Materials and Methods:

Experimental protocols and functional studies: All animal procedures were approved by the Animal Studies Subcommittee (IACUC) of the San Francisco Veterans Administration Medical Center and performed in accordance with their guidelines. Since hesperidin is not soluble in 100% ethanol, 70% ethanol was used as vehicle. Since the turnover time for hairless is about 8-9.5 days in normal young mice (Potten et al., 1987), we chose to treat aged mice for 9 days. Both flanks of 12-15 month old mice were treated topically with 60 μl of 2% hesperidin or 70% ethanol twice daily for 9 days. Basal epidermal permeability barrier function was assessed by measuring transepidermal water loss (TEWL) using TM300 connected to MPA5 (C&K, Cologne, Germany)(Mao-Qiang et al., 2004; Man et al., 2012). For barrier recovery, TEWL was measured using an electrolytic water analyzer (Meeco, Warrington, PA) at 0, 2 and 4 hours after tape stripping (10-fold increase in TEWL), and percent barrier recovery was calculated as described earlier (Liu et al., 2010; Mao-Qiang et al., 2004; Man et al., 2012).

Keratinocyte Culture: Second-passage keratinocytes isolated from adult human (donor aged 60-65 year old) were cultured in serum-free keratinocyte growth medium containing 0.07 mM calcium (Clonetics, San Diego, California). Cells at 60%–70% confluence were switched to a medium containing 1.2 mM calcium and treated with either 0.02% hesperidin or vehicle alone (0.02% ethanol). After 24 and 48 hrs of treatment, keratinocytes were collected for Q-PCR analysis (Hou et al., 2013).

Immunohistochemistry: Immunohistochemical staining for assessing changes in epidermal differentiation was performed as described earlier (Hou et al., 2013; Mao-Qiang et al., 2004). Briefly, 5 μm paraffin sections were incubated with the primary antibodies (Covance, Emeryville, CA) overnight at 4°C. After washes ×3, sections were incubated with the secondary antibody for

30 minutes. Staining was detected with ABC-peroxidase kit from Vector Lab (Burlingame, CA). Sections were examined with a Zeiss fluorescence microscope (Jena, Germany) and digital images were captured with AxioVision software (Carl Zeiss Vision, Munich, Germany).

Q-PCR for mRNA expression： Total RNA was isolated from cultured human keratinocytes using TRI Reagent (Sigma). First strand cDNA was synthesized from 1ug of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The real-time PCR contained 20 ng of reversed transcribed total RNA, 450 nM forward and reverse primers, and 10 μl of 2× LightCycler 480 SYBR Green I Master in a final volume of 20 μl in 96-well plates using Mx3000P™ Real-time PCR System (Stratagene, La Jolla, CA). Quantification was performed by the comparative C_T method with 36B4 or Cyclophilin A used for normalization. The primers for lipid synthetic enzymes such as 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCoA), serine–palmitoyl transferase 1 (SPT1), fatty acid synthase (FAS), lipid transporters (ATP-binding cassette A12 (ABCA12)), mouse beta defensin 3 (mBD3), sodium-hydrogen exchanger 1(NHE1), secretary phospholipase A2g2f (sPLA2g2f), filaggrin, involucrin, loricrin and Cyclophilin A are listed in supplemental table 1. Relative expression of the mRNAs compared to control mRNA was calculated. Data are expressed as percentage of control (as 100%) (Hou et al., 2013).

Electron Microscopy: Skin biopsies from both vehicle and hesperidin-treated mice were taken for electron microscopy (Bourguignon et al., 2006; Hou et al., 2012 and 2013). Briefly, samples were minced to <0.5 mm^3, fixed in modified Karnovsky's fixative overnight, and post-fixed in either 0.2% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide. After fixation, all samples were dehydrated in a graded ethanol series, and embedded in an Epon-epoxy mixture. Ultrathin sections were examined, with or without further

contrasting with lead citrate, in a Zeiss 10A electron microscope (Carl Zeiss, Thornwood, NJ), operated at 60 kV.

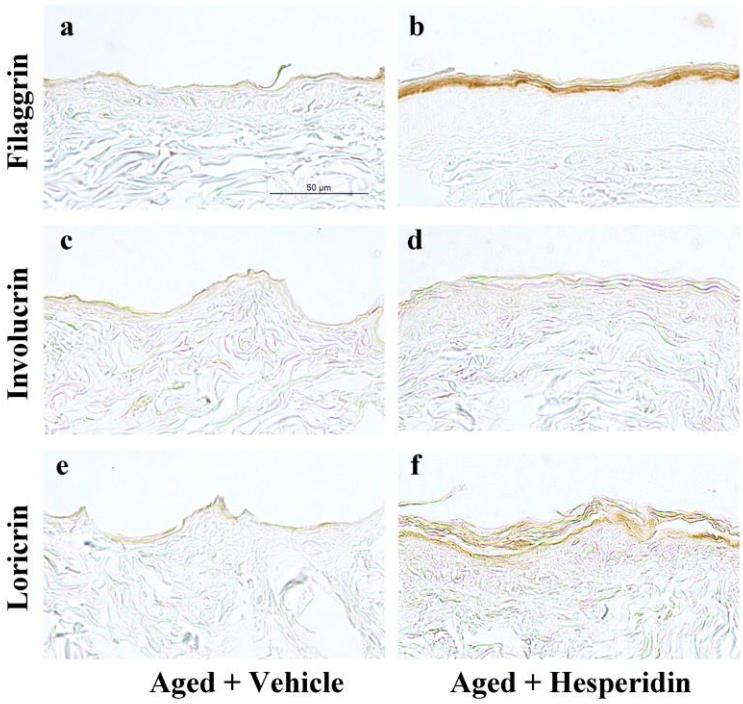
Measurement of Lamellar Body Density and Secretion: LB numbers were determined in granular cells two to three layers below the stratum granulosum-stratum corneum(SG–SC) junction as previously described (Elias et al., 1983). The number of LBs was counted at 4800 magnification using a calibrated grid. Total 10 random pictures from each biopsy sample were assessed. For quantification of LB secretion, number of LB protrusion at the SG–SC junction were measured at a magnification of 5800 and correlated with the length of the bottom surface of the first SC layer on 10 random images at 5800 magnification.

Statistics

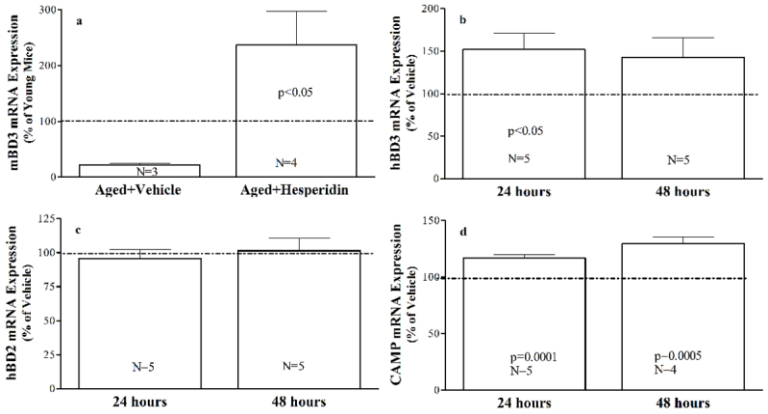
Data are expressed as the mean $\pm$ SEM. GraphPad Prism 4 software (San Diego, CA, USA) was used for all statistical analyses. Unpaired two-tailed student's *t*-test with Welch's correction was used to determine the statistical significances when two groups were compared. One-Way ANOVA with Tukey correction was used when three or more groups were compared.

