

RESEARCH ARTICLE

Forkhead Box C1 Regulates Human Primary Keratinocyte Terminal Differentiation

Lianghua Bin^{1,2*}, Liehua Deng¹, Hengwen Yang¹, Leqing Zhu¹, Xiao Wang¹, Michael G. Edwards³, Brittany Richers², Donald Y. M. Leung^{2,4}

1 The First Affiliated Hospital, Biomedical Translational Research Institute, the International Immunology Center and the Key Laboratory of Antibody Engineering of Guangdong Province, Jinan University, Guangzhou, Guangdong Province, China, **2** Department of Pediatrics, National Jewish Health, Denver, Colorado, United States of America, **3** Division of Pulmonary Sciences and Critical Care Medicine, Department of Medicine, University of Colorado Denver, Aurora, CO, United States of America, **4** Guangdong Provincial Key Laboratory of Allergy & Clinical Immunology, The State Key Clinical Specialty in Allergy, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong Province, China

* Binl@njhealth.org



OPEN ACCESS

Citation: Bin L, Deng L, Yang H, Zhu L, Wang X, Edwards MG, et al. (2016) Forkhead Box C1 Regulates Human Primary Keratinocyte Terminal Differentiation. *PLoS ONE* 11(12): e0167392. doi:10.1371/journal.pone.0167392

Editor: Tsutomu Kume, Feinberg Cardiovascular Research Institute, Northwestern University, UNITED STATES

Received: April 11, 2016

Accepted: November 14, 2016

Published: December 1, 2016

Copyright: © 2016 Bin et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The RNA-seq data of this manuscript are held in NCBI Gene Expression Omnibus (Access number: GSE73305 and GSE76647).

Funding: This work was supported by the National Natural Science Foundation of China (No. 81371718)(LB); NIH grants R01 AR41256 (DL); Overall Atopic Dermatitis Research Network (NIH/NIAID 1U19 AI 117673-01). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

Abstract

The epidermis serves as a critical protective barrier between the internal and external environment of the human body. Its remarkable barrier function is established through the keratinocyte (KC) terminal differentiation program. The transcription factors specifically regulating terminal differentiation remain largely unknown. Using a RNA-sequencing (RNA-seq) profiling approach, we found that forkhead box c 1 (FOXC1) was significantly up-regulated in human normal primary KC during the course of differentiation. This observation was validated in human normal primary KC from several different donors and human skin biopsies. Silencing FOXC1 in human normal primary KC undergoing differentiation led to significant down-regulation of late terminal differentiation genes markers including epidermal differentiation complex genes, keratinization genes, sphingolipid/ceramide metabolic process genes and epidermal specific cell-cell adhesion genes. We further demonstrated that FOXC1 works down-stream of ZNF750 and KLF4, and upstream of GRHL3. Thus, this study defines FOXC1 as a regulator specific for KC terminal differentiation and establishes its potential position in the genetic regulatory network.

Introduction

Epidermal keratinocyte (KC) terminal differentiation is essential for the acquisition of the normal skin barrier function and homeostasis [1]. Aberrant barrier function is associated with various skin diseases, including atopic dermatitis, psoriasis, ichthyosis vulgaris, etc [2,3]. Several transcription factors have been identified to regulate the late differentiation and barrier formation, including KLF4, ZNF750, GATA3, GRHL3, and PRDM1 (Blimp-1)[4–8]. Recently, two additional transcription factors, MAF and MAFB, were identified as important KC terminal differentiation regulators [9]. MAF and MAFB working together to regulate KLF4, ZNF750, GRHL3 and PRDM1. These studies suggest there is a regulatory network of

Competing Interests: Lianghua Bin is a member of the advisory committee for Astellas Pharma Inc. The authors declare no financial conflict of interest. This does not alter our adherence to PLOS ONE policies on sharing data and materials.

Abbreviations: ChIP, Chromatin immunoprecipitation; DSC1, Desmocollin 1; FLG, Filaggrin; FOXC1, Forkhead box C1; IVL, Involucrin; KC, Keratinocytes; KLK, Kallikrein; KRT, Keratin; LCE, Late cornified envelope; LOR, Loricrin; RNA-seq, RNA-sequencing; RPKM, Reads Per Kilobase of transcript per Million mapped reads; siRNA, Small interference RNA; UD, Un-differentiated keratinocytes.

transcription factors involved in KC terminal differentiation. Due to the high complexity of this regulatory program, it is likely that additional important regulators in this process remain to be identified.

Through an exploratory gene profiling analyses of human normal primary KC during the progression of calcium-induced differentiation, we found that Forkhead box C1 (FOXC1) is significantly up-regulated in differentiated KC (GSE 73305). *FOXC1* is a member of the forkhead box transcription factors. These proteins are distinguished by a characteristic 100-amino acid DNA-binding motif that is highly homologous to *Drosophila* forkhead genes. FOX proteins are critical regulators in the development of tissue differentiation, embryogenesis and tumorigenesis [10]. Here, in this study, we first validate the discovery from our gene profiling analyses that FOXC1 is induced in differentiated KC in cultured human primary KC and human skin biopsies. We further demonstrate that FOXC1 is necessary for human KC terminal differentiation through inhibiting FOXC1 gene expression and FOXC1 over-expression experiments. In the KC terminal differentiation regulatory network, FOXC1 is a target gene of ZNF750 and KLF4, and works up-stream of GRHL3.

Results

FOXC1 is up-regulated in differentiated human primary KC

To discover novel effectors for human KC differentiation, we profiled gene expression of undifferentiated human KC and differentiated KC under Ca^{2+} -induced differentiation over a 5-day time course (S1 Appendix). The gene profile data have been deposited in the NCBI Gene Expression Omnibus (access number: GSE 73305). We searched for previously uncharacterized transcription factors that were significantly up-regulated during the course of KC differentiation. This effort led us to find that FOXC1 is significantly up-regulated during the progression of KC differentiation (S1 Table).

We first validated the results by real-time PCR and western blots using human primary keratinocytes from different human donors. The cells were differentiated by elevation of calcium concentration in culture media up to 5 days. As shown in Fig 1A, the mRNA of FOXC1 was significantly up-regulated during the course of KC differentiation, and its protein expression was also gradually increased in differentiated KC (Fig 1B). A recent paper reported that FOXC1 is expressed in mouse hair follicles but absent in mouse epidermis [11]. In our study, however, we find that FOXC1 is expressed not only in human hair follicles, but also in the differentiated KC of inner hair root sheath that surrounds the hair shaft and the epidermis upper layers of KC (Fig 1C). These data provide *in vivo* evidence that FOXC1 is associated with KC differentiation in humans.

Up-regulation of FOXC1 is necessary for KC terminal differentiation

To test for a potential functional role for FOXC1 in KC terminal differentiation, FOXC1 siRNA (FOXC1i) and scrambled siRNA (Control) duplexes were transfected into undifferentiated KC. The cells were then allowed to undergo Ca^{2+} -induced differentiation. Although the early differentiation markers keratin 1 and 10 were unaffected (data not shown), silencing FOXC1 resulted in reduced induction of late differentiation genes, including filaggrin (FLG), involucrin (IVL), and loricrin (LOR) in both mRNA and protein levels (Fig 2A & 2B). However, loss of FOXC1 did not change the protein expression of desmocollin 1 (DSC1), a desmosome cell-cell adhesion protein (Fig 2B). Inhibition of FOXC1 gene expression did not change cyclin D protein levels as compared to the control cells in Ca^{2+} -driven differentiated KC. We also examined and compared the cell cycle parameters of undifferentiated KC transfected with scrambled siRNA and FOXC1 siRNA, and found no difference between normal cells and

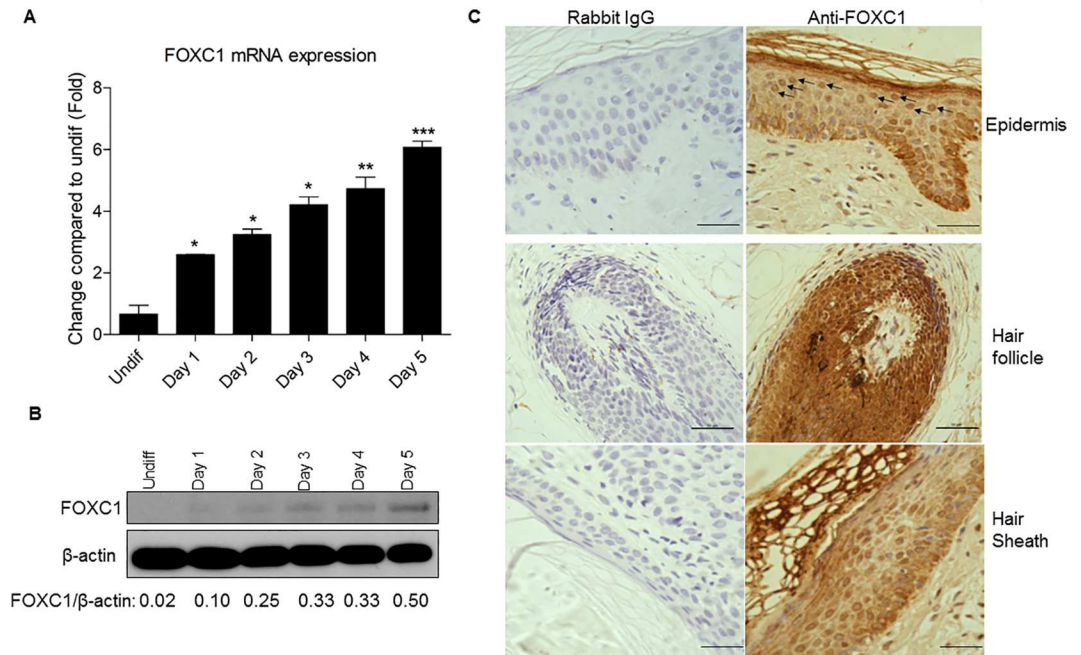


Fig 1. FOXC1 is induced in differentiated keratinocytes. A) mRNA levels of FOXC1 is increasing during the course of KC differentiation. Data presented as mean \pm SEB. “undiff” is the abbreviation of undifferentiated KC. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. The data are representative of four independent experiments. B) FOXC1 protein is increasing along with KC differentiation degree. The data represent one of the three independent experiments. C) Immunohistochemistry staining of FOXC1 protein expression in human epidermis, hair follicle and the differentiated inner hair root sheath. FOXC1 positive staining is presented as brown color in cell nuclear (Scale bar, 50 μ m). The arrows point to examples of typical positive staining.

doi:10.1371/journal.pone.0167392.g001

FOXC1 deficient KC (S1 Fig). These data suggest that FOXC1 doesn’t change the cell cycle of undifferentiated and differentiated KC. We also over-expressed FOXC1 in proliferative undifferentiated KC. In contrast to loss of expression, over-expression of FOXC1 led to increased mRNA levels of FLG, LOR and IVL in the absence of Ca^{2+} induction (Fig 3). Due to technical difficulty of growing primary human KC on glass cover slides for immunostaining, we couldn’t test the protein expression of FLG, LOR and IVL in the same cells with FOXC1 over-expression. Nevertheless, these data along with FOXC1 positive staining in human epidermis upper layer KC demonstrate that FOXC1 plays a role in KC terminal differentiation.

Transcriptome Analysis of FOXC1-regulated Genes

To identify the specific FOXC1 targeting genes among the global gene sets identified in KC differentiation, we profiled FOXC1 silenced KC versus control using a RNA-seq approach. We plotted the transcriptional data in three-dimensional space using principal component analysis (PCA) as implemented by Partek Genomics Suite using a standard correlation method, observing clear separation of FOXC1 silenced KC and control KC after differentiation (Fig 4A). In contrast, loss of FOXC1 expression in undifferentiated KC did not separate significantly from control cells.

Down-regulated genes in FOXC1 siRNA treated cells represent targets that are positively regulated by FOXC1. Among the top 50 most significantly differentially expressed genes across the four experimental groups (False discovery rate < 0.01), 42 genes were down-regulated in FOXC1 silenced differentiated KC (Fig 4B), including KC terminal differentiation markers of

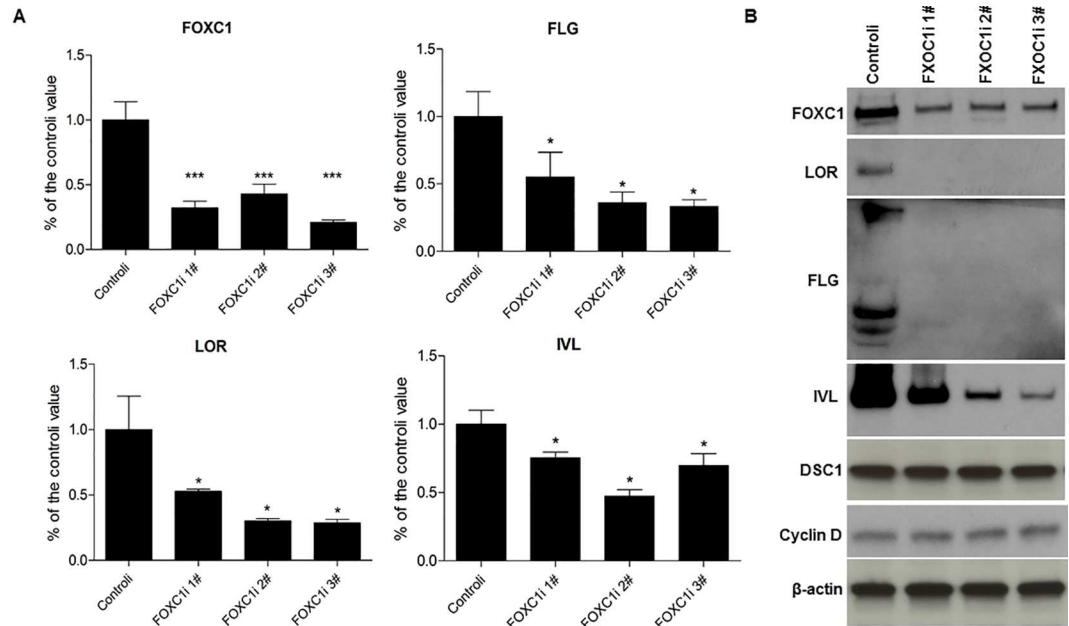


Fig 2. Silencing FOXC1 leads to decreased gene expression of KC differentiation markers. A) mRNA expression of FLG, IVL and LOR in FOXC1 silenced differentiated KC and control cells evaluated by real-time PCR. Data represented as mean \pm SEB. The data are representative of four independent experiments. B) Protein expression of FLG, IVL, LOR, DSC1 and Cyclin D in FOXC1 silenced differentiated KC and control cells evaluated by western -blot. The data represent one of the three independent experiments.

doi:10.1371/journal.pone.0167392.g002

periplakin (PPL)[12], keratinocyte proline-rich protein (KPRP)[13] and Epsin 3 (EPN3)[14], lipids synthesis genes of serine palmitoyltransferase (SPTSSB) and sphingomyelin phosphodiesterase 3 (SMPD3) and FLG maturation related protease gene caspase 14 (CASPD14)[15–17]. Additionally, the majority of genes located at epidermal differentiation complex (EDC) of 1p21 [18] were decreased in differentiated KC upon FOXC1 silencing (Table 1). Aside from the EDC genes, kallikrein genes, that are important proteases functioning in desquamation and skin barrier homeostasis [19,20], are targets positively regulated by FOXC1 (Table 1).

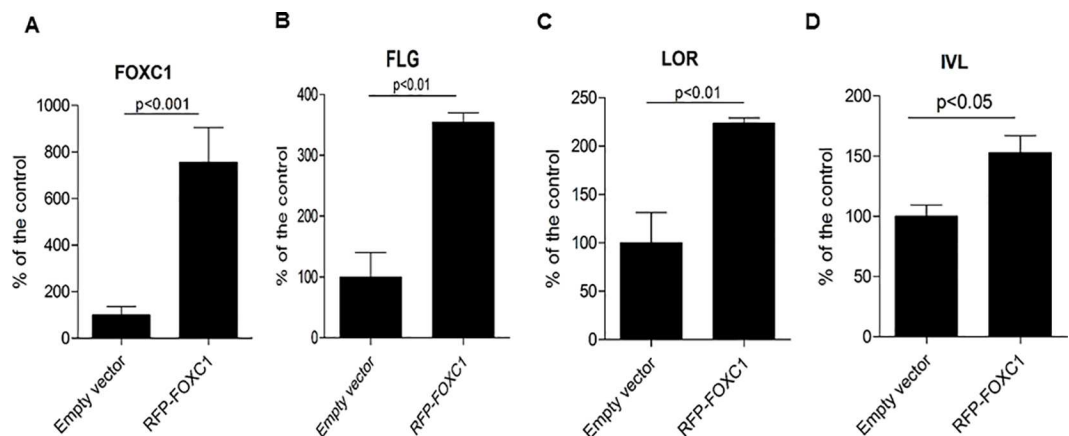


Fig 3. Over-expression of FOXC1 leads to increased gene expression of KC differentiation markers. mRNA expression of FLG, IVL and LOR in FOXC1 over-expressed KC and control cells evaluated by real-time PCR. Data represented as mean \pm SEB. The data are representative of three independent experiments.

doi:10.1371/journal.pone.0167392.g003

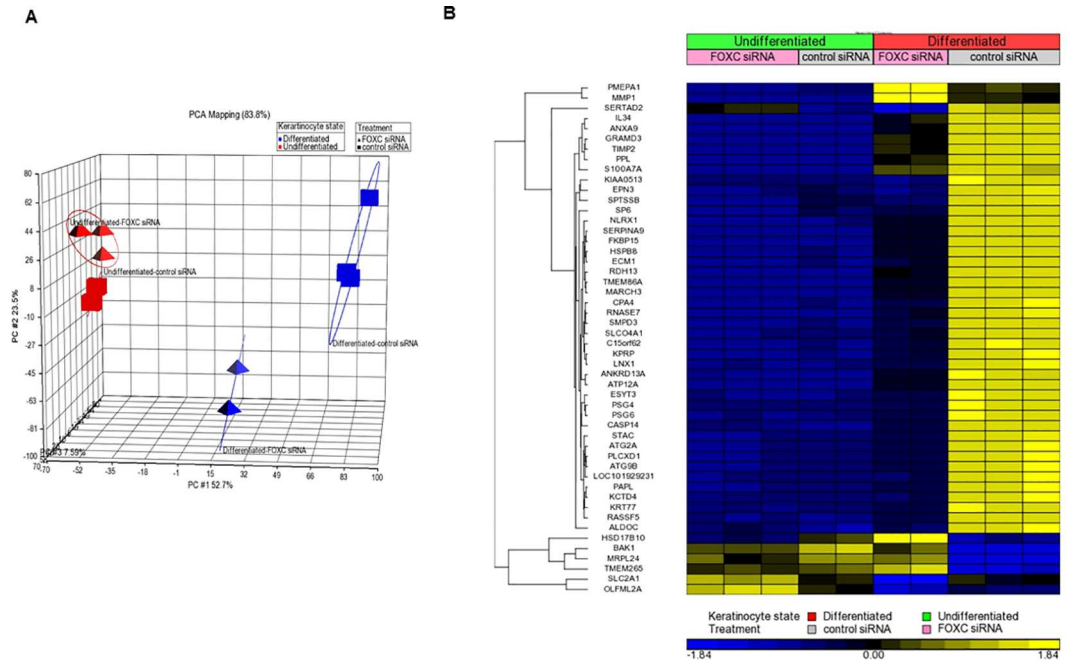


Fig 4. Transcription profiles of FOXC1 silenced KC are significantly different from control cells upon differentiation. A) A principle component assay shows separation of differentiated FOXC1 silenced KC from control cells. B) Heatmap shows the top 50 most differentially expressed genes in FOXC1 silenced undifferentiated and differentiated KC as compared to scrambled siRNA silenced undifferentiated and differentiated KC (FDR<0.01).

doi:10.1371/journal.pone.0167392.g004

Table 1. Down-regulated Keratinocytes differentiation markers in FOXC1 silenced KC.

LCE family		SPRR family		S100 fuse genes		KLK family		Lipid synthesis and transport and other gene	
Gene-Symbol	Log2 Ratio*	Gene-Symbol	Log2 Ratio*	Gene-Symbol	Log2 Ratio*	Gene-Symbol	Log2 Ratio*	Gene-Symbol	Log2 Ratio*
LCE1A	-2.21	SPRR2G	-1.25	FLG	-3.43	KLK10	-1.09	ABCA12	-1.25
LCE1B	-2.94	SPRR3	-1.70	FLG2	-4.70	KLK12	-0.72	ACER1	-1.33
LCE1C	-2.52	SPRR4	-2.93	TCHH	-0.86	KLK13	-1.30	ACER2	-1.73
LCE1D	-2.35			TCHHL1	-2.84	KLK14	-1.07	SMPD3	-2.28
LCE1E	-2.41			RPTN	-2.74	KLK4	-1.32	SPTSSB	-2.50
LCE1F	-2.35			CRNN	-4.76	KLK5	-0.95		
LCE2A	-1.58			HRNR	-1.47	KLK6	-1.80		
LCE2B	-2.05					KLK7	-0.90		
LCE2C	-1.57					KLK9	-3.80		
LCE2D	-2.04								
LCE3C	-3.65								
LCE3D	-1.70								
LCE3E	-2.10								
LCE5A	-2.27								
LCE6A	-2.60								

*Gene expression ratio in FOXC1 silenced differentiated KC versus in Scrambled siRNA silenced differentiated KC.

doi:10.1371/journal.pone.0167392.t001

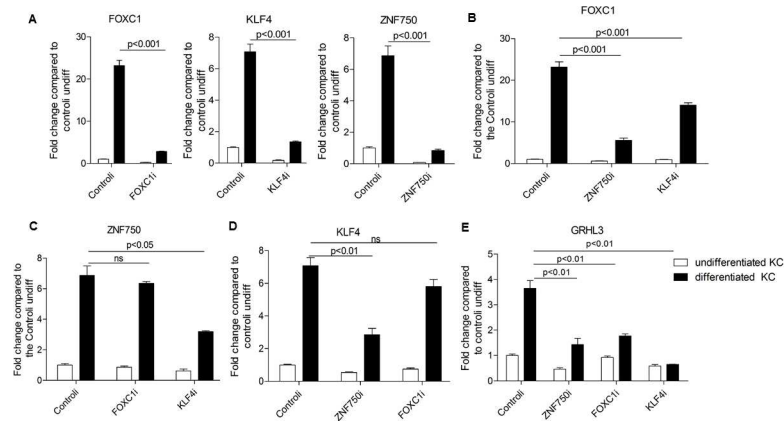


Fig 5. FOXC1 acts down-stream of ZNF750 and KLF4, and up-stream of GRHL3. A) FOXC1 expression in ZNF750 and KLF4 silenced KC. B) ZNF750 expression in FOXC1 and KLF4 silenced KC. C) KLF4 expression in ZNF750 and FOXC1 silenced KC. D) GRHL3 expression in ZNF750, KLF4 and FOXC1 silenced KC. Gene expression was evaluated by real time PCR. Data represented as mean \pm SEB. The data are representative of three independent experiments.

doi:10.1371/journal.pone.0167392.g005

ABCA12, a gene with function in keratinocyte lamellar body lipid transportation and its loss-of-function mutations are associated with harlequin ichthyosis [21–23], was also significantly reduced in FOXC1-silenced KC (Table 1). The identity of FOXC1-regulated genes is consistent with a crucial role for FOXC1 in promoting KC differentiation.

The hierarchy role of FOXC1 in KC regulatory network

ZNF750 and KLF4 are target genes of P63 and MAF/MAFB, and they are also essential regulators of epidermal differentiation [5,9]. To determine the hierarchy cascade of ZNF750, KLF4 and FOXC1, we knocked-down ZNF750 and KLF4 in KC progenitor cells that were then induced to differentiation. As shown in Fig 5A, FOXC1, KLF4 and ZNF750 were significantly inhibited by siRNA silencing. As compared to control cells, FOXC1 gene expression was significantly down-regulated in ZNF750 and KLF4 silenced KC (Fig 5B). In contrast, gene expression of ZNF750 and KLF4 were not affected by FOXC1 knockdown (Fig 5C & 5D). We found that ZNF750 silencing led to reduced gene expression of KLF4, reciprocally, KLF4 silencing resulted in reduced gene expression of ZNF750 (Fig 5C & 5D). Additionally, the gene expression of GRHL3 was down-regulated in ZNF750, KLF4 and FOXC1-silenced KC (Fig 5E). These results demonstrated that FOXC1 acts down-stream of ZNF750 and KLF4, up-stream of GRHL3, and ZNF750 and KLF4 reciprocally regulate each other.

To further validate whether FOXC1 is regulated by ZNF750 and KLF4 in keratinocytes, we used chromatin immune-precipitation (ChIP) to determine whether ZNF750 and KLF4 protein interact with FOXC1 promoter. We found that both ZNF750 and KLF4 bind to the promoter region of FOXC1: KLF 4 binds to two regions of -873bp to -643 bp and -663bp to -493 bp upstream of the start codon; whereas ZNF750 only binds to the -663bp to -493bp region up-stream of the FOXC1 start codon (Fig 6).

Discussion

In our current study, we defined a previously unidentified role for the transcription factor FOXC1 in regulating the differentiation gene program in KC. Through siRNA knockdown experiments and transcriptome analysis as well as over-expression experiments, plus real-time PCR and western-blot validation, we demonstrated that FOXC1 activates an array of genes

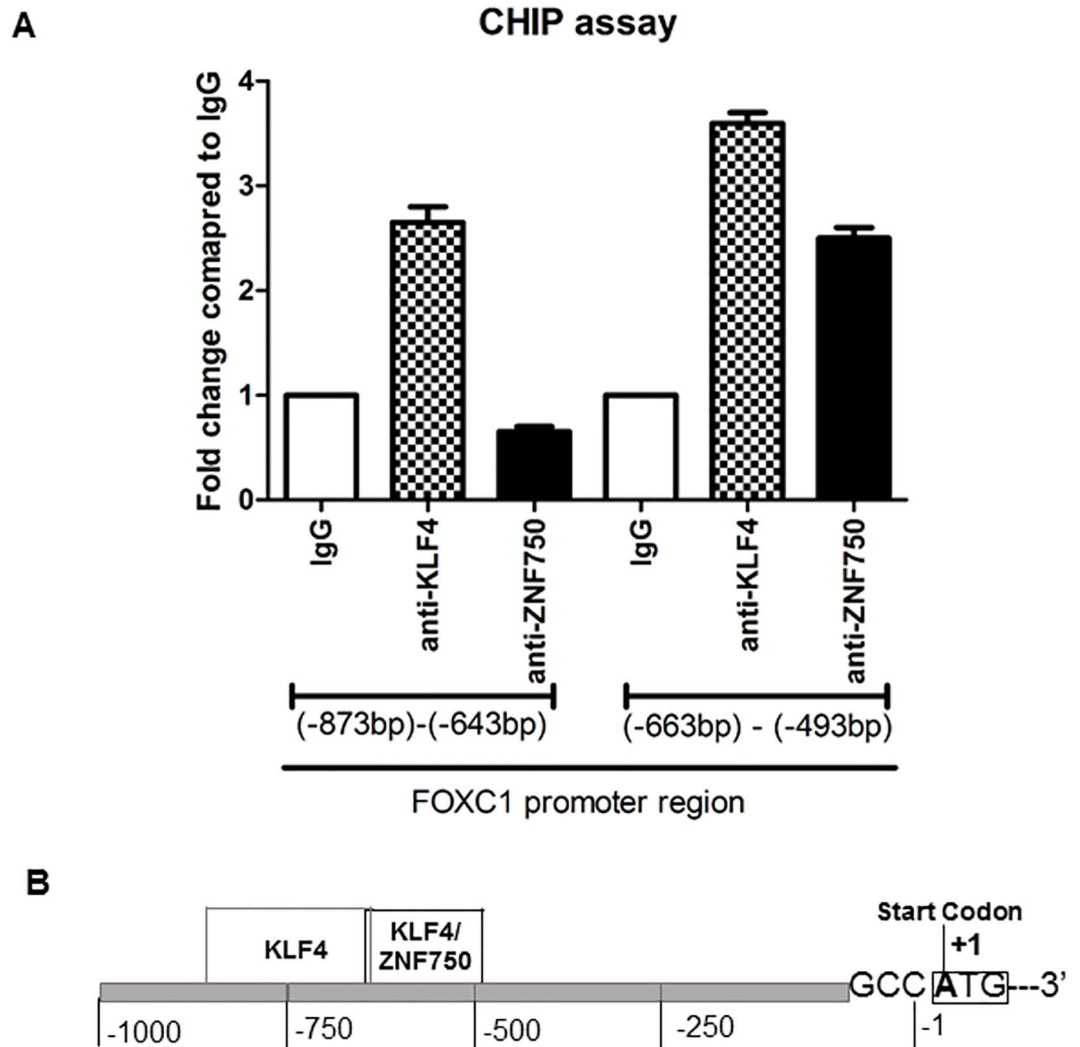


Fig 6. ZNF750 and KLF4 bind to the promoter region of FOXC1 gene. A) ChIP assays were performed using Ca^{2+} -driven differentiated keratinocytes. 9 pairs of primers covering 2 kilobase region of the upstream of FOXC1 start codon. Increased binding activity was found in the regions of -873bp to -643bp and -663bp to -493bp upstream of the start codon. Data represented as mean \pm SEB. B) The Schematic diagram of FOXC1 promoter region. KLF4 and ZNF750 binding sites are indicated.

doi:10.1371/journal.pone.0167392.g006

representing the terminal differentiation stage of human epidermal KC. Although a previous large scale multi-technique study of KC terminal differentiation mentioned the increased mRNA expression of FOXC1 in the granular layer of KC in human epidermis [24], our study is the first to demonstrate the regulatory function of FOXC1 in human somatic epidermal KC differentiation. FOXC1 knockout mice die prenatally with hydrocephalus, eye defects, and multiple skeletal abnormalities, demonstrating its critical role in the development of these tissues and organs [25]. Its function, however, in epidermal differentiation after birth has never been studied. Although FOXC1 mutations were found in human Axenfeld-Rieger syndrome which has no skin barrier disorder, the Axenfeld-Rieger syndrome is resulted from FOXC1 haploinsufficiency, which may not functionally damage enough to cause epidermal symptoms. Recently, while we were revising our submission, Cui et al published elegant work on the Journal of Investigative Dermatology reporting that specifically disrupted FOXC1 gene expression

in mouse sweat gland epithelium led to symptoms of hypohidrosis; loss of FOXC1 in mouse sweat gland epithelium led to enhanced gene expression of *Spr2a/b* and *KRT8* which formed a plug to block sweat gland secretion ducts[26]. However, similar to Lay et al's report[11], this article by Cui et al did not show FOXC1 gene expression in mouse epidermis. Comparing our data generated from human primary KC and human skin biopsies with data from Lay et al and Cui et al using murine models, we think FOXC1 may function differently in human epidermis from mouse epidermis.

Although FOXC1 over-expression is found in some types of breast cancer cells, liver cancer cells, acute myeloid leukemia, and increased FOXC1 in these cancer cells is associated with proliferation [27–29], there is no study reported FOXC1 over-expression in keratinocyte-derived cancer. A previous study found that cervical cancer derived cell line, HeLa cells, lost FOXC1 gene expression due to gene mutation. When reintroduction of FOXC1 into HeLa cells restores the cell growth inhibition [30]. The contradictory results obtained from different cancer cells and primary normal human KC suggest that FOXC1 has pleiotropic functions.

We show here the hierarchy position of FOXC1 in the transcription factor network regulating epidermal differentiation. Recently, a few studies began to study the transcriptional regulatory network for KC terminal differentiation. Transcription factor p63 is a master regulator that is required both for the development and homeostasis of stratified epithelial tissues[31]. ZNF750 is a p63 target gene. It is induced during the progression of KC differentiation, and binds to another KC terminal differentiation transcription factor KLF4. The research results from Sen et al suggested that ZNF750 worked up-stream of KLF4 [5]. They therefore concluded that ZNF750 is a p63 target gene that induces KLF4 to drive terminal epidermal differentiation. A more recent study found that KLF4, ZNF750 and GRHL3 are not the only targets of p63, but also down-stream of MAF and MAFB [9]. In our study, through multiple siRNA silencing experiments, we found that KLF4 and ZNF750 are regulating their expression reciprocally. FOXC1 is the target gene for both KLF4 and ZNF750. To validate our data, we used ChIP assay to analyze whether ZNF750 and KLF4 directly bind to the promoter region of FOXC1. Our results confirmed that ZNF750 and KLF4 indeed bind to -873bp to -643 bp and -663bp to -493 bp upstream of the start codon of FOXC1 (Fig 6). Furthermore, we define that GRHL3 is the target gene of FOXC1, therefore silencing any one of ZNF750, KLF4 and FOXC1 resulted the reduced gene expression of GRHL3. Indeed, the sets of target genes of ZNF750, KLF4, FOXC1 and GRHL3 are overlapped, supporting the assumption that these genes organize as a hierarchy network.

In summary, our work supports a previously unappreciated role for FOXC1 in promoting the human epidermal KC terminal differentiation program; it acts down-stream of ZNF750 and KLF4 and up-stream of GRHL3.

Materials and Methods

Human normal KC culture and differentiation

Primary human neonatal foreskin KC from 6 different donors were purchased from Thermo Fisher Scientific and maintained in EpiLife Medium containing 0.06 mM CaCl_2 and S7 supplemental reagent under standard tissue culture conditions. The cells were seeded in 24 well-dishes at 2×10^5 /well to form a confluent monolayer. The following day, the cells were subjected to differentiation by increasing CaCl_2 to 1.3mM in the culture media. The cells were harvested for RNA extraction before differentiation, differentiation for 24 hours (D1), 48 hours (D2), 72 hours (D3), 96 hours (D4) and 120 hours (D5). Then RNA and protein were prepared from the cells.

siRNA knockdown gene expression

FOXC1 siRNA duplexes, ZNF750 siRNA duplexes, KLF4 siRNA and control non-targeting scrambled siRNA duplexes were purchased from Life Technologies. The sequence for KLF4 siRNA are: sense: UGACCAGGCACUACCGUAAtt; antisense: UUACGGUAGUGCCUG GUCAtt. The sequence for ZNF750 siRNA are: sense: CCUCA AUGUUGUGAACGGAtt; antisense: UCCGUUCACAACA AUUGAGGct. The sequence for FOXC1 siRNA are: sense 1: GAAAGUCGCUUUCUUUUUAtt; antisense: UAAAAAGAAAGCGACUUUCat. Sense 2: ACUCUCCAGUGAACGGGAAtt, antisense: UCCCCGUUCACUGGAGAGUtg; Sense 3: AGAGGAUCGGCUUGAACAAAtt; anti-sense: UUGUUCAAGCCGAUCCUCUgt. KCs were plated in 24 well plates at 1×10^5 per well the day before transfection. Cells were transfected with siRNA duplexes at final concentration of 10 nM using lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). After 24 hours incubation, the cells were replaced with EpiLife supplemented either with 0.06mM or 1.3 mM calcium for up to 120 hours. Then the cells were used for total RNA and protein extraction.

Total RNA extraction and real-time PCR

Total RNA was extracted using RNeasy mini kit according to the manufacture's guideline (QIAGEN, MD). RNA was then reverse transcribed into cDNA using superScript® III reverse transcriptase from Invitrogen (Portland, OR) and analyzed by real time RT-PCR using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA) as previously described (Nomura *et al*, 2003). Primers and probes for human *FOXC1* (Hs00559473_s1), *FLG* (Hs00856927_g1), *IVL* (Hs00846307_s1), *LOR* (Hs01894962_s1), *ZNF750* (Hs01563263_g1), *KLF4* (Hs00358836_m1), *GRHL3* (Hs00297962_m1) and *18S* (Hs99999901_s1) were purchased from Applied Biosystems (Foster City, CA). Quantities of all target genes in test samples were normalized to the corresponding 18S levels.

Western-blot protein expression

Cells were lysed in protein lysis buffer (20mM Tris-HCl pH7.4, 150 mM NaCl, 1mM EDTA, 1% Triton-X100) supplemented with protease inhibitors. After collecting the aqueous soluble protein, the un-soluble portion were solubilized in 6M urea supplemented with proteinase inhibitors. The proteins were then used for western-blot assay following the standard procedure. The antibodies against human FOXC1, IVL, LOR, FLG, DSC1 and cyclin D were purchased from Abcam (Cambridge, MA). The mouse monoclonal antibody against human β -actin was purchased from Sigma Aldrich (St. Louis, MO).

Immunohistochemistry staining of normal human skin

Normal human skin were obtained as discarded materials from plastic surgeries after approval of the human research committee of the first affiliated hospital of Jinan University at Guangzhou, China. Written informed consents were obtained from participating human subjects. Rabbit polyclonal anti-FOXC1 antibodies (recognize the region of amino acids 250–300 of FOXC1 protein) were purchased from Abcam (Catalog number ab115201, Cambridge, MA). After sequential steps of deparafinization, antigen retrieval, and inactivation of endogenous peroxidase, normal human skin sections (5 μ m) were blocked with 5% BSA, then incubated with primary antibody at 40C overnight. The following day, skin sections were washed by 1xPBS, incubated with secondary antibody conjugated HRP for 30 minutes, and then developed with 3, 3'-Diaminobenzidine. Hematoxylin solution was used to stain the cell nucleus. Pictures were acquired using a Leica DM6000 microscope system.

Overexpression of FOXC1 in undifferentiated primary KC

pLenti-C-RFP and pCMV6-entry-FOXC1(RC215629) plasmids were purchased from Origene Technologies (Rockville, MD). Full-length cDNA of FOXC1 was cut by Sgf I and Mlu I from pCMV6-entry-FOXC1 plasmid and then inserted into pLenti-C-RFP backbone to construct pLenti-C-RFP-FOXC1. The lentiviral particles of pLenti-C-RFP and pLenti-C-RFP-FOXC1 were generated in 293FT cells by co-transfection with packaging plasmids using Lenti-vpak packing kit (Origene Technologies, Rockville, MD). The viral supernatants were harvested and infected undifferentiated primary KC. 5 days after infection, the cells were harvested for RNA extraction and real-PCR.

RNA-Sequencing of transcriptome profiling

Total RNA were extracted KC transfected with scrambled siRNA and FOXC1 siRNA duplexes under undifferentiated conditions and differentiated for 5 days using RNeasy mini kit (QIAGEN, MD). RNA purity and concentration was measured on an Agilent Bioanalyzer (Agilent Technol., Palo Alto, CA). A total of 1 µg total RNA was used to prepare the Illumina HiSeq libraries according to manufacturer's instructions of the TruSeq RNA kit. Library preparation and HiSeq sequencing was performed at BGI technologies in China (Shenzhen, China).

Transcript levels were quantified in reads per kilobase of exon per million mapped reads (RPKM). The RPKM reflects the molar concentration of a transcript in the starting sample by normalizing for gene length and for the total read number in the measurement. This allows comparison of transcript levels both within and between experiments. The sequencing reads were analyzed using the computational pipeline of Bowtie, Tophat, and Cufflinks [32]. The mapped and aligned files of this study, along with processed data of RPKM for all detected transcripts have been deposited in the NCBI Gene Expression Omnibus (Access number: GSE76647).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed using Pierce Agarose ChIP kit (Thermo Scientific, prod#26156)

Following the manufacture's protocol. Briefly, human primary KC were differentiated by elevated the calcium to 1.3mM and cultured for 3 days. The cells were then incubated with 1% formaldehyde for 10 minutes at 37°C, followed by incubation with 1x Glycine solution for 5 minutes in room temperature. The cells were then collected, lysed and digested by MNase. Protein-chromatin complexes were immunoprecipitated with antibodies against KLF4, ZNF750, or rabbit IgG (negative control). Anti-KLF4, ZNF750 and rabbit IgG were purchased from Abcam (Cambridge, MA). Complexes were separated by incubation with ChIP grade proteinA/G agarose. Chromatin DNA fragments were then eluted and purified for quantitative real-time PCR. The primers used for amplification of specific regions of FOXC1 promoter were described in supplemental materials.

Statistical and Bioinformatic Analysis

Partek Genomics Suite (www.partek.com) was used to calculate the normalized gene expression levels and run the statistical analysis on the RNA-seq data. Partek's transcript algorithm is similar to the one in Xing et al [33] except Partek quantifies expression across the whole genome at the same time rather than each gene separately, and accounts for the fragmentation step in RNA-seq by normalizing by transcript length (http://www.partek.com/Tutorials/microarray/User_Guides/RNASEQ.pdf). A two-way ANOVA model was applied to the experimental groups and corrected for multiple testing at FDR<0.01. The statistical analysis of real-

time qPCR data was conducted using Graph Pad prism, version 5.03 (San Diego, CA). Comparisons of expression levels were performed using ANOVA techniques and independent sample *t* tests as appropriate. Differences were considered significant at $P < 0.05$.

Supporting Information

S1 Appendix. Supplemental materials and methods.
(DOCX)

S1 Fig. Cell cycle analysis of KC transfected with scrambled siRNA and FOXC1 siRNA duplexes.
(PDF)

S1 Table. The top 10 most up-regulated TFs during the course of KC differentiation.
(DOCX)

Acknowledgments

Lianghua Bin is the advisory board member of Astellas Pharma Inc. The authors wish to thank the Edelstein Family Foundation for their generous support of this work.

Author Contributions

Conceptualization: LB.

Formal analysis: ME.

Funding acquisition: LB DYML.

Investigation: LB LZ XW BR.

Methodology: LB.

Project administration: LB.

Resources: LD HY.

Supervision: LB DYML.

Validation: LB.

Visualization: LB.

Writing – original draft: LB.

Writing – review & editing: LB DYML.

References

1. Candi E, Schmidt R, Melino G (2005) The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol* 6: 328–340. doi: [10.1038/nrm1619](https://doi.org/10.1038/nrm1619) PMID: [15803139](https://pubmed.ncbi.nlm.nih.gov/15803139/)
2. Czarnowicki T, Krueger JG, Guttman-Yassky E (2014) Skin barrier and immune dysregulation in atopic dermatitis: an evolving story with important clinical implications. *J Allergy Clin Immunol Pract* 2: 371–379; quiz 380–371. doi: [10.1016/j.jaip.2014.03.006](https://doi.org/10.1016/j.jaip.2014.03.006) PMID: [25017523](https://pubmed.ncbi.nlm.nih.gov/25017523/)
3. Stawczyk-Macieja M, Szczerkowska-Dobosz A, Rebala K, Purzycka-Bohdan D (2015) Genetic background of skin barrier dysfunction in the pathogenesis of psoriasis vulgaris. *Postepy Dermatol Alergol* 32: 123–126. doi: [10.5114/pdia.2014.44003](https://doi.org/10.5114/pdia.2014.44003) PMID: [26015782](https://pubmed.ncbi.nlm.nih.gov/26015782/)
4. Segre JA, Bauer C, Fuchs E (1999) Klf4 is a transcription factor required for establishing the barrier function of the skin. *Nat Genet* 22: 356–360. doi: [10.1038/11926](https://doi.org/10.1038/11926) PMID: [10431239](https://pubmed.ncbi.nlm.nih.gov/10431239/)

5. Sen GL, Boxer LD, Webster DE, Bussat RT, Qu K, Zarnegar BJ, et al. (2012) ZNF750 is a p63 target gene that induces KLF4 to drive terminal epidermal differentiation. *Dev Cell* 22: 669–677. doi: [10.1016/j.devcel.2011.12.001](https://doi.org/10.1016/j.devcel.2011.12.001) PMID: [22364861](https://pubmed.ncbi.nlm.nih.gov/22364861/)
6. de Guzman Strong C, Wertz PW, Wang C, Yang F, Meltzer PS, Andl T, et al. (2006) Lipid defect underlies selective skin barrier impairment of an epidermal-specific deletion of Gata-3. *J Cell Biol* 175: 661–670. doi: [10.1083/jcb.200605057](https://doi.org/10.1083/jcb.200605057) PMID: [17116754](https://pubmed.ncbi.nlm.nih.gov/17116754/)
7. Yu Z, Lin KK, Bhandari A, Spencer JA, Xu X, Wang N, et al. (2006) The Grainyhead-like epithelial transactivator Get-1/Grhl3 regulates epidermal terminal differentiation and interacts functionally with LMO4. *Dev Biol* 299: 122–136. doi: [10.1016/j.ydbio.2006.07.015](https://doi.org/10.1016/j.ydbio.2006.07.015) PMID: [16949565](https://pubmed.ncbi.nlm.nih.gov/16949565/)
8. Magnusdottir E, Kalachikov S, Mizukoshi K, Savitsky D, Ishida-Yamamoto A, Panteleyev AA, et al. (2007) Epidermal terminal differentiation depends on B lymphocyte-induced maturation protein-1. *Proc Natl Acad Sci U S A* 104: 14988–14993. doi: [10.1073/pnas.0707323104](https://doi.org/10.1073/pnas.0707323104) PMID: [17846422](https://pubmed.ncbi.nlm.nih.gov/17846422/)
9. Lopez-Pajares V, Qu K, Zhang J, Webster DE, Barajas BC, Sipsrshvili Z, et al. (2015) A LncRNA-MAF: MAFB transcription factor network regulates epidermal differentiation. *Dev Cell* 32: 693–706. doi: [10.1016/j.devcel.2015.01.028](https://doi.org/10.1016/j.devcel.2015.01.028) PMID: [25805135](https://pubmed.ncbi.nlm.nih.gov/25805135/)
10. Kaufmann E, Knochel W (1996) Five years on the wings of fork head. *Mech Dev* 57: 3–20. PMID: [8817449](https://pubmed.ncbi.nlm.nih.gov/8817449/)
11. Lay K, Kume T, Fuchs E (2016) FOXC1 maintains the hair follicle stem cell niche and governs stem cell quiescence to preserve long-term tissue-regenerating potential. *Proc Natl Acad Sci U S A* 113: E1506–1515. doi: [10.1073/pnas.1601569113](https://doi.org/10.1073/pnas.1601569113) PMID: [26912458](https://pubmed.ncbi.nlm.nih.gov/26912458/)
12. Kalinin AE, Idler WW, Marekov LN, McPhie P, Bowers B, Steinert PM, et al. (2004) Co-assembly of envoplakin and periplakin into oligomers and Ca(2+)-dependent vesicle binding: implications for cornified cell envelope formation in stratified squamous epithelia. *J Biol Chem* 279: 22773–22780. doi: [10.1074/jbc.M313660200](https://doi.org/10.1074/jbc.M313660200) PMID: [15033990](https://pubmed.ncbi.nlm.nih.gov/15033990/)
13. Lee WH, Jang S, Lee JS, Lee Y, Seo EY, You KH, et al. (2005) Molecular cloning and expression of human keratinocyte proline-rich protein (hKPRP), an epidermal marker isolated from calcium-induced differentiating keratinocytes. *J Invest Dermatol* 125: 995–1000. doi: [10.1111/j.0022-202X.2005.23887.x](https://doi.org/10.1111/j.0022-202X.2005.23887.x) PMID: [16297201](https://pubmed.ncbi.nlm.nih.gov/16297201/)
14. Spradling KD, McDaniel AE, Lohi J, Pilcher BK (2001) Epsin 3 is a novel extracellular matrix-induced transcript specific to wounded epithelia. *J Biol Chem* 276: 29257–29267. doi: [10.1074/jbc.M101663200](https://doi.org/10.1074/jbc.M101663200) PMID: [11359770](https://pubmed.ncbi.nlm.nih.gov/11359770/)
15. Zhao L, Spassieva S, Gable K, Gupta SD, Shi LY, Wang J, et al. (2015) Elevation of 20-carbon long chain bases due to a mutation in serine palmitoyltransferase small subunit b results in neurodegeneration. *Proc Natl Acad Sci U S A* 112: 12962–12967. doi: [10.1073/pnas.1516733112](https://doi.org/10.1073/pnas.1516733112) PMID: [26438849](https://pubmed.ncbi.nlm.nih.gov/26438849/)
16. Marchesini N, Luberto C, Hannun YA (2003) Biochemical properties of mammalian neutral sphingomyelinase 2 and its role in sphingolipid metabolism. *J Biol Chem* 278: 13775–13783. doi: [10.1074/jbc.M212262200](https://doi.org/10.1074/jbc.M212262200) PMID: [12566438](https://pubmed.ncbi.nlm.nih.gov/12566438/)
17. Jung M, Choi J, Lee SA, Kim H, Hwang J, Choi EH (2014) Pyrrolidone carboxylic acid levels or caspase-14 expression in the corneocytes of lesional skin correlates with clinical severity, skin barrier function and lesional inflammation in atopic dermatitis. *J Dermatol Sci* 76: 231–239. doi: [10.1016/j.jdermsci.2014.09.004](https://doi.org/10.1016/j.jdermsci.2014.09.004) PMID: [25315296](https://pubmed.ncbi.nlm.nih.gov/25315296/)
18. Kypriotou M, Huber M, Hohl D (2012) The human epidermal differentiation complex: cornified envelope precursors, S100 proteins and the ‘fused genes’ family. *Exp Dermatol* 21: 643–649. doi: [10.1111/j.1600-0625.2012.01472.x](https://doi.org/10.1111/j.1600-0625.2012.01472.x) PMID: [22507538](https://pubmed.ncbi.nlm.nih.gov/22507538/)
19. Caubet C, Jonca N, Brattsand M, Guerrin M, Bernard D, Schmidt R, et al. (2004) Degradation of corneodesmosