The Polyamine Regulator AMD1 Upregulates Spermine Levels to Drive Epidermal Differentiation

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Maintaining tissue homeostasis depends on a balance between cell proliferation, differentiation, and apoptosis. Within the epidermis, the levels of the polyamines putrescine, spermidine, and spermine are altered in many different skin conditions, yet their role in epidermal tissue homeostasis is poorly understood. We identify the polyamine regulator, Adenosylmethionine decarboxylase 1 (AMD1), as a crucial regulator of keratinocyte (KC) differentiation. AMD1 protein is upregulated on differentiation and is highly expressed in the suprabasal layers of the human epidermis. During KC differentiation, elevated AMD1 promotes decreased putrescine and increased spermine levels. Knockdown or inhibition of AMD1 results in reduced spermine levels and inhibition of KC differentiation. Supplementing AMD1-knockdown KCs with exogenous spermidine or spermine rescued aberrant differentiation. We show that the polyamine shift is critical for the regulation of key transcription factors and signaling proteins that drive KC differentiation, including KLF4 and ZNF750. These findings show that human KCs use controlled changes in polyamine levels to modulate gene expression to drive cellular behavior changes. Modulation of polyamine levels during epidermal differentiation could impact skin barrier formation or can be used in the treatment of hyperproliferative skin disorders.


INTRODUCTION

Epidermal homeostasis is dependent on a balance between keratinocyte (KC) proliferation, apoptosis, and differentiation (Fuchs, 2018; Fuchs and Raghavan, 2002). During differentiation, KCs in the epidermal basal layer stop proliferating and gradually differentiate as they move up through the spinous and granular layers to form the stratum corneum. Perturbed control of this process can result in hyperproliferation and the development of skin disorders such as skin neoplasia, atopic dermatitis, psoriasis, and squamous cell carcinoma (Belokhvostova et al., 2018; Darido et al., 2016).

Putrescine (Put), spermidine (Spd), and spermine (Spm) are naturally occurring polyamines that are present in all eukaryotes and are essential for cellular function (Kusano and Suzuki, 2015). Put is generated from the decarboxylation of ornithine by ODC1. Adenosylmethionine decarboxylase 1 (AMD1) decarboxylates S-adenosylmethionine to decarboxylated S-adenosylmethionine to provide the aminopropyl donor groups to form Spd from Put and Spm from Spd. Intracellular polyamine levels are tightly controlled through a combination of synthesis, catabolism, and transportation (Park and Igarashi, 2013; Pegg, 2016). Polyamines predominantly exist bound to RNA and/or DNA, and changes in their levels can influence RNA and DNA structure (Igarashi and Kashiwagi, 2015; Lightfoot and Hall, 2014). Polyamines can also influence epigenetic regulation through the modulation of histone methylation and acetylation (Arruabarrena-Aristorena et al., 2018; Hobbs and Gilmour, 2000; Murray-Stewart et al., 2014; Wei et al., 2007). The levels and ratios of polyamines can vary between different cell types and cellular contexts, and it has been suggested that changes in polyamine levels can have a regulatory role (Igarashi and Kashiwagi, 2019; Lim et al., 2018; Pegg, 2016; Zhang et al., 2012).

High polyamine levels in the epidermis are associated with hyperproliferative skin disorders, including psoriasis and cancer (Broshtilova et al., 2012; Nowotarski et al., 2015). Increased epidermal ODC1 levels play a role in skin tumorigenesis by promoting high Put levels, which disrupts the
These data suggest that during epidermal differentiation, differentiated KCs (Figure 1c). To study whether AMD1 is regulated on epidermal differentiation, we stained healthy human abdominal skin sections to analyze AMD1 protein (n = 6) and RNA (n = 3) expression in the epidermis. AMD1 protein was highly expressed in the epidermal-differentiated layers and, consistent with previous observations, was colocalized with the late differentiation marker, involucrin (IVL) (Lim et al., 2018). AMD1 was expressed as a gradient in the epidermis where it was present at low levels in the basal layer, increased in the stratum spinosum, and was at high levels in the stratum granulosum. RNAscope in situ hybridization showed that AMD1 RNA was equally expressed in all layers of the epidermis (Figure 1a). These data suggest that AMD1 is post-transcriptionally regulated during epidermal differentiation.

We determined the expression of AMD1 in cultured human primary KCs undergoing calcium-induced differentiation. We confirmed KC differentiation by the upregulation of differentiation markers keratin (K) 10 and IVL (Figure 1b and c). Similar to human skin, we found that AMD1 protein was upregulated on differentiation, whereas AMD1 mRNA remained constant (Figure 1b and Supplementary Figure S1a). We made similar observations after 6 days of calcium-induced differentiation of N/TERT-1 human immortalized KCs (Figure 1c).

