is Rho-Kinase (ROK-also called Rho-binding kinase) which is a serine-threonine kinase that interacts with RhoA in a GTP-dependent manner [25–27]. RhoA-activated ROK participates in a number of HA/CD44-mediated cellular functions [28–31]. HA also promotes CD44 interaction with Rac1 signaling, leading to altered cytoskeleton-mediated cell functions [32, 33]. In contrast to RhoA, different cellular targets have been identified as downstream effectors for Rac1 signaling. One such target is protein kinase N- (PKN) (also called PRK2) which belongs to a family of serine-threonine kinases known to interact with Rac1 in a GTP-dependent manner [34–36]. In keratinocytes, Rho-activated PKN has been found to be involved in Fyn/Src kinase-regulated cell-cell adhesion during Ca^{2+} -induced differentiation [37]. The results of our previous study indicated that HA promotes CD44-mediated Rac1-PKN kinase signaling and downstream effectors, including PLC- β -1-mediated Ca^{2+} mobilization and cortactin-actin interaction. This pathway, in turn, regulates keratinocyte cell-cell adhesion and differentiation [17]. Whether the binding of HA (HA_S vs. HA_L) to CD44 activates RhoA-ROK and/or Rac1-PKN signaling in keratinocytes is an additional focus of this investigation.

In this study we determined that changes in HA-size distribution are closely associated with marked alterations in epidermal proliferation and differentiation with deleterious consequences for the permeability barrier function in aged epidermis. A topical application regimen consisting of HA_S or HA_L or sequential HA ($\text{HA}_S \rightarrow \text{HA}_L$) treatment selectively restored keratinocyte activities (e.g., proliferation and/or differentiation), increased skin thickness and improved the permeability barrier function in aged skin. The fact that both HA_S and HA_L failed to correct the epidermal defects observed in aged CD44 knock-out (k/o) mice demonstrate the requirement of CD44 in HA (HA_S vs. HA_L)-mediated regulation of epidermal functions in aged mice. Since treatment of cultured human keratinocytes (CHK) or mouse skin with ROK (Y27632) or PKN (Ro31-8220) inhibitors effectively reduced the expected HA_S or HA_L -mediated changes in epidermal functions, both RhoA-ROK and Rac1-PKN play an important role in HA (HA_S vs. HA_L)-mediated regulation of keratinocyte activities and epidermal function. Taken together, this information could point to new HA signaling-based therapies for the treatment of age-related skin diseases.

MATERIALS AND METHODS

Antibodies and Reagents

Monoclonal rat anti-human CD44 antibody (Clone: 020; Isotype: IgG_{2b}; obtained from CMB-TECH, Inc., San Francisco, CA.) used in this study recognizes a common determinant of the CD44 class of glycoproteins. Polyclonal mouse anti-involucrin and polyclonal mouse anti-profilaggrin were purchased from Covance Inc. (Princeton, NJ) and Zymed Laboratories Inc (South San Francisco, CA), respectively. Mouse Anti-PCNA antibody and ABC peroxidase reagents were obtained from Caltag Labs (Burlingame, CA) and Vector Labs (Burlingame, CA), respectively. A panel of immune-reagents such as mouse anti-RhoA antibody, mouse anti-Rac1, rabbit anti-PKN, goat anti-ROK antibody and goat anti-actin antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For the preparation of polyclonal rabbit anti-HAS1 antibody, rabbit anti-HAS2 antibody, rabbit anti-HAS3 antibody, rabbit anti-Hyal-1, and rabbit anti-Hyal-2- specific synthetic peptides [~15–17 amino acids unique for the HAS1 or HAS2 or HAS3 or Hyal-1 or Hyal-2 sequences] were prepared by the Peptide Laboratories using an Advanced Chemtech automatic synthesizer (model ACT350). All polyclonal antibodies were prepared using conventional DEAE-cellulose chromatography and tested to be monospecific (by immunoblot assays).

HA Preparations

Large HA (HA_L) (molecular mass ~700,000–1,000,000 dalton) was prepared from Healon HA polymers (purchased from Pharmacia & Upjohn Company, Kalamazoo, MI) using gel filtration column chromatography-Sephacryl S1000 column. Small HA (HA_S) fragments (molecular mass ~27,000 dalton) was obtained by digesting HMW-HA with bovine testicular hyaluronidase (PH20) according to the method described previously [9]. Briefly, intact healon HA polymers (500mg) was dissolved in 50ml of 0.1M acetate buffer (pH 5.4) containing 0.15M NaCl and digested with 20,000 units of bovine testicular hyaluronidase (PH20) (Wyeth Laboratories Inc. Philadelphia, PA) at 37°C. Ten milliliter aliquots were removed after 2, 4, 6, 8, and 24h intervals, and the reaction was terminated by adding trichloroacetic acid at 10% final concentration (v/v). After incubating at 4°C for at least 4h, any precipitate was removed by centrifugation at $2,500 \times g$ for 30min. The supernatants were then pooled, dialyzed extensively against distilled water, recentrifuged, and freeze-dried. The HA fragment preparation was dissolved in 10ml of 0.1M acetic acid and applied to a column (2.0 × 150cm) of Sephadex G-50. The column was eluted in 0.1M acetic acid at the flow of 10ml/h, and 5-ml fractions were collected. Each fraction was assayed for hyaluronan content, and size ranges of the fragments were determined as described previously [9]. The purity of both HA_S fragments and HA_L polymers used in our experiments was further verified by anion exchange high-performance liquid chromatography (HPLC) followed by protein and endotoxin analyses using BCA protein assay kit (Pierce Co., Rockford, IL) and an *in vitro* Limulus Amebocyte Lysate (LAL) assay (Cambrex Bio Science Walkersville Inc., Walkersville, MD), respectively. No protein or endotoxin contamination was detected in these HA_S and HA_L preparations. Both HA_S and HA_L were then analyzed by using 4–25% polyacrylamide gradient gel electrophoresis followed by Alcian blue 8GX and silver staining. Both Select-HATM High Ladder (in the range of Mr ~500,000 dalton to Mr 100,000,000 dalton) and Select-HATM LowLadder (in the range of Mr ~27,000 dalton to Mr 495,000 dalton) obtained from Hyalose (Oklahoma City, OK) were used as HA standards. Both Y27632 and Ro31-8220 were purchased from EMD (San Diego, CA).

Cultured Human Keratinocytes (CHK)

Normal human keratinocytes were isolated from neonatal human foreskins and grown in serum-free keratinocyte growth medium (KGM, Clonetics, San Diego, CA) as described previously [16, 17].

Animal Model Systems

Both 10 week-old (young) and 24 month-old (aged) male CD44 knock-out (k/o) and wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All procedures were performed according to protocols approved by the University of California Committee on Research (San Francisco, CA) and SFVA Animal Research Subcommittee.

Topical Application of HA (HA_S, HA_L and HA_S→HA_L) on Mouse Skin

To examine the effects of different HA fragments on epidermal functions of mouse skin *in vivo*, groups of 10 young mice (10 week-old) and 10 aged mice (24 month-old) were routinely used in each set of the experiments and the hair of the back skin was shaved before topical application of HA fragments as described below. Specifically, HA [e.g., HA_S (50µg/ml) or HA_L (50µg/ml) or HA_S (50µg/ml) plus Y27632 (5µM) or HA_L (50µg/ml) plus Ro31-8220 (5µM)] or a sequential small/large HA (HA_S→HA_L) treatment [a concentration ratio of HA_S: HA_L (1:1); HA_S for 3-days followed by HA_L for 3-days in the presence or absence of Y27632 (5µM) or Ro31-8220 (5µM)] or vehicle cream (Liposomal) samples were applied twice daily for three different time intervals (e.g. 1, 2, 3 and 5 days) to the dorsal

skin of these mice. Animals were then be euthanized after the last application. Both skin biopsies and epidermal layers were collected for various experiments described below.

Extraction of Total RNA and Real-Time Reverse Transcriptase PCR Analysis

Mouse skin biopsies obtained from mouse skin topically treated with HA (e.g., HA_S, HA_L or HA_S->HA_L) were collected and epidermis from these samples was separated from whole skin by 1 mol/L NaCl in sterile water at 4°C for 2 hours. Total RNA from both mouse epidermis and human CHK were isolated using Tripure Isolation Reagent kits (Roche Applied Science, Indianapolis, IN). First-stranded cDNAs were synthesized from RNA using Superscript First-Strand Synthesis system (Invitrogen, Carlsbad, CA). Gene expression was quantified using probe-based Sybr Green PCR Master Mix kits, ABI PRISM 7900HT sequence detection system, and SDS software (Applied Biosystems, Foster City, CA). The Q-PCR primers and probes specific for mouse differentiation-specific protein genes including involucrin, filaggrin, and control housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β -actin were obtained from Taqman Gene Expression Assay (Applied Biosystems) (Probe ID; Mm00515219_s1, Mm01716522_m1, Mm99999915_g1, and Mm00607939_s1). Differences between the mean CT values of involucrin, filaggrin and those of GAPDH or β -actin were calculated. The Q-PCR primers used for detecting gene expression of human involucrin and filaggrin were as follows: Specifically, forward primer Involucrin: TCCTCCAGTCAATACCCATCAG; and reverse primer Involucrin: GCAGTCATGTGCTTTTCCTCTTG as well as forward primer Filaggrin: TTTCGGCAAATCCTGAAGAAT; and reverse primer Filaggrin: GCCAACTTGAATACCATCAGA were used. The primers for human PCNA (QT00024633) were obtained from Qiagen (QuantiTect Primer Assays) (Valencia, CA). A cycle threshold (minimal PCR cycles required for generating a fluorescent signal exceeding a preset threshold) was determined for each gene of interest and normalized to a cycle threshold for a housekeeping gene GAPDH (QT00073247) (Qiagen, QuantiTect Primer Assays). The PCR reaction was performed at 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of amplification of melting at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds, respectively. The PCR reaction was performed in duplicate, with 4~5 samples in each group ($n=4\sim5$). The expression levels of each gene were normalized against housekeeping genes using the comparative C_T method, and expressed as percentage of control, with the control as 100%.

DNA Synthesis/Keratinocyte Proliferation Assay

CHK cells were grown on 48-well Costar cluster culture plates. When cells reached 60% confluence, the medium was replaced by serum-free KGM. Small HA (HA_S) (50 μ g/ml) or large HA (HA_L) (50 μ g/ml) was added to the CHK pretreated with normal rat IgG (50 μ g/ml) or rat anti-CD44 antibody (50 μ g/ml). The control cells received no HA. After 24 h at 37 °C, cells were pulsed with [methyl-³H]thymidine (1 μ Ci/ml) for another 2 h. The [methyl-³H]thymidine incorporation was stopped by washing twice with 10% trichloroacetic acid and once with ethanol/ether (2:1, v/v). The radioactivity was measured by dissolving the cells in 0.2 N NaOH and counting in a scintillation counter. The HA-dependent incorporation of [methyl-³H]thymidine was determined by subtracting the [methyl-³H]thymidine incorporation of the control without HA.

Measurement of RhoA and Rac1 Activation

Cultured human keratinocytes (CHK) ($\sim 5.0 \times 10^5$ cells) were incubated in a buffer containing 118 mM KCl, 5 mM NaCl, 0.4 mM CaCl₂, 1 mM EGTA, 1.2 mM Mg-acetate, 1.2 mM KH₂PO₄, 25 mM Tris-HCl (pH 7.4), 20 mg/ml BSA followed by adding [³⁵S]GTP S (12.5 μ Ci). Subsequently, CHK were electroporated at 25 microfarads and 2.0

kV/cm followed by incubating with 50µg/ml HA [in the presence or absence of rat anti-CD44 antibody (50µg/ml)] or without any HA treatment at 37°C for 10 min. [³⁵S]GTP S labeled cells were then solubilized by NP-40 followed by incubating with mouse anti-RhoA IgG or mouse anti-Rac1 IgG (5µg/ml) plus goat anti-mouse conjugated beads, respectively. The amount of [³⁵S]GTP S-RhoA or [³⁵S]GTP S-Rac1 associated with anti-RhoA or anti-Rac1-conjugated immuno-beads was measured by a gamma-counter.

Measurement of ROK Activity Assay *In Vitro*

CHK [pretreated with normal rat IgG (50µg/ml) or anti-CD44 antibody (50µg/ml), or a ROK inhibitor, Y27632 (5µM) or a PKN inhibitor, R031-8220 (5µM) or vesicle control treatment] were treated with HA (50µg/ml) (or no HA) for various time intervals (e.g., 10min, 30min, 1h or 24h) at 37°C. These cells were then immediately lysed in NP-40 buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl₂, 1% NP-40, 1 mM Na₃VO₄, 1 mM NaF, Complete Protease Inhibitor cocktail (Roche), 1 mM PMSF, 1x Halt™ Phosphatase inhibitor cocktail (Pierce)] at 4 °C and centrifuged to obtain the lysates. Equal amount of total lysates (~10µg), or immunoprecipitation-purified ROK by preincubating lysates (~100µg) with a rabbit anti-ROK antibody and agarose-conjugated anti-rabbit secondary antibody, were assayed for Rho-kinase activity using a kit from CycLex (Japan, Cat# CY-1160), following a protocol provided by the vendor. Basically, samples were incubated with a Kinase Reaction Buffer with 0.1 mM ATP at 30 °C for 45 min in plates pre-coated with a Rho kinase substrate corresponding to the C terminus of recombinant MBS (Myosin-Binding Subunit of myosin phosphatase), which contains a threonine residue that can be phosphorylated, and the product was detected by an HRP-conjugated antibody AF20 recognizing Thr696 of MBS as described previously (49). HRP-mediated color reaction was then measured in a spectrophotometric plate reader at dual wavelengths of 450/540 nm. The absorbance data were analyzed. Controls include solvent control (no protein lysate), and inhibitor control (5µM Y-27632 with protein lysate).

Measurement of PKN γ Activity Assay *In Vitro*

The PKN kinase reaction was carried out in CHK pretreated with normal rat IgG (50µg/ml) or rat anti-CD44 antibody (50µg/ml) prior to the addition of 50µg/ml HA_S, HA_L or no HA for various time intervals (e.g., 10min, 30min, 1h or 24h) at 37°C. PKN kinase (~2µg) was then isolated using anti-PKN -conjugated Sepharose beads) followed by incubating with the reaction mixture containing 40mM Tris-HCl (pH 7.5), 2mM EDTA, 1mM DTT, 7mM MgCl₂, 0.1% CHAPS, 0.1µM calyculin A, 10µCi of [-³²P]ATP (5000Ci/mmol), and 1µg purified cortactin (isolated from CHK using anti-cortactin-conjugated Sepharose beads). After 30min at 30°C, reactions were terminated by adding 20% cold trichloroacetic acid (TCA); and 2mg/ml BSA was then added as a carrier. TCA precipitated proteins were spotted on 3MM filter papers followed by extensive wash with 10% TCA. The radioactivity associated with TCA-precipitated materials was analyzed by liquid scintillation counting.

In some cases, CHK was pretreated with 5µM R031-8220 (a PKN inhibitor) or 5µM Y27632 (a ROK inhibitor) for 1h followed by the addition of 50µg/ml HA_S, HA_L or no HA for various time intervals (e.g., 10min, 30min, 1h or 24h) at 37°C. Subsequently, PKN activity was measured according to the procedures described above.