Supplemental Table 1. Primer Sequences

		Sequences
Mouse HMGCoA	Forward	5’ GCCGTGAACTGGGTCGA 3’
	Reverse	5’ GCATATATAGCAATGTCTCCTGC 3’
Mouse SPT1	Forward	5’ AGGGTTCTATGGCACATTTGATGT 3’
	Reverse	5’ TGGCTTCTTCGGTCTTCATAAAC 3’
Mouse FAS	Forward	5’GCTGCGGAAACTTCAGGAAAT3’
	Reverse	5’AGAGACGTGTCACTCCTGGACTT3’
h/m ABCA12	Forward	5’ ACAGGAATGGCCTTCATCAC 3’
	Reverse	5’ AACATGGTGCCCTGAGAAAC 3’
Human Filaggrin	Forward	5’ CCATCATGGATCTGCGTGG 3’
	Reverse	5’ CACGAGAGGAAGTCTCTGCGT 3’
Mouse Filaggrin	Forward	5’ TGACAGCCAAGTCCATTCTG 3’
	Reverse	5’ TATCCTCCCTGACCACTTGC 3’
Human Involucrin	Forward	5’ CTGCCTGAGCAAGAATGTGA 3’
	Reverse	5’ TGCTCTGGGTTTTCTGCTTT 3’
Mouse Involucrin	Forward	5’ AAGGGCTTTCCCAAACATGA 3’
	Reverse	5’ TGCTGGTGCTCACACTTTTGA 3’
Mouse Loricrin	Forward	5’ GTGGAAAGACCTCTGGTGGA 3’
	Reverse	5’ TGGAACCACCTCCATAGGAA 3’
Human BD3	Forward	5’ CCTAGCAGCTATGAGGATCCAT 3’
	Reverse	5’ CTTCGGCAGCATTTTCG 3’
Mouse BD3	Forward	5’ TCTGTTTGCATTTCTCCTGGT 3’
	Reverse	5’ GGAACTCCACAACTGCCAAT 3’
Human CAMP	Forward	5’ GCTAACCTCTACCGCCTCCT 3’
	Reverse	5’ GGTCACTGTCCCCATACACC 3’
Mouse CAMP	Forward	5’ TGAGCCCCAAGGGGACGAGG 3’
	Reverse	5’ GCCGGGTTCAGGGTGACTGC 3’
Human CycloA	Forward	5’ TCTCCTTTGAGCTGTTTGCAG 3’
	Reverse	5’ CACCACATGCTTGCCATC 3’
h/m 36B4	Forward	5’ GCGACCTGGAAGTCCAACTAC 3’
	Reverse	5’ ATCTGCTGCATCTGCTTGG 3’
Nouse NHE1	Forward	5’- TTTCCCCGATTTCCTTCTCT - 3’
	Reverse	5’- GCGTGTAAGACCTGGGACAT – 3’
Mouse sPLA2g2f	Forward	5’- CCCCATCCAGTCCTTAGTCA -3’
	Reverse	5’- ACTTCTGGGCAGGAGTCAGA -3’
Human GAPDH	Forward	5’CGAGTCAACGGATTTGGTCGTA3’
	Reverse	5’GCAACAATATCCACTTTACCAGAGTTAA3’



Supplemental Figure 1. Topical Hesperidin Stimulates Filaggrin and Loricrin Expression in Aged Mouse Epidermis: 5μm paraffin sections were incubated with respective primary and secondary antibodies (see methods). The sections were visualized with a Zeiss microscope. Magnifications are the same for all figures. Magnification bars represent 50 μm (a–f). Suppl Fig. 1a and b are filaggrin staining; c and d are involucrin staining; e and f are loricrin staining. Suppl Fig. 1a, c and e are vehicle-treated samples, and b, d and f are hesperidin-treated samples.



Supplemental Figure 2. Hesperidin Stimulates Antimicrobial Peptide mRNA Expression in Aged Skin and in Adult Human Keratinocytes: Suppl Fig. 2a represents mBD3 mRNA expression in mice. Significance and numbers of mice are indicated in the figures. Suppl Fig. 2b-d show the changes in the levels of hBD3, hBD2, and CAMP mRNA expression in human keratinocyte cultures. Data are expressed as % of vehicle treated keratinocytes, setting the levels of vehicle treated as 100% (dotted line). Significances and number of samples are indicated in the figures.