To determine whether altered mRNA translation was responsible for the changes in protein level seen on differentiation of KCs, we determined the ribosomal load of AMD1 before and after differentiation by polysome profiling. Cell extracts were separated on sucrose gradients and fractionated to separate mRNAs on the basis of their ribosomal load. Quantitative real-time transcriptase–PCR analysis showed that AMD1 RNA was highly enriched in the fractions (F) representing the less highly translated RNAs (F5, 6) before differentiation. On differentiation, AMD1 RNA shifted to the highly translated fractions (F7, 8, Supplementary Figure S1b). These data suggest that on epidermal differentiation, AMD1 is translationally upregulated (as indicated by the increased ribosomal load), thus increasing AMD1 protein levels in differentiated KCs.

**RESULTS**

**AMD1 is post-transcriptionally upregulated during human epidermal differentiation**

To determine whether AMD1 is regulated on epidermal differentiation, we stained healthy human abdominal skin sections to analyze AMD1 protein (n = 6) and RNA (n = 3) expression in the epidermis. AMD1 protein was highly expressed in the epidermal-differentiated layers and, consistent with previous observations, was colocalized with the late differentiation marker, involucrin (IVL) (Lim et al., 2018). AMD1 was expressed as a gradient in the epidermis where it was present at low levels in the basal layer, increased in the stratum spinosum, and was at high levels in the stratum granulosum. RNAscope in situ hybridization showed that AMD1 RNA was equally expressed in all layers of the epidermis (Figure 1a). These data suggest that AMD1 is post-transcriptionally regulated during epidermal differentiation.

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**AMD1 upregulation is required for KC differentiation and stratification**

To determine the function of AMD1 during KC differentiation, we used two independent short hairpin (sh) RNAs, shAMD1–3 and shAMD1–4, which knocked down AMD1 mRNA and protein by 80% in N/TERT-1 KCs compared with that in the scrambled shRNA controls (Figure 2a and Supplementary Figure S2a and b). After inducing differentiation, shAMD1 KCs showed a significant decrease in the RNA and protein expression of differentiation markers K10, FLG, and loricrin (LOR). IVL RNA and protein expression were only marginally decreased in shAMD1 cells compared with that in the scrambled shRNA controls (Figure 2b and c). These data suggest that AMD1 is required for efficient KC differentiation. We determined whether AMD1 knockdown affected cell confluence. We seeded equal numbers of shAMD1 and scrambled shRNA cells at the start of differentiation, and on counting, we found no significant difference in cell number on day 0, day 3, or day 6 of differentiation (Supplementary Figure S2d), suggesting that increased AMD1 levels are required for efficient KC differentiation, independent of the impact of AMD1 on KC proliferation.

Increased AMD1 will lead to increased synthesis of Spd and Spm, so we measured the levels of Put, Spd, and Spm in undifferentiated and differentiated KCs with and without AMD1 knockdown. After 6 days of KC differentiation, we saw significant changes in the Put-to-Spd-to-Spm ratios, with Put decreasing and Spm increasing (Figure 2d and Supplementary Figure S2e). Spd was also decreased likely owing to its more rapid conversion to Spm when AMD1 levels are high. This change in ratios was dependent on AMD1 because in the shAMD1–3 cells, Spm levels were reduced, and the ratios of the three polyamines were more similar to those of the undifferentiated cells.

To determine whether the shAMD1 differentiation phenotype was due to decreased rates of Spd and/or Spm synthesis, we attempted to rescue the differentiation phenotype by supplementing KCs with exogenous Spd and Spm. KCs were viable in up to 1 mM of Spd and/or Spm (Supplementary Figure S3a). We differentiated shAMD1–3 cells in the presence of 1 mM Spd and Spm and found that RNA expression of differentiation markers FLG and LOR were rescued and that K10 was partially rescued on supplementation with Spd and/or Spm. The protein expression of FLG, LOR, and K10 was fully rescued with Spd and/or Spm supplementation (Figure 2b and c). Similar phenotypes were observed with shAMD1–4 knockdown KCs (Supplementary Figure S2c). In addition to rescuing the AMD1 knockdown phenotype, Spd and/or Spm supplementation alone significantly increased the mRNA and protein expression levels of differentiation markers K10, FLG, and LOR in the control cells (Figure 2b and c). This finding shows that Spd and/or Spm supplementation can enhance KC differentiation.

To confirm that reducing AMD1 inhibits KC differentiation, we treated KCs with an irreversible AMD1 inhibitor, ethylglyoxal bis(guanilhydrazone) (EGBG) (Igarashi et al., 1984). After an MTT assay (Supplementary Figure S3b), we selected 25 μM and 50 μM EGBG to inhibit AMD1 activity. AMD1 levels are elevated by day 3 of KC differentiation, so we
Figure 1. AMD1 is upregulated during epidermal differentiation. (a) RNAscope in situ hybridization of AMD1 mRNA expression and immunofluorescence staining of AMD1 in the human Ep. The dotted lines represent the basement membrane, and the nuclei are stained with DAPI. Enlarged images are shown on the right. Bar = 50 μm. (b) Western blot (left) and QRT-PCR (right) analysis of K10, IVL, and AMD1 in human primary keratinocytes and (c) western blot (left) and QRT-PCR (right) analysis of K10, IVL, and AMD1 in N/TERT-1 cells during 6 days of calcium-induced differentiation. GAPDH or β-actin was used as a loading control. mRNA expression was normalized to the housekeeping gene, RPL13A, and the results represent the means ± SEM of three independent biological replicates. *P < 0.05 and **P < 0.01. d, day; D, Dermis; Ep, Epidermis; IVL, involucrin; K, keratin; QRT-PCR, quantitative real-time reverse transcriptase–PCR.
added EGBG from day 3 and harvested it on day 6. On day 6 of differentiation, there was no observable increase in cell death, and cell confluence was comparable between the samples (data not shown). To confirm the inhibition of AMD1, we measured the levels of Put, Spd, and Spm. As seen previously, on KC differentiation when AMD1 levels are high, Spm levels are increased, and Put and Spd levels are decreased (Figure 3a). Inhibiting AMD1 with EGBG resulted in decreased Spm levels on day 6 of differentiation, whereas Spd levels did not change (Figure 3a and Supplementary Figure S3c). Exposure to EGBG on day 3 of differentiation resulted in a significant decrease in IVL and LOR expression by day 6. As before, we could rescue this effect by adding 1 mM Spd and/or Spm to the culture (Figure 3b and c). A similar day 6 differentiation phenotype was observed when EGBG was added at the start of KC differentiation (day 0), and this was rescued through the supplementation of 1 mM Spd and/or Spm (Supplementary Figure S4a and b).

To better recapitulate the human epidermis, we used reconstructed human skin equivalent models to determine the effect of AMD1 inhibition by EGBG on stratification. Human primary KCs were cultured and stratified at the air–liquid interface for 6 days with and without 10 or 50 μM EGBG from day 3 onward. In addition, we attempted to rescue the EGBG AMD1 inhibition phenotype by supplementation with Spd and/or Spm (Figure 3d). Whereas efficient epidermal stratification was observed with untreated KCs, those treated with EGBG showed reduced stratification; decreased epidermal thickness; and decreased FLG, K10, and LOR staining. This phenotype was stronger in the 50 μM EGBG–treated samples than in the 10 μM EGBG–treated samples. Ki67 staining showed a marginal decrease in staining in the basal layer in 50 μM EGBG–treated samples but showed no significant change in the 10 μM EGBG–treated samples (Supplementary Figure S3d). EGBG–treated cultures supplemented with 100 μM Spd and/or Spm showed restored epidermal thickness and FLG, K10, and LOR staining that was comparable with those in the control-ununtreated cells. These data suggest that inhibition of AMD1 with EGBG inhibits epidermal differentiation in three-dimensional cultures and that this is rescued by supplementation with Spd and/or Spm. Taken together, these data indicate that increased Spd and/or Spm levels as a result of AMD1 upregulation are required for KC differentiation and epidermal barrier formation.
Figure 3. Inhibition of AMD1 prevents epidermal differentiation in 2D and 3D cultures. (a) HPLC data showing the relative levels of Put, Spd, and Spm in undifferentiated and differentiated keratinocytes with and without the addition of the AMD1 inhibitor, EGBG. (b) Western blot and (c) QRT-PCR analysis of the differentiation markers IVL and LOR in d0 undifferentiated and d6 differentiated keratinocytes in response to increasing EGBG concentrations with and without 1 mM Spd and/or Spm. GAPDH was used as a loading control for the western blot, and gene expression levels were normalized to that of RPL13A for QRT-PCR. Results are presented as the means ± SEM of three independent biological replicates; *P < 0.05, **P < 0.01, and ***P < 0.001. Bar = 50 μm. (d) H&E (top), FLG (Red), K10 (Green) (middle), and LOR (bottom) staining of reconstructed human skin equivalent models treated with and without the AMD1 inhibitor (10 and 50 μM EGBG) and rescued with Spd and/or Spm; (n = 3). Bar = 50 μm. 2D, two-dimensional; 3D, three-dimensional; d, day; EGBG, ethylglyoxal bisguanylhydrazone; IVL, involucrin; K, keratin; LOR, loricrin; Put, putrescine; QRT-PCR, quantitative real-time reverse transcriptase–PCR; Spd, spermidine; Spm, spermine; SS, spermidine/spermine.
These data confirm that reduction in AMD1 activity with shRNAs or through enzyme inhibition prevents the upregulation of KC differentiation markers, demonstrating that high AMD1 levels are required for efficient KC differentiation.

**AMD1 is upstream of key transcription factors and signaling molecules that drive KC differentiation**

To identify the downstream targets of AMD1 during KC differentiation, we performed a microarray analysis of differentiated KCs with and without EGBG treatment and Spd and/or Spm rescue. A total of 3,050 genes were differentially expressed (>2 fold) in KCs between day 0 (undifferentiated cells) and day 6 of differentiation. Of these genes, 777 were reduced by >2 fold in EGBG-treated cells, and of these, the expression of 675 genes could be rescued on the addition of Spd and/or Spm. These data show that 22.2% (675) of genes that are differentially expressed during epidermal differentiation are sensitive to reduced AMD1 levels and can be rescued by exogenous Spd and/or Spm (Figure 4a and b).

Gene ontology analysis of the 675 AMD1-sensitive genes showed a strong enrichment for genes involved in epidermal barrier formation and gene expression control among those that were upregulated on differentiation (Figure 4c and Supplementary Figure S5). These genes included a significant number of genes within the epidermal differentiation complex (Figure 4d) (Mischke et al., 1996; Peterlowicz et al., 2017; Segre, 2006). These genes were upregulated on epidermal differentiation, and this upregulation was inhibited by AMD1 inhibitor EGBG. Importantly, Spd and/or Spm supplementation restored the gene expression to levels comparable with those of differentiation on day 6 in normal KCs. These data demonstrate that AMD1 functions as an upstream regulator of epidermal differentiation and promotes, at least in part, the high Spd and/or Spm levels that are required to drive the expression changes required for KC differentiation.

One of the gene ontology categories dependant on AMD1 was positive regulation of gene expression (Figure 4c). Genes in this category included transcriptional regulators, many of which direct gene expression changes during epidermal differentiation, including KLF4, JUNB, ZNF750, and GRHL3 (Cangkrama et al., 2013; Kretz, 2013; Kyririotou et al., 2012) (Figure 4e). ZNF750 promotes the levels of the long non-coding RNA TINCR, which is required for epidermal differentiation (Kretz et al., 2013). We show in our analyses that TINCR, along with its targets ALOX3, ALOX12B, and CASP14, are AMD1 sensitive on epidermal differentiation (Supplementary Table S1).

We performed quantitative real-time reverse transcriptase–PCR on day-0 and day-6 KCs differentiated with or without EGBG and rescued with Spd and/or Spm (Figure 5a). We confirmed that transcriptional regulators JUNB, KLF4, HOXA9, and ZNF750 and their target TINCR were AMD1 sensitive. We also confirmed that genes encoding for signaling molecules important for KC differentiation—EGF, KLK9, S100A8/9, CALML5, and AQP3—were all AMD1 sensitive. Western blot analysis showed that IVL, KLF4, KLK7, AQ83, and S100B/9 were all upregulated on day 6 of differentiation and that upregulation was inhibited with EGBG and rescued after supplementation with Spd and Spm. Downregulation of NOTCH1 was inhibited on differentiation and rescued after supplementation with Spd and Spm (Figure 5b).

We have shown that if normal KCs are supplemented with Spd and/or Spm during differentiation, the efficiency of differentiation is enhanced as shown by an increase in differentiation markers (Figure 2b and c). On analysis of the genome-wide gene expression data, we see that supplementation of differentiating KCs with Spd and/or Spm (without EGBG) further enhances the gene expression changes seen on differentiation of 258 of the 675 AMD1-sensitive genes (Figure 6a). Gene ontology analysis of these genes shows enrichment for genes involved in barrier formation and includes a large number of proteins from the epidermal differentiation complex (Figure 6b and c). Together, these data support the notion that Spd and Spm supplementation can enhance KC differentiation.

The data presented in this paper show that AMD1 functions on differentiation by altering the ratios and levels of Put, Spd, and Spm such that Spm levels are increased. This increase in Spm then drives the expression of key epidermal differentiation transcription factors, including KLF4 and ZNF750, to promote KC differentiation (Figure 6d).

**DISCUSSION**

This study shows that the polyamine regulator, AMD1, is essential for KC differentiation. AMD1 protein is expressed in a gradient within the epidermis from high expression in the differentiated suprabasal layers to low expression in the basal layers. Conversely, AMD1 RNA is evenly expressed throughout the epidermal layers. We show that the upregulation of AMD1 is essential for KC differentiation and that it functions to promote high levels of Spm that are required for differentiation. Finally, genome-wide expression analysis revealed that AMD1, through the promotion of high Spm levels, has an essential role upstream of key epidermal differentiation regulators, including KLF4 and ZNF750 (Darido et al., 2016).

Our data suggest that AMD1 protein is upregulated during KC differentiation through the translational upregulation as shown by the increased ribosomal load on the mRNA on differentiation. Although these data do indicate that AMD1 mRNA is translationally upregulated, it is also possible that the protein is further stabilized to promote the significant increase in AMD1 protein seen on differentiation.

AMD1 is rate limiting for the conversion of Put to Spd and Spd to Spm. During KC differentiation, we detected an increase in AMD1 that was accompanied by an increase in Spm but not with Spd levels. We speculate that newly synthesized Spd is rapidly converted to Spm during KC differentiation and thus contributes to the increased Spm levels. Spd is needed to form the modified amino acid hypusine that is required for EIF5A function (Park and Wolff, 2018); therefore, it is possible that Spd levels do not increase owing to an increased rate of hypusination during differentiation.
Our microarray analysis identified a large number of mRNAs that are dependent on high AMD1 levels for their expression during KC differentiation; however, we do not confirm whether these are direct polyamine targets. It is likely that these mRNAs represent a mixture of indirect and direct polyamine targets. Changes in polyamine levels can promote histone methylation and acetylation epigenetic changes (Arruabarrena-Aristorena et al., 2018; Hobbs and Gilmour, 2000; Murray-Stewart et al., 2014; Wei et al., 2007), in addition to changes in DNA and RNA structure and function (Igarashi and Kashiwagi, 2019, 2015; Lightfoot and Hall, 2014). It is likely that on epidermal differentiation, polyamines function to modulate gene expression at the epigenetic, transcription, and RNA stability levels. Additional controls at the levels of
**Figure 5.** Validation of AMD1-sensitive genes. (a) QRT-PCR showing the gene expression changes of AMD1-sensitive genes on epidermal differentiation. Keratinocytes were differentiated for 6 d and were treated with or without EGBG. Samples rescued with Spd and/or Spm are also shown. Gene expression was normalized to the housekeeping gene *RPL13A*. Results are presented as the means ± SEM of four independent biological replicates, and *P*-value < 0.05, **P** < 0.01, and ***P** < 0.001 were considered statistically significant. (b) Western blot analysis showing the protein changes of AMD1-sensitive genes on epidermal differentiation. Keratinocytes were treated as in a, and antibodies to IVL, KLF4, KLK7, AQP3, S100A8/9, and NOTCH1 were used. GAPDH was used as a loading control for all blots. d, day; EGBG, ethylglyoxal bis(guanylhydrazone); IVL, involucrin; KLK, kallikrein; QRT-PCR, quantitative real-time reverse transcriptase-PCR; Spd, spermidine; Spm, spermine; SS, spermidine/spermine.
translation likely exist through the modulation of RNA structure (Igarashi and Kashiwagi, 2019).

Increased ODC1 and Put levels have been linked to increased tumorigenesis in mice, whereas increased AMD1 has been shown to inhibit tumor formation. Increased polyamine catabolic enzyme SAT1 leads to increased Put levels and aberrant barrier formation (Gilmour, 2007; Nowotarski et al., 2015; Pietilä et al., 2005; Shi et al., 2012). These results highlight the complexity of polyamine regulation within the epidermis. Our data show that AMD1 upregulation in normal human skin during differentiation drives a change in polyamine ratios that is required for normal skin barrier formation. We suggest that high Put and low Spm levels are present in the basal proliferative cells of the epidermis. On differentiation, this ratio shifts, such that Put is decreased and Spm is increased as a result of increased AMD1, and this drives KC differentiation. Our data suggest that maintaining this balance among polyamines is essential to maintain the balance between proliferation and differentiation in the epidermis.
We have reported previously that AMD1 protein is rapidly upregulated on wounding and that this upregulation is required for cell migration (Lim et al., 2018). In this study, we report that on epidermal differentiation, AMD1 is also upregulated. We propose that the upregulation of AMD1 alone is not sufficient to induce cells to migrate or differentiate and that the consequence of AMD1 upregulation and modulation of polyamine levels will be dependent on the cellular context at the time. The majority of polyamines in the cell are bound to RNA, and so it is likely that they mediate their effect on cellular behavior at least in part through the modulation of RNA stability or translation. As such, they would mediate their effect through the RNAs that are present in the cell. This would explain why AMD1 upregulation plays a different role in KC differentiation from the role in KC migration on wounding.

The findings presented in this paper have important implications in the context of skin disease and aging where polyamine levels are altered (Nishimura et al., 2006; Rittié and Fisher, 2015). A further understanding of how AMD1 and the polyamines are misregulated in these skin conditions could provide new targets for the development of more effective strategies to treat aging and hyperproliferative skin disorders.

MATERIALS AND METHODS

Cell culture

The N/TERT-1 human immortalized KC cell line was obtained from James Rheinwald (Dickson et al., 2000). N/TERT-1 cells were maintained and differentiated as previously described (Lim et al., 2018; Rheinwald et al., 2002). AMD1 inhibitor, EGBG, was used at concentrations of 5, 10, 25, or 50 μM and was added to the cells on day 0 or day 3 of KC differentiation. Spd and Spm (Sigma-Aldrich, St. Louis, MO) were used at 1 mM or 100 μM for cultured KCs and organotypic cultures, respectively. shAMD1 knockdown lines were generated previously (Lim et al., 2018).

Human tissue sectioning, immunofluorescence staining, and RNAscope

Human abdominal skin biopsies were sourced from the National University Hospital, Singapore. Written, informed consent was obtained from all the patients, and all experiments were performed in accordance with the declaration of Helsinki and were approved by the Singapore National Healthcare Group ethics review board. Frozen skin sections were stained with anti-AMD1 antibody and imaged as previously described (Lim et al., 2018). For the RNAscope analysis, formalin-fixed, paraffin-embedded skin samples were sectioned and stained using AMD1 and control probes with the RNAscope kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturers’ instructions.

Organotypic skin equivalents and immunofluorescence

A human epidermis model (EPI-200-3S; MatTek Life Sciences, Ashland, MA) was used following the manufacturer’s instructions. Cultures were treated with 10 or 50 μM EGBG and/or 100 μM Spd and/or Spm on day 2, day 3, and day 5 of an airlift. Tissues were harvested on day 7 after the airlift, fixed in formalin for 4 hours, and then processed into wax blocks. Tissue sections were stained with H&E. Immunofluorescence was performed with anti-FLG, anti-LOR, anti-K10 (Abcam, Cambridge, United Kingdom), or anti-Ki67 (Agilent-Dako, Santa Clara, CA) antibodies followed by AlexaFluor 488/568 (Invitrogen, Carlsbad, CA) before imaging.

Western blotting and quantitative real-time reverse transcriptase–PCR

Protein was run on SDS-PAGE gels as described (Lim et al., 2018). Antibodies targeting K10, IVL, FLG, LOR, AMD1, GAPDH, and β-actin were used for western blotting. RNA was harvested with TRIzol, purified on RNeasy columns, and reverse transcribed, and quantitative real-time reverse transcriptase–PCR was performed using Luminaris Colour HiGreen Hi ROX (Thermo Fisher Scientific) master mix with gene-specific primers.

Measurement of polyamine levels

Polyamine measurements were made by HPLC, as previously described (Igarashi et al., 1986). Polyamine levels are presented as the ratios of the total Put, Spd, and Spm content.

Microarray analysis

Biotin-labeled cRNA (from 500 ng of total RNA) was hybridized onto the HumanHT-12 v4 Expression BeadChip (Illumina, San Diego, CA). Raw expression data were obtained using the GenomeStudio Software and were then further normalized across all the samples with cross-correlation method (Chua et al., 2006).

Statistical and bioinformatic analyses

Statistical comparisons were performed using the two-tailed Student’s t-test between two groups or two-way ANOVA test within multiple groups, followed by Benjamini–Hochberg’s posthoc test. All data were obtained from at least three independent biological replicates, and the values are represented as the means ± SEM. *P-values < 0.05, **P < 0.01, and ***P < 0.001 were considered to be statistically significant.

A detailed description of materials and methods is provided in the Supplementary Materials and Methods.

Data availability statement

Datasets related to this article can be found at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164881, hosted at National Center for Biotechnology Information Gene Expression Omnibus (accession number GSE164881).

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: LAV; Formal Analysis: LAV, ABR, CYRT, TU, JHS, HKL, SB, KI, LJ, HY; Funding Acquisition: LAV, RP, SB, KI, HY; Investigation: ABR, HKL, AB Rahim et al.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2021.01.039

REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Cell culture
The N/TERT-1 human immortalized keratinocyte (KC) cell line was obtained from James Rheinwald (Dickson et al., 2000). N/TERT-1 cells were maintained in KC serum-free media (Gibco, Life Technologies, Carlsbad, CA), supplemented with 25 μg/ml bovine pituitary extract, 0.2 ng/ml EGF, and 0.3 mM calcium chloride at ~50–60% confluency. Differentiation was induced at high confluence by switching to high calcium (1.2 mM calcium chloride) media, as previously described (Rheinwald et al., 2002). The AMD1 inhibitor, ethglyoxyal bis(guanylhydrazone) (EGBG), was used at concentrations of 5, 10, 25, or 50 μM and was added to the cells on day 0 or day 3 of KC differentiation. Spermidine (Spd) and spermine (Spm) (Sigma-Aldrich, St. Louis, MO) were used at 1 mM or 100 μM for cultured KCs and organotypic cultures, respectively. Short hairpin AMD1 knockdown lines were generated as previously described (Lim et al., 2018). Cell viability after EGBG or Spd and/or Spm treatment was determined using the MTS One Solution Assay (Promega, Madison, Wisconsin). Cell proliferation assays after EGBG treatment during differentiation were performed by plating an equal number of cells and assessing the cell number on 3 and 6 days after treatment (n = 3).

Human tissue sectioning, immunofluorescence staining, and RNAScope
Human abdominal skin biopsies were sourced from the National University Hospital, Singapore. Written, informed consent was obtained from all the patients, and all experiments were performed in accordance with the declaration of Helsinki and were approved by a local scientific ethics review board. Freshly obtained skin was dissected and snap frozen in Tissue-Tek optimal cutting temperature formulation (Sakura Finetek USA, Torrance, CA). Skin biopsies were sectioned at 7 μm thickness and incubated with a mouse monoclonal anti-AMD1 antibody (sc-390073; Santa Cruz Biotechnology, Dallas, TX) as previously described (Lim et al., 2018). Immunofluorescence images were captured under a Zeiss Axio Imager GU microscope (Carl Zeiss, Cambridge, United Kingdom). AMD1 protein expression level was validated in six patient samples, and RNA expression level was validated in three patient samples. All patient samples were from different donors. For the RNAScope analysis, formalin-fixed, paraffin-embedded skin samples were sectioned and stained using the AMD1 and control probes with the RNAScope kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s instructions.

Organotypic skin equivalents and immunofluorescence
A human epidermis model (EPI-200-3S; MatTek Life Sciences, Ashland, MA) derived from newborn foreskin was used to assay the effects of EGBG ± Spm and/or Spd on three-dimensional organotypic models. Briefly, submerged KCs cultured on the inserts were air lifted and stratified to full thickness according to the manufacturer’s instructions in a humidified atmosphere with 5% carbon dioxide at 37 °C. EPI-200-3S cultures were treated with 10 or 50 μM EGBG and/or 100 μM Spd and/or Spm on day 2, day 3, and day 5 of the airlift. Tissues were harvested on day 7 after the airlift period. Tissues were fixed in 10% neutral buffered formalin for 4 hours and were then processed into wax blocks before further histological analyses. To evaluate the morphological structure of the three-dimensional organotypic models, 5-μm-thick sections were deparaffinized and stained with H&E. Paraffin samples were dewaxed in xylene and rehydrated through a descending ethanol to water series (3 minutes per solution). Epitope retrieval was performed at 121 °C for 10 minutes using Citrate Buffer, pH 6.0, Antigen Retriever (Electron Microscopy Sciences, Hatfield, PA), and the slides were cooled before three washes in 0.05% Tween 20 and/or PBS. The sections were then incubated with 10% goat serum (Dako, Glostrup, Denmark) in PBS for 30 minutes before exposure to anti-FLG (1:200; Abcam, Cambridge, United Kingdom), anti-iorcin (1:500, ab176322), anti-keratin 10 (1:400, ab76318), or anti-Ki67 (1:200, M7240; Agilent-Dako, Santa Clara, CA) for 1 hour. Samples were thoroughly washed with water for 10 minutes and 0.05% Tween 20 and/or PBS for 5 minutes followed by 30-minute incubation with goat antirabbit and/or mouse IgG (H+L) AlexaFluor 488/568 (1:1,000; Invitrogen, Carlsbad, CA). Then, the samples were washed in water and 0.05% Tween 20 and/or PBS before incubation with 1 μg/ml DAPI (Sigma-Aldrich) for 10 minutes. Finally, the sections were rinsed in water before mounting using Hydromount (Electron Microscopy Sciences). All images were acquired using a Zeiss Axio Imager Z1 upright motorized microscope attached to an AxioCam MRc5 digital camera (Carl Zeiss) and Zen Imaging software (Carl Zeiss).

Western blotting
Cells were lysed in RIPA buffer, and 30–90 μg of protein was run on 10% or 4–20% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA) as previously described (Lim et al., 2018). Antibodies targeting keratin 10 (ab76318; Abcam), involucrin (ab53112; Abcam), FLG (ab17808; Abcam), loricrin (ab176322; Abcam), AMD1 (sc-390073; Santa Cruz Biotechnology), GAPDH, and β-actin were used. Membranes were incubated with horseradish peroxidase–labeled antimouse or antirabbit IgG (Santa Cruz Biotechnology) secondary antibodies and were developed using enhanced chemiluminescent substrate (Thermo Fisher Scientific).

Measurement of polyamine levels
Polyamine measurements were made by HPLC, as previously described (Igarashi et al., 1986). Polyamine levels are presented as the ratios of the total putrescine, spermidine, and spermine content.

Quantitative real-time reverse transcriptase–PCR
RNA was harvested from cultured KCs with TRIzol (Life Technologies) and purified on RNeasy columns (Qiagen, Hilden, Germany) before being reverse transcribed to cDNA (RevertAid cDNA synthesis kit, Thermo Fisher Scientific). Quantitative real-time reverse transcriptase–PCR was performed on an ABI PRISM 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific) with transcript-specific primers and Luminaris Colour HiGreen Hi ROX (Thermo Fisher Scientific) master mix. Primers were either purchased from Integrated DNA Technologies (Coralville, IA) or designed and validated. CT values were normalized to RPL13A.
**Microarray analysis**

Total RNA (500 ng) was reverse transcribed using ArrayScript Reverse Transcriptase (Illumina, San Diego, CA) with T7 oligo primer for 2 hours at 42 °C. This was followed by an in vitro transcription of cDNA to biotin-labeled cRNA by T7 RNA polymerase in the presence of biotinylated ribonucleotides (Enzo Life Sciences, Farmingdale, NY) at 37 °C for 4 hours using the Illumina TotalPrep RNA Amplification kit (Applied Biosystems, Foster City, CA). cRNA (750 ng) was hybridized to HumanHT-12 v4 Expression BeadChip (Illumina) for 16 hours at 58 °C. Arrays were then washed and stained with Streptavidin-Cy3 (GE Healthcare, Chalfont Saint Giles, United Kingdom), followed by scanning using Illumina BeadArray Reader (Illumina) at 25 scan factor 1. Raw expression data were obtained using the GenomeStudio Software and were then further normalized across all samples with the cross-correlation method (Chua et al., 2006). The normalized data were log₂ transformed before identification of differential expression. Using the day 6 (untreated) data as control, differential expression was performed with the following cut-off values: fold change of 2 and \( P \)-value of 0.05. Before heatmap generation, log₂ transformed and normalized data were subtracted by the mean values of all untreated replicates on day 6. Heatmaps were generated using hierarchical clustering. Gene ontology pathway analysis was performed on the basis of differentially expressed genes using DAVID (https://david.ncifcrf.gov/).

**SUPPLEMENTARY REFERENCES**


Supplementary Figure 1. Specific AMD1 expression in human abdominal skin sections. (a) Immunofluorescence showing AMD1 expression in the suprabasal layers of the human epidermis in the absence and presence of an AMD1 blocking peptide. In the presence of the blocking peptide the AMD1 signal is no longer detectable confirming the specificity of this antibody for AMD1. Dotted line represents the basement membrane, scale bar = 50 μm. (b) Left panel: Polysome profiles of day 0 (D0), undifferentiated (blue) and D6, differentiated (red) N/TERT-1 keratinocytes. Fractions 1-4 are nontranslated, fractions 5-11 are translated polysomal fractions. Right Panels: QRT-PCR data showing the percentage mRNA abundance of AMD1 across 12 fractions. TBP is shown as a control. AMD1, adenosyl methionine decarboxylase 1; QRT-PCR, quantitative real-time reverse transcriptase-PCR.
**Supplementary Figure 2. AMD1 is required for keratinocyte differentiation.** (a) mRNA and (b) protein levels of AMD1 in shAMD1-4 keratinocytes shows a significant 80% knockdown throughout the course of keratinocyte differentiation. (c) Protein expression of differentiation makers, FLG and LOR were reduced with AMD1 knockdown and rescued with the supplementation of 1mM Spd/Spm. Either GAPDH or β-actin was used as a loading control. shAMD1-4 knock down keratinocytes had a milder differentiation phenotype than shAMD1-3 likely due to the decreased efficiency of AMD1 protein knockdown. (d) Cell count during keratinocyte differentiation with shScram and shAMD1 knockdown keratinocytes. No significant difference in cell number was seen at day 3 (D3) and day 6 (D6) of monolayer keratinocyte differentiation using shScram and shAMD1 knockdown cells. (e) HPLC data showing the levels of Put, Spd and Spm in undifferentiated and differentiated shScram and shAMD1 keratinocyte lines. Polyamine levels are presented as nmol/mg of protein. Note, differentiated N/TERT-1 keratinocytes are larger have significantly more protein per cell than undifferentiated keratinocytes. All results are represented as the means ± SEM of three independent biological replicates. ***P < 0.001. AMD1, adenosyl methionine decarboxylase 1; LOR, loricrin; shScram, scrambled short hairpin RNA; spd, spermidine; spm, spermine.
Supplementary Figure 3. N/TERT-1 cell viability assay using Spd/Spm and AMD1 inhibitor, EGBG. (a) MTS assay showing the tolerable range of Spd/Spm for N/TERT-1 keratinocytes after 48 hours of treatment. (b) Graph depicting the cell viability of N/TERT-1 keratinocytes cultured at increasing concentrations of AMD1 inhibitor, EGBG for 48 hours. Results are represented as ± SEM of three independent biological replicates. (c) HPLC data showing the levels of Put, Spd and Spm in undifferentiated and differentiated keratinocytes with and without addition of the AMD1 inhibitor EGBG. Polyamine levels are presented as nmol/mg of protein. Note differentiated N/TERT-1 keratinocytes are larger have significantly more protein per cell than undifferentiated keratinocytes. (d) K10 (Red) and Ki67 (Green) staining of reconstructed human skin equivalent models treated with and without AMD1 inhibitor EGBG (10 and 50 μM) and rescued with Spd and Spm; (n = 3). Scale bar = 50 μM. AMD1, adenosyl methionine decarboxylase 1; EGBG, ethylyglyoxal bisguanylhydrazone; spd, spermidine; spm, spermine.
Supplementary Figure 4. AMD1 inhibition from D0 post-confluence perturbs keratinocyte differentiation and is rescued by the addition of 1-mM Spd/Spm. (a) mRNA and (b) protein expression levels of early differentiation marker K10, and late differentiation markers IVL and FLG decreases with the addition of EGBG at the start of differentiation and are rescued by the addition of 1mM Spd/Spm. Results are represented as the means ± SEM of three independent biological replicates, *P < 0.05, **P < 0.01, and ***P < 0.001. AMD1, adenosyl methionine decarboxylase 1; EGBG, ethylglyoxal bisguanlyhydrzone; IVL, involucrin; spd, spermidine; spm, spermine.

Supplementary Figure 5. Gene ontology of AMD1-sensitive down-regulated genes.