Immunoblotting Techniques

CHK [pretreated with normal rat IgG (50µg/ml) or rat anti-IgG (50µg/ml) or 5µM R031-8220 (a PKN inhibitor) or 5µM Y27632 (a ROK inhibitor) or vesicle control] was incubated with HA_S (50µg/ml), HA_L (50µg/ml) or no HA for 36h and/or 48h. These cells were then solubilized in 50mM HEPES (pH 7.5), 150mM NaCl, 20mM MgCl₂, 1.0% Nonidet P-40 (NP-40), 0.2mM Na₃VO₄, 0.2mM phenylmethylsulfonyl fluoride, 10µg/ml

leupeptin, and 5µg/ml aprotinin and immunoblotted with various immuno-reagents [e.g. mouse anti-involucrin (5µg/ml), or mouse anti-profilaggrin (5µg/ml), or mouse anti-actin (5µg/ml)] followed by incubating with horseradish peroxidase (HRP)-labeled HRP-labeled goat anti-mouse IgG.

In some cases, mouse skin biopsies obtained from mouse skin (young vs. aged mice) were collected and epidermis from these samples was separated from whole skin by 1 mol/L NaCl in sterile water at 4°C for 2 hours. Isolated epidermis samples were then processed for immunoblotting with anti-HAS1 antibody (5µg/ml), or anti-HAS2 antibody (5µg/ml), or anti-HAS3 antibody (5µg/ml), or anti-Hyal-1 antibody (5µg/ml), or anti-Hyal-2 antibody (5µg/ml), respectively. These samples were then incubated with followed by incubating with horseradish peroxidase (HRP)-labeled HRP-labeled goat anti-mouse IgG as described above. The blots were then developed by the ECL system (Amersham Co.). Routinely, we apply 20µg of proteins per well on the SDS-PAGE for immunoblotting analyses.

Immunohistochemistry Staining Techniques

Paraffin-embedded skin tissues [isolated from CD44 k/o or wild type mice treated with HA [HA_S alone, HA_L alone or a sequential small/large HA (HA_S->HA_L)] were cut in 5µm sections. After deparaffinization and rehydration, the sections were stained H & E or incubated with various reagents (e.g. rat anti-CD44, biotin-conjugated HA binding protein, anti-PCNA, anti-involucrin and anti-profilaggrin) followed by adding ABC peroxidase reagent. Subsequently, peroxidase activity was localized with diaminobenzidine substrate (Vector Labs, Burlingame, CA). As controls, skin sections were incubated with preimmune serum followed by incubating with ABC peroxidase reagents. Epidermal thickness was measured on hematoxylin and eosin stained sections using 20X objectives and an ocular micrometer (final magnification × 100). Experimental thickness was defined as the distance between the basement lamina and the apical surface of the uppermost nucleated keratinocytes as described previously [38]. To quantitate changes in epidermal proliferation, the numbers of PCNA-positive cells/unit length of epidermis was assessed in randomized, coded digital images as described previously [38].

Assessment of Permeability Barrier Function and Barrier Recovery After Acute Perturbations

The integrity of the permeability barrier of CD44 k/o and wild-type skin treated with HA [HA_S alone, HA_L alone or a sequential small/large HA (HA_S->HA_L)] was assessed by transepidermal water loss (TEWL) using an electrolytic water analyzer (Meeco Co., Warrington, PA., USA) as described previously [16]. The barrier was disrupted by either sequential cellophane tape stripping or acetone treatment until TEWL levels exceeded 5mg/cm² per hr, and measurements of TEWL were then repeated at 1h, 3h and 6h following tape stripping. Samples of skin were also obtained for electron microscopy prior to and 1h, 3h and 6h following barrier disruption later to evaluate cellular basis for recovery. All studies were approved by the Animal Use Committee at the Veteran Affairs Medical Center, San Francisco.

RESULTS

Alterations in HA Metabolism and Keratinocyte Proliferation/Differentiation Markers in Aged Murine Epidermis

Skin aging is associated with thinning of the epidermis and impairment of a variety of epidermal functions [3]. Using histologic sections and H & E staining, we have demonstrated substantial alterations, including a noticeable thinning of the epidermis in aged (24-month-old) compared to young (10-week-old) mice (Fig. 1a). Epidermal thinning can be

attributed largely to loss of the rete ridges, resulting in flattening of the epidermal-dermal interface, although decreased epidermal proliferation and differentiation also appear to be involved. In order to ascertain the basis for epidermal changes in aged mice, we next compared epidermal proliferation and differentiation in young vs. aged mice. Immunocytochemical staining of PCNA (a proliferation marker) (Fig. 1b), involucrin and filaggrin (two differentiation markers) (Fig. 1c and 1d) in aged mouse skin reveals significant reduction of the expression of both proliferation and differentiation markers compared to young mouse skin which could contribute to the epidermal thinning and dysfunction in aged mice.

Our previous work showed that HA plays an important role in CD44-mediated keratinocyte activities and epidermal functions [16, 17]. To investigate the role of CD44 and its ligand (HA) in regulating in epidermal structure and function during epidermal aging, we analyzed the expression of HA in the epidermis of young vs. aged mice. Our results indicate that HA is present in all of the nucleated layers of young mouse epidermis (Fig. 2A). In contrast, reduced endogenous HA is detected in the epidermis of aged mouse skin (Fig. 2A). HA staining appears to be located at the extracellular matrix of the entire epidermis of either young or aged mouse skin (Fig. 2A). Further analyses indicate that both small HA (HA_S) (~27,000 daltons) and large HA (HA_L) (~500,000–1,000,000 daltons) are significantly accumulated in the epidermis of young mice (Fig. 2B-lane 1). In contrast, the amount of HA_S (~27,000 daltons) and some HA_L (~500,000–1,000,000 daltons) [to a lesser extent the intermediate-size HA (~200,000 daltons)] is significantly reduced in the epidermis of aged mice (Fig. 2B-lane 2) as compared to that in young mouse epidermis (Fig. 2B-lane 1). The fact that both HA_S and HA_L detected by agarose gel electrophoresis can be readily digested by hyaluronidase treatment (Fig. 2B-lane 3 and lane 4) indicates that our HA measurement is specific.

HA is synthesized by several HA synthases (e.g., HAS-1, HAS-2 and HAS-3) [39, 40] and modified/degraded by hyaluronidases [41]. Our data indicate that the expression of both HAS2 and HAS3 (but not HAS1) is significantly reduced in aged murine epidermis as compared to young murine epidermis (Fig. 2C). However, no significant difference in the expression of hyaluronidases (e.g., Hyal-1 or Hyal-2) between young and aged murine epidermis is evident (Fig. 2C). Apparently, abnormal HA synthesis (to a lesser extent HA degradation) is involved in the changes in HA-size distribution observed in epidermis during skin aging. Our results are consistent with previous findings showing that alterations of HA bulk production and size distribution in aged murine and human skin [11, 12]. Thus, abnormal HA metabolism could contribute to the alterations of epidermal functions that occur during the skin aging processes.

Analyze HA (HA_S vs. HA_L)-mediated Signaling and Keratinocyte Functions in Cultured Human Keratinocytes (CHK)

In order to further investigate the cellular and molecular processes underlying HA (HA_S vs. HA_L)-induced keratinocyte functions, both HA_L (~500,000–1,000,000 dalton) and HA_S (27,000 dalton) were prepared and used for the functional analyses as described below. Previous studies indicate that both RhoA and Rac1 signaling are involved in keratinocyte functions [17, 22, 24, 37]. In this study we found that the binding of HA_S or HA_L to cultured human keratinocytes (CHK) selectively activates RhoA and Rac1 (Table 1). In particular, HA_S (to a lesser extent HA_L) promotes RhoA activation leading to a stimulation of RhoA-dependent kinases such as ROK (Table 2). Treatment of CHK cells with HA_S also induces PCNA gene expression and cell proliferation (but not differentiation marker expression) (Table 3). The fact that both ROK inhibitor, Y27632 and anti-CD44 antibody (to a lesser extent a PKN inhibitor, Ro31-8220) significantly block HA_S-mediated ROK activation, PCNA gene expression and cell proliferation (Table 2A, Table 2B, Table 3A and Table 3B)

suggests that HA_S stimulates keratinocyte proliferation in a ROK-specific and CD44-dependent manner in CHK.

We also observed that the addition of HA_L to CHK stimulates Rac1 activation (Table 1) and Rac1-mediated PKN activity (Table 2). In contrast, only a low level of Rac1 activation (Table 1) and Rac1-mediated PKN activity (Table 2) was detected in keratinocytes treated with HA_S or vehicle (no HA). These findings indicate that HA_L preferentially promotes Rac1 activation and Rac1-mediated PKN activity. Treatment of CHK with a PKN inhibitor, Ro31-8220 (to a lesser extent Y27632) or anti-CD44 antibody effectively inhibits HA_L-mediated PKN activity (Table 2A and Table 2B). In addition, we observed that HA_L (to a lesser extent HA_S) promotes the expression of differentiation markers such as involucrin and filaggrin at both gene and protein levels (Table 3A and Fig. 3). Pretreatment of anti-CD44 antibody (Fig. 3A) or a PKN inhibitor, Ro31-8220 (but not Y27632) also blocks HA_L-mediated differentiation marker gene/protein expression (Table 3B and Fig. 3). These findings suggest that both PKN and CD44 are involved in the regulation of keratinocyte differentiation in CHK following HA_L treatment.

Topical Application of HA (HA_L vs. HA_S) on Aged Mouse Skin

Keratinocyte Proliferation and Skin Thickness—Next, we investigated the effects of HA (HA_L vs. HA_S)-mediated epidermal function in aged murine (wild-type, CD44^{+/+}) skin. Our results indicate that topical application of HA_S upregulates keratinocyte proliferation (as shown by PCNA staining) and increases skin thickness (Fig. 4B-a) in aged mouse skin, as compared with those detected in the aged mouse epidermis without any HA treatment (Fig. 4A-a). In contrast, HA_L fails to stimulate either keratinocyte proliferation or alter skin thickness in aged wild-type (CD44^{+/+}) mouse skin (Fig. 4C-a). These results indicate the critical importance of selective HA fragment to affect keratinocyte activity and epidermal physiology in aged mouse skin. In contrast, neither HA_L nor HA_S can upregulate proliferation marker (PCNA) expression or skin thickness in aged CD44 k/o (CD44^{-/-}) mice (Fig. 4B-d vs. Fig. 4A-d or Fig. 4C-d). These findings suggest that CD44 is required for the selective HA effects on keratinocyte proliferation and skin thickness in aged mouse skin. Our results are consistent with a previous study showing HA fragments can reverse skin atrophy in aged mouse skin by a CD44-dependent mechanism [42].

Keratinocyte Differentiation and Permeability Barrier Recovery—Normal permeability barrier homeostasis requires the differentiation-dependent generation of corneocytes embedded in a lipid-enriched extracellular matrix forming the stratum corneum (SC) [43, 44]. To assess epidermal differentiation in young vs. aged mice we determined that the expression of involucrin and filaggrin each was greatly reduced in aged mouse skin compared to young mouse skin (Fig. 1c and d). These observations indicate decreased differentiation in aged epidermis, which would inevitably result in the formation of effete or structurally weakened corneocytes. To assess whether reduction of differentiation marker expression in the epidermal aging process influences permeability barrier recovery, acute disruption with sequential cellophane tape stripping was conducted to raise transepidermal water loss (TEWL) levels until the rates exceeded >5mg/cm²/hr (Nm=0.2mg/cm²/hr). Our results showed that barrier recovery is delayed in aged murine skin as compared to the young murine skin after barrier abrogation by taped stripping (Fig. 6A). These findings suggest that changes in HA-size distribution/production (Fig. 2C) and reduced differentiation marker expression (Fig. 1) could contribute to the abnormality in permeability barrier homeostasis in aged epidermis. Our data are consistent with previous findings showing that aged epidermis is often characterized by abnormal keratinocyte proliferation/differentiation and delayed permeability barrier recovery.

Further analyses indicate that a topical application of HA_L effectively restores keratinocyte differentiation [as shown by involucrin (Fig. 5I-C-a) and filaggrin staining (Fig. 5II-C-a)] and improves permeability barrier function in aged skin (Fig. 6B), as compared with those detected in the aged wild-type (CD44^{+/+}) mouse epidermis with no HA treatment (Fig. 5I-A-a; and Fig. 5II-A-a). However, HA_S fails to significantly promote either keratinocyte differentiation [as shown by involucrin (Fig. 5I-B-a) and filaggrin staining (Fig. 5II-B-a)] or permeability barrier repair in aged wild-type (CD44^{+/+}) mouse skin (Fig. 6B). The fact that neither HA_L nor HA_S can correct epidermal defects (reduced differentiation marker expression and slow permeability barrier recovery) observed in aged CD44 knock-out (k/o) mice (Fig. 5I-C-d; Fig. 5II-C-d and Fig. 6C) demonstrate the importance of CD44 in HA_L-mediated epidermal functions observed in aged mice.

Effects of ROK and PKN_Y Inhibitors on Aged Mouse Skin Following HA (HA_L vs. HA_S) Treatments

To further assess the roles of RhoA-activated ROK and Rac1-mediated PKN in regulating HA effects on aged mouse skin, we conducted a topical treatment of a ROK inhibitor, Y27632 [30, 31] on aged wild-type (CD44^{+/+}) mouse skin together with HA (HA_L vs. HA_S) addition. Our results indicate that treatment of Y27632 effectively reduces both the number of PCNA-positive cells and epidermal thickness (Fig. 4B-b and Table 4). In contrast, aged wild-type (CD44^{+/+}) mice similarly treated with a PKN inhibitor, Ro31-8220 [45–47] do not display noticeable changes in HA_S-induced PCNA-positive cell numbers and subsequent changes in epidermal thickness (Fig. 4B-c and Table 4). These results suggest that ROK (to a lesser extent PKN) plays an important role in keratinocyte proliferation and epidermal thickness in aged skin following HA_S treatment.

We also found that the expression of keratinocyte differentiation markers (e.g., involucrin and filaggrin) is greatly decreased in the epidermis from aged wild-type (CD44^{+/+}) mouse skin treated with a PKN inhibitor, Ro31-8220 [45–47] in the presence of HA_L treatment (Fig. 4C-c vs. Fig. 4A-c). Since topical treatment of aged wild-type (CD44^{+/+}) mice with a ROK inhibitor, Y27632 treatment fails to significantly inhibit HA_L-induced differentiation marker expression (Fig. 4C-b and Table 4), the HA_L effects on keratinocyte differentiation appear to be ROK-independent. In addition, we found that a topical application of aged murine skin with a PKN inhibitor, Ro31-8220 significantly delays HA_L-mediated permeability barrier recovery in wild-type (CD44^{+/+}) mouse skin (Fig. 6D). Most importantly, we found that a sequential topical treatment regimen [consisting of small HA followed by large HA (HA_S→HA_L)] not only increases keratinocyte proliferation/skin thickness but also upregulates differentiation in aged mouse skin (Table 4). Sequential HA (HA_S→HA_L) treatment also fully restores the permeability barrier function in aged murine skin to that observed in young murine skin (Fig. 6E). Downregulation of ROK and PKN by treating the mouse skin with Y27632 and Ro31-8220, respectively greatly reduces sequential HA (HA_S→HA_L)-mediated epidermal functions and permeability barrier recovery (Fig. 6F). Taken together, these findings suggest that RhoGTPase-regulated ROK and PKN are closely involved in aged mouse epidermal functions (e.g., proliferation, differentiation, and permeability barrier recovery) during HA_L or HA_S or sequential HA (HA_S→HA_L) treatment. These findings suggest that new HA signaling-based therapies may possibly counteract aberrant HA-induced epidermal dysfunction and age-associated skin disease.

Taken together, we would like to propose that in epidermis, HA_S binding to CD44 promotes RhoA-ROK signaling resulting in proliferation and epidermal thickness in aged skin; whereas HA_L interaction with CD44 stimulates Rac-PKN activation leading to differentiation and subsequent restoration of epidermal permeability barrier function in aged mouse skin. Both skin aging and CD44 deficiency in k/o mice cause abnormal epidermal

structure and function (Fig. 7). Our newly discovered HA/CD44-signaling strategies and HA (HA_S vs. HA_L or HA_S->HA_L)-based therapeutic approaches could prove beneficial for use in the treatment of human patients suffering a number of aging-related skin diseases (e.g., skin atrophy, psoriasis, atopic dermatitis, actinic keratoses, and chronic non-healing wounds). In addition, signaling perturbation agents (e.g. Y27623, a ROK inhibitor) may be applied to certain skin diseases displaying upregulation of keratinocyte proliferation (such as psoriasis and actinic keratoses) in order to correct the imbalance between RhoA-ROK signaling and Rac1-PKN activation during epidermal aging and various skin diseases.

DISCUSSION

Although the process of skin aging is not well understood, reduction in epidermal functions appears to be hallmarks of aging skin. Hyaluronan (HA, hyaluronic acid) is one of the major component of the extracellular matrix (ECM) in the epidermis, and HA level is known to decline during aging process [11, 12]. Since the biology of skin HA has not been thoroughly studied in aged skin, investigating the metabolism of HA, its functions within skin, and the interactions of HA with its receptor (CD44) shall enhance understanding of skin aging.

The epidermis comprises multiple layers of keratinocytes displaying a variety of different functions (e.g. proliferation, migration, differentiation, and permeability barrier formation) [13, 42–44]. After terminal differentiation, keratinocytes are lost from the outermost epidermal layers, but they are replaced as the basal layer keratinocytes cease proliferating, undergo suprabasal differentiation, apical migration, ultimately forming the outermost cornified layers [43, 44]. Normal permeability barrier epidermal homeostasis requires the balanced activities of proliferation, migration, differentiation and cornification. In this study we observed that both keratinocyte proliferation and differentiation, and permeability barrier function are significantly impaired in aged mouse epidermis as compared to these parameters in young epidermis. Our data are consistent with previous findings showing that aged epidermis is often characterized by abnormal keratinocyte proliferation and delayed permeability barrier recovery. However, at the present time, the cellular and molecular mechanisms accounting for epidermal dysfunction during skin aging are not well understood.

Several studies indicate that HA (HA_S and HA_L) is abundant in stratified squamous epithelia, including the epidermis, and that it regulates multiple epidermal functions and integrity [4, 5]. We found here that changes in HA-size distribution and production occur in aged as compared to young mouse epidermis. In particular, the amount of HA_S (~27,000 daltons) is significantly reduced in the epidermis of aged mice as compared to young mouse skin. Although some HA_L (~200,000–1,000,000 daltons) still can be detected in the epidermis of aged mouse skin, the overall levels of HA_L in aged mouse skin appears to decline in the young mouse epidermis (Fig. 2). Our results are consistent with previous findings showing that alterations of HA bulk production and size distribution in aged murine and human skin. Thus, abnormal HA metabolism could contribute to the alterations of epidermal functions that occur during the skin aging processes.

HA interacts with CD44, a ubiquitous, abundant and functionally important receptor expressed on the surface of many different types of cells, including normal and transformed keratinocytes [15–17]. The crystal structure of the HA-CD44 complex has been reported recently and a single HA binding site has been identified [48]. Different sizes of HA polymers appear to contribute to the onset of distinct CD44 signaling pathways and biological activities. For example, large HA (HA_L) polymers (~1,000,000 dalton) inhibits cell proliferation [50], while small HA (HA_S) polymers promote certain sets of genes that stimulates cell proliferation, migration, secretion, angiogenesis and chemosensitivity [7, 9,

15, 49, 50]. Our previous studies indicated a correlation between high affinity binding of HA_S to cells expressing CD44 and the occurrence of a mitogenic response [9] in several different cell type. Since anti-CD44 antibodies inhibit both HA_L and HA_S-mediated downstream functions, it is generally accepted that CD44 serves as a critical HA receptor in HA-mediated functions [9, 15, 50].

Epidermal proliferation regulates skin thickness [42], while keratinocyte differentiation contributes to the function of the cutaneous permeability barrier [43, 44]. In the current study, we reported selective HA_S and HA_L effects on keratinocyte activities in cultured human keratinocytes (CHK). Specifically, HA_S stimulates keratinocyte proliferation, whereas HA_L selectively upregulates the expression of keratinocyte differentiation markers such as involucrin, and filaggrin, but not other structural proteins, including actin. Using an anti-CD44 antibody (known to block HA binding sites located at the N-terminal region of CD44), we show here that blockage of the interaction between HA (HA_S vs. HA_L) and CD44 inhibits keratinocyte proliferation and the expression of certain differentiation markers. These findings suggest that CD44 is directly involved in HA_S and HA_L-mediated functions in CHK *in vitro*.

Because of the above results, we predicted that topical application of the appropriate HA fragments should override the age-related decline in HA production. Accordingly, topical applications of HA_S (to a lesser extent HA_L) to aged mouse epidermis stimulated keratinocyte proliferation (as indicated by PCNA staining) and increased skin thickness, while administration of HA_L (to a lesser extent HA_S) enhanced differentiation marker expression, and restored normal permeability barrier function in aged mouse skin (Fig. 6B). These results clearly demonstrate not only that HA fragments (HA_S vs. HA_L) can selectively induce diverse epidermal processes (e.g., epidermal proliferation, skin thickness, differentiation and epidermal barrier formation), but also that they can override age-related deterioration in epidermal functions. Our preliminary data indicate that topical applications of fluorescent-labeled HA fragments [e.g., FITC-labeled large HA (HA_L) or FITC-labeled small HA (HA_S)] can penetrate to the keratinocytes through the epidermal barrier. Our observation is consistent with a previous study by Brown et al showed that topical application of HA fragments (labeled by [³H]-hyaluronan) can penetrate through both mouse and human skin via active transport (not passive diffusion) [53]. As such, the penetration of HA fragments in mouse skin does not present a technical problem for our studies. Therefore, it is possible to design corrective HA-based therapeutic strategies for the treatment of skin aging-related diseases.

To verify the CD44-specificity of the observed HA effects in aged skin, we assess changes in CD44 k/o (CD44^{-/-}) aged (vs. young) mice. Our previous data showed that CD44 deficiency is accompanied by reduction in HA staining in CD44 knock out (k/o) mouse skin leading to a marked thinning of epidermis vs. wild-type mouse skin and barrier recovery (following acute barrier disruption) is delayed in CD44 k/o vs. wild-type mouse skin [16]. In this study, we found that neither HA_S nor HA_L treatment could stimulate keratinocyte proliferation, differentiation marker expression or permeability barrier homeostasis in CD44 k/o (CD44^{-/-}) aged mice. These findings strongly support the contention that CD44 plays an important role in regulating HA (HA_S vs. HA_L)-mediated keratinocyte activities and epidermal functions not only young, but also aged epidermis.

Because RhoGTPases (RhoA vs. Rac1) have been closely associated with remodeling of cortical actin [17], Ca²⁺ signaling [17, 29], cell-cell adhesion [17], cell proliferation and differentiation [16, 17], in this study we are investigating that RhoGTPase signal cellular events in response to either HA_S vs. HA_L/CD44-mediated ligand binding. Several lines of evidence indicate that CD44 selects its unique downstream effectors and coordinates

downstream, intracellular signaling pathways that influence multiple cellular functions [15, 19]. Previous work showed that HA promotes the interaction between CD44 and several RhoA-specific regulators, thereby up-regulating RhoA (a member of the Rho subclass of the Ras superfamily), leading to altered biological functions [15, 19]. A number of studies also indicate that HA-CD44 interaction promotes RhoA-mediated ROK activation [28–31]. Inhibition of RhoA-activated ROK by Y27632 treatment effectively blocks the HA/CD44-induced cellular signaling and functions [17, 18].

Here, we observed a selective HA_S and HA_L effects on the activation of RhoGTPase signaling in cultured human keratinocytes (CHK) (Table 1). Specifically, HA_S (to a lesser extent HA_L) stimulates RhoA-mediated ROK activities in CHK. Treatment of CHK with a ROK inhibitor, Y27632 effectively blocks RhoA-ROK activation and the subsequent keratinocyte proliferation *in vitro*. Topical administration of a ROK inhibitor, Y27632 followed by HA_S also reduces ROK-associated proliferation pathways (as indicated by PCNA staining) and decreases skin thickness (Figs. 3–5 and Table 4). These observations clearly suggest that RhoA-ROK is closely linked to keratinocyte proliferation and skin thickness. A number of studies indicate that HA_S (but not HA_L-mediated) activation of Toll-like receptors (TLR2/4) and MyD88 play an important role in stimulating pro-inflammatory gene expression leading to cytokine/chemokine production following tissue injury [51, 52] or cancer progression [10]. Our preliminary data indicate that HA_S interacts with both CD44 and TLR2/4 directly leading to MyD88-dependent nuclear factor- κ B (NF- κ B) signaling and keratinocyte survival (but not inflammation) (data not shown).

HA also induces CD44 interaction with several Rac1-specific regulators, thereby up-regulating PKN which has been found to be involved in Fyn/Src kinase-regulated cell-cell adhesion during Ca²⁺-induced keratinocyte differentiation [17]. PKN shares a great deal of sequence homology with protein kinase C in the C-terminal region [35, 36]. The N-terminal region of PKN contains three homologous sequences of approximately 70aa (relatively rich in charged residues), which form an antiparallel coiled-coil fold (ACC domain) [35, 36]. In keratinocytes, this ACC domain has been shown to interact with RhoGTPases such as Rac1 (and to a lesser extent with RhoA and Cdc42) [17]. The C-terminal region contains the C2-like region which functions as an auto-inhibitory domain [35, 36]. Both the ACC and the C2-like domains, together with the catalytic domain, are conserved among the PKN family members [35, 36]. One of the Rac1-PKN-specific downstream targets is the cytoskeletal protein, cortactin. Our previous study indicated that one of the Rac1-PKN-specific downstream targets is the cytoskeletal protein, cortactin which is involved in cell-cell adhesion and differentiation [17]. Inhibition of Rac1-PKN by Ro31-8220 treatment significantly reduces cellular signaling and functions [45]. In this study we found that HA_L (to a lesser extent HA_S) stimulates Rac1-PKN activities in CHK. Treatment of CHK with a PKN inhibitor, Ro31-8220 greatly downregulates HA_L-mediated Rac1-PKN activation and keratinocyte differentiation *in vitro*. Topical administration of a PKN inhibitor, Ro31-8220 followed by HA_L treatment also decreases PKN-associated differentiation marker expression (as indicated by involucrin and filaggrin staining) and permeability barrier functions. These findings suggest that Rac1-PKN is closely involved in HA-CD44-mediated keratinocyte differentiation and permeability barrier recovery.

In addition, we found that sequential topical treatment regimen [consisting of small HA followed by large HA (HA_S→HA_L)] not only enhances keratinocyte proliferation/skin thickness but also promotes differentiation in aged mouse skin (Table 4). Most importantly, sequential HA (HA_S→HA_L) treatment fully restores the permeability barrier function in aged murine skin to that observed in young murine skin (Fig. 6E). Downregulation of ROK and PKN by treating the mouse skin with Y27632 and Ro31-8220, respectively also greatly reduces sequential HA (HA_S→HA_L)-mediated epidermal functions and permeability barrier

recovery (Fig. 6F). Taken together, our findings suggest that activation of CD44 signaling (via HA_S vs. HA_L or HA_S→HA_L) induces pathway (RhoA-ROK vs. Rac-PKN)-specific effects on diverse epidermal processes (e.g., epidermal proliferation, skin thickness, differentiation and epidermal barrier formation) in aged mouse epidermis (Fig. 7). Our newly discovered HA/CD44-signaling strategies and HA (HA_S vs. HA_L or HA_S→HA_L)-based therapeutic approaches could be considered for use in the treatment of human patients suffering a number of aging-related skin diseases (e.g., skin atrophy, psoriasis, atopic dermatitis, actinic keratoses, and chronic non-healing wounds). In addition, signaling perturbation agents (e.g. Y27623, a ROK inhibitor) may be applied to certain skin diseases displaying upregulation of keratinocyte proliferation such as psoriasis and actinic keratoses in order to correct the imbalance between RhoA-ROK signaling and Rac1-PKN activation during epidermal aging and various skin diseases.

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Fig. 1.

Immunohistochemical analyses of young and aged wild-type (CD44+/+) mouse epidermis (using the skin biopsies obtained from the back of the mice).

Light microscopic analyses of young vs. aged wild-type (CD44+/+) mouse epidermis using H & E staining (a); Detection of PCNA (b) Involucrin (c) and Filaggrin (d) staining in young vs. aged wild-type (CD44+/+) mouse epidermis using anti-PCNA/anti-Involucrin/anti-Filaggrin-mediated immunoperoxidase staining, respectively.

Fig. 2.

Analyses of HA staining, HA sizes and HA synthases/hyaluronidases in young and aged wild-type (CD44+/+) mouse epidermis.

A: Detection of HA staining in young and aged wild-type (CD44+/+) mouse epidermis using HA-binding protein-mediated immunoperoxidase staining.

B: HA size distribution in wild-type (CD44+/+) mouse epidermis [without hyaluronidase digestion (lane 1, young; lane 2, aged) or with hyaluronidase digestion (lane 3, young; Lane 4, aged)] was analyzed by using 4–25% polyacrylamide gradient gel followed by Alcian blue 8GX and silver staining. Both Select-HA™ HighLadder (in the range of molecular mass ~100,000,000 dalton to 500,000 dalton) (Lane 1) and Select-HA™ LowLadder (in the range of molecular mass ~500,000 dalton to 27,000 dalton) (Lane 1) were used as HA-size marker standards.

C: Mouse epidermis was solubilized by 1% Nonidet P-40 (NP-40) buffer and immunoblotted with anti-HAS1 antibody, anti-HAS2 antibody, anti-HAS3 antibody, anti-Hyal-1 antibody and anti-Hyal-2 antibody in young (lane 1) and aged (lane 2) wild-type (CD44+/+) mouse epidermis.

Fig. 3.

Detection of differentiation marker (Involucrin and Filaggrin) expression in CHK.

A: CHK [untreated (no HA) (lane 1) or treated with HA_S (lane 2), HA_L (lane 3) or HA_L plus anti-CD44 antibody (lane 4)] were solubilized by 1% Nonidet P-40 (NP-40) buffer and immunoblotted with anti-Involucrin (a) or anti-Profilaggrin (b) or anti-actin (as a loading control) (c).

B: CHK [untreated (no HA) (lane 1) or treated with HA_L (lane 2) or HA_L plus Ro31-8220 or HA_L plus Y27632 (lane 4)] were solubilized by 1% Nonidet P-40 (NP-40) buffer and immunoblotted with anti-Involucrin (a) or anti-Profilaggrin (b) or anti-actin (as a loading control) (c).

Fig. 4.

Detection of proliferation marker (PCNA) expression in aged wild-type (CD44^{+/+}) and CD44 k/o (CD44^{-/-}) mouse epidermis using immunohistochemical staining.

A: Anti-PCNA-mediated immunoperoxidase staining of the wild-type (CD44^{+/+}) (vehicle control without HA treatment) (a) or treated with Y27632 without HA addition (b) or treated with Ro31-8220 without HA addition (c) vs. CD44 k/o (CD44^{-/-}) (vehicle control without HA treatment) (d).

B: Anti-PCNA-mediated immunoperoxidase staining of the wild-type (CD44^{+/+}) treated with HA_S (a) or treated with Y27632 plus HA_S addition (b) or treated with Ro31-8220 plus HA_S addition (c) vs. CD44 k/o (CD44^{-/-}) treated with HA_S (d).

C: Anti-PCNA-mediated immunoperoxidase staining of the wild-type (CD44^{+/+}) treated with HA_L (a) or treated with Y27632 plus HA_L addition (b) or treated with Ro31-8220 plus HA_L addition (c) vs. CD44 k/o (CD44^{-/-}) treated with HA_L (d).

Fig. 5.

Detection of differentiation marker [Involucrin (I) and Filaggrin (II)] expression in aged wild-type (CD44+/+) and CD44 k/o (CD44-/-) mouse epidermis using immunohistochemical staining.

A: Anti-Involucrin-mediated (I) or anti-Filaggrin-mediated immunoperoxidase staining of the wild-type (CD44+/+) (vehicle control without HA treatment) (a) or treated with Y27632 without HA addition (b) or treated with Ro31-8220 without HA addition (c) vs. CD44 k/o (CD44-/-) (d) (vehicle control without HA treatment).

B: Anti-Involucrin-mediated (I) or anti-Filaggrin-mediated immunoperoxidase staining of the wild-type (CD44+/+) treated with HA_S (a) or treated with Y27632 plus HA_S addition (b) or treated with Ro31-8220 plus HA_S addition (c) vs. CD44 k/o (CD44-/-) treated with HA_S (d).

C: Anti-Involucrin-mediated (I) or anti-Filaggrin-media immunoperoxidase staining of the wild-type (CD44+/+) treated with HA_L (a) or treated with Y27632 plus HA_L addition (b) or treated with Ro31-8220 plus HA_L addition (c) vs. CD44 k/o (CD44-/-) treated with HA_L (d).

Fig. 6.

Analyses of HA effects on permeability barrier recovery in wild-type (CD44^{+/+}) and CD44 k/o (CD44^{-/-}) mouse epidermis using Transepidermal water loss (TEWL) measurement.

A: TEWL in young (n=10) and aged wild-type (CD44^{+/+}) mouse (n=10) epidermis (with no HA treatment) was measured after 1, 3 and 6h after tape stripping (The error bars enclosed mean $\pm$ S.D). All numbers are statistically significant [$p < 0.05$ (1h), $p < 0.02$ (3h) and $p < 0.03$ (6h), respectively; analysis of variance; $n=5$] as compared with control samples (e.g., young mouse control).

B: TEWL in aged wild-type (CD44^{+/+}) mouse epidermis treated with HA (HA_S or HA_L) or no HA was measured after 1, 3 and 6h after tape stripping (The error bars enclosed mean $\pm$ S.D). All numbers are statistically significant [$p < 0.01$ (1h), $p < 0.01$ (3h) and $p < 0.05$ (6h), respectively; analysis of variance; $n=5$] as compared with control samples (e.g., no HA-treated control).

C: TEWL in aged CD44 k/o (CD44^{-/-}) mouse epidermis treated with HA (e.g., HA_S or HA_L) or no HA was measured after 1, 3 and 6h after tape stripping (The error bars enclosed mean $\pm$ S.D). All numbers are statistically significant [$p < 0.02$ (1h), $p < 0.05$ (3h) and $p < 0.04$ (6h), respectively; analysis of variance; $n=4$] as compared with control samples (e.g., no HA-treated control).

D: TEWL in aged wild-type (CD44^{+/+}) mouse epidermis treated with various agents (e.g., HA_S, or HA_L or Y27632 plus HA_S or Ro31-8220 plus HA_L or no HA) was measured after 1, 3 and 6h after tape stripping (The error bars enclosed mean $\pm$ S.D). All numbers are

statistically significant [$p<0.04$ (1h), $p<0.05$ (3h) and $p<0.04$ (6h), respectively; analysis of variance; $n=5$] as compared with control samples (e.g., no HA-treated control).

E: TEWL in young and aged wild-type (CD44+/+) mouse epidermis treated with HA (HA_S ->HA_L) or no HA was measured after 1, 3 and 6h after tape stripping (The error bars enclosed mean $\pm$ S.D). All numbers are statistically significant [$p<0.03$; (1h), $p<0.01$ (3h) and $p<0.05$ (6h), respectively; analysis of variance; $n=5$] as compared with control samples (e.g., no HA-treated control).

F: TEWL in aged wild-type (CD44+/+) mouse epidermis treated with various agents (e.g., HA_S->HA_L or Y27632 plus HA_S->HA_L or Ro31-8220 plus HA_S->HA_L or a combination of Y27632 and Ro31-8220 plus HA_S->HA_L) was measured after 1, 3 and 6h after tape stripping (The error bars enclosed mean $\pm$ S.D). All numbers are statistically significant [$p<0.01$ (1h), $p<0.03$ (3h) and $p<0.04$ (6h), respectively; analysis of variance; $n=5$] as compared with control samples (e.g., no drug-treated control).

A Model for Selective Effects of HA_S vs. HA_L on CD44-mediated Epidermal Functions During Skin Aging

Fig. 7.

A proposed model for illustrating CD44 interaction with HA (HA_S or HA_L) in regulating aged epidermal structure and function.

In epidermis, HA_S binding to CD44 promotes RhoA-ROK signaling resulting in proliferation and epidermal thickness in aged skin; whereas HA_L interaction with CD44 stimulates Rac-PKN_γ activation leading to differentiation and subsequent restoration of epidermal permeability barrier function in aged mouse skin. [Both skin aging and CD44 deficiency in k/o mice cause abnormal epidermal structure and function.

Table 1
Selective HA (HA_S vs. HA_L) effects on RhoGTPase signaling in cultured human keratinocytes (CHK)

HA (HA_S vs. HA_L)-induced RhoGTPase activation in cultured human keratinocytes (CHK).

Samples	[³⁵ S]GTP •RhoA (cpm) (% of control) *	[³⁵ S]GTP •Rac1(cpm) (% of control) *
No HA (control)	100 ± 3	100 ± 5
+ HA _S	278 ± 12^a	106 ± 4 ^b
+ HA _L	105 ± 6 ^a	269 ± 10^b

* Cultured human keratinocytes (CHK) (~5.0 × 10⁶ cells) were preloaded with [³⁵S]GTP S (12.5 µCi) using electroporation methods as described in the Materials and Methods. These cells were incubated with 50µg/ml HA_S or HA_L at 37°C for 10 min. Subsequently, [³⁵S]GTP S labeled cells were solubilized in 1.0% NP-40 and incubated with either mouse anti-RhoA IgG (5µg/ml) or mouse anti-Rac1 IgG (5µg/ml) plus goat anti-mouse conjugated beads, respectively. The amount of [³⁵S]GTP S-Rac1 associated with anti-RhoA IgG or anti-Rac1-conjugated immuno-beads was measured using a gamma counter. The values expressed in Table 1 represent an average of triplicate determinations of 6 experiments with a standard deviation less than ±5%.

^a & ^b Statistically significant ($p < 0.001$; analysis of variance; $n=6$) as compared with control samples [e.g., No HA-treated cells (vehicle control)].

Table 2

Effects of various agents on ROK and PKN activities in HA (HA_S vs. HA_L)-treated cultured human keratinocytes (CHK).

A: Effects of various drugs on RhoA-ROK and Rac1-PKN activities:		
Samples	Relative ROK activity (% of control)*	Relative PKN activity (% of control)*
No HA (vehicle control)	100± 5	100± 3
+ HA _S	280±15^a	120± 6 ^b
+ HA _S + Y27632	65± 5 ^a	105± 4 ^b
+ HA _S + Ro31-8220	188±10 ^a	98± 3 ^b
+ HA _L	132± 6 ^a	272±12^b
+ HA _L + Y27632	76± 4 ^a	200±14 ^b
+ HA _L + Ro31-8220	92± 5 ^a	60± 4 ^b

B: Effects of anti-CD44 antibody on HA-mediated ROK and PKN activities:		
Samples	Relative ROK activity (% of control)*	Relative PKN activity (% of control)*
Normal Rat IgG (No HA)	100± 4	100± 5
Normal Rat IgG (+HA _S)	254±16^c	110± 10 ^d
Normal Rat IgG (+HA _L)	90± 3 ^c	262±12^d
Anti-CD44 antibody (No HA)	85± 4 ^c	92± 5 ^d
Anti-CD44 antibody (+HA _S)	72± 3 ^c	94± 4 ^d
Anti-CD44 antibody (+HA _L)	88± 5 ^c	75± 6 ^d

* *In vitro* Rho kinase (ROK) and PKN activity in human CHK [untreated or pretreated with normal rat IgG or rat anti-CD44 antibody or Y27632 (5μM) or Ro31-8220 (5μM) or vehicle control] followed by 50μg/ml HA (HA_S or HA_L) addition] was measured as described in the Materials and Methods. The activity of ROK or PKN in untreated cells (Table 2A-vehicle control) or normal IgG-treated cells without HA (Table 1B-control) was designated as 100%. The values expressed in this table represent an average of triplicate determinations of 5 experiments. All data represent mean ±SEM (with n=5) of the ROK or PKN activity detected in each sample.

^{a & b} Statistically significant ($p<0.001$; analysis of variance; $n=5$) as compared with control samples [e.g., no HA-treated cells (control)].

^{c & d} Statistically significant ($p<0.005$; analysis of variance; $n=5$) as compared with control samples [e.g., Normal Rat IgG (no HA)-treated cells (control)].

Table 3

Selective HA (HA_S vs. HA_L) effects on DNA synthesis and gene expression of PCNA, Involucrin and Filaggrin in cultured human keratinocytes (CHK).

A: Effects of various drugs on HA-mediated DNA synthesis and gene expression of PCNA, Involucrin and Filaggrin in CHK:				
Samples	[3H]-Thymidine Incorporation* cpm (% of control)	PCNA Gene Expression** mRNA level (% of control)	Involucrin Gene Expression** mRNA level (% of control)	Filaggrin Gene Expression** mRNA level (% of control)
No HA (control)	6,523 ± 130 (100%)	100±2	100±5	100±4
+ HA _S	18,244 ± 500 (280%)^a	265±10^b	95±3 ^c	90±2 ^d
+ HA _S + Y27632	6,659 ± 100 (102%) ^a	105±4 ^b	90±2 ^c	85±3 ^d
+ HA _S + Ro31-8220	10,197 ± 304 (156%) ^a	160±8 ^b	88±4 ^c	85±2 ^d
+ HA _L	6,478 ± 115 (99%) ^a	90±2 ^b	273±12^c	267±15^d
+ HA _L + Y27632	5,871 ± 102 (90%) ^a	85±3 ^b	183±10 ^c	170±13 ^d
+ HA _L + Ro31-8220	6,197 ± 108 (95%) ^a	92±4 ^b	98±4 ^c	102±5 ^d

B: Effects of anti-CD44 antibody on HA-mediated DNA synthesis and gene expression of PCNA, Involucrin and Filaggrin in CHK:				
Samples	[3H]-Thymidine Incorporation* cpm (% of control)	PCNA Gene Expression** mRNA level (% of control)	Involucrin Gene Expression** mRNA level (% of control)	Filaggrin Gene Expression** mRNA level (% of control)
Normal Rat IgG (No HA)	5,615 ± 130 (100%)	100±5	100±6	100±3
Normal Rat IgG (+HA _S)	16,754 ± 425 (298%)^e	258±12^f	101±5 ^g	102±4 ^h
Normal Rat IgG (+HA _L)	5,500 ± 90 (98%) ^e	105±4 ^f	249±12^g	236±13^h
Anti-CD44 antibody (No HA)	5,290 ± 104 (94%) ^e	160±8 ^f	104±5 ^g	95±2 ^h
Anti-CD44 antibody (+HA _S)	5,559 ± 98 (99%) ^e	90±2 ^f	105±6 ^g	102±5 ^h
Anti-CD44 antibody (+HA _L)	5,498 ± 102 (98%) ^e	85±3 ^f	103±8 ^g	100±4 ^h

* DNA synthesis was measured by the amount of [methyl-³H]thymidine incorporation in CHK preincubated with normal rat IgG or anti-CD44 antibody prior to HA (HA_S or HA_L) addition as described in the Materials and Methods. Each experiment was performed in quadruplicate and the results represent mean±S.D.

** The expression of PCNA, Involucrin and Filaggrin genes was measured using specific primers and Q-PCR in mouse epidermis according to the procedures described in the Materials and Methods. Relative mRNA expression levels of PCNA, Involucrin or Filaggrin in various treatments were calculated after normalization with GAPDH mRNA levels as determined by Involucrin or Filaggrin gene expression from untreated cells (no HA, vehicle control) is designated as 100%. Each experiment was performed in quadruplicate and the results represent mean ± S.D.

a, b, c, d. Statistically significant ($p<0.005$; analysis of variance; $n=5$) as compared with control samples [e.g., No HA-treated cells (vehicle control)];

e, f, g & h. Statistically significant ($p<0.001$; analysis of variance; $n=5$) as compared with control samples [e.g., Normal Rat IgG (No HA)-treated cells (vehicle control)].

Table 4

Effects of various drugs on HA (HA_S vs. HA_L) or sequential HA (HA_S → HA_L)-mediated epidermal thickness, PCNA-positive cells and gene expression of Involucrin and Filaggrin in aged Wild-type (CD44+/+) mouse skin.

Animals	Epidermal thickness μm per mm [*]	PCNA-positive cells per mm ^{**}	Involucrin Gene Expression mRNA level (% of control) ^{***}	Filaggrin Gene Expression mRNA level (% of control) ^{***}
No HA (vehicle control) (n=6)	6 ± 1	35±2	100±3	100±4
+ HA _S (vehicle control) (n=6)	18± 3^a	93±4^b	115±5 ^c	122±4 ^d
+ HA _S +Y27632 (n=6)	4 ± 1 ^a	23±2 ^b	86±6 ^c	75±5 ^d
+ HA _S +Ro31-8220 (n=6)	12± 4 ^a	62±8 ^b	104±5 ^c	95±2 ^d
+ HA _L (vehicle control) (n=6)	9± 2 ^a	38±4 ^b	275±16^c	287±12^d
+ HA _L +Y27632 (n=6)	6± 2 ^a	37±3 ^b	102±8 ^c	105±6 ^d
+ HA _L +Ro31-8220 (n=6)	5± 1 ^a	35±2 ^b	153±10 ^c	160±14 ^d
+ HA _S → HA _L (vehicle control) (n=6)	12± 3^a	80±5^b	180±18^c	187±12^d
+ HA _S → HA _L +Y27632 (n=6)	5± 2 ^a	37±5 ^b	162±8 ^c	165±6 ^d
+ HA _S → HA _L +Ro31-8220 (n=6)	5± 1 ^a	35±4 ^b	123±10 ^c	130±12 ^d
+ HA _S → HA _L +Y27632 +Ro31-8220 (n=6)	3± 1 ^a	15±2 ^b	83±6 ^c	80±4 ^d

* Data are presented as mean ± SEM. The total length of the epidermis analyzed varied between 8 mm and 20 mm for each sample. At least five measurements of epidermal thickness were made per specimen.

** Data are presented as mean number of PCNA-positive cells ±SEM per mm. The total length of the epidermis analyzed varied between 10 mm and 16 mm for each sample.

*** The expression of Involucrin and Filaggrin genes was measured using specific primers and Q-PCR in mouse epidermis according to the procedures described in the Materials and Methods. Relative mRNA expression levels of Involucrin or Filaggrin in various treatments were calculated after normalization with mRNA levels as determined by Involucrin or Filaggrin gene expression from untreated cells (no HA, vehicle control) is designated as 100%. Each experiment was performed in quadruplicate and the results represent mean ± S.D.

a, b, c & d Statistically significant ($p < 0.001$; analysis of variance; $n=6$) as compared with control samples [e.g., No HA-treated cells (vehicle control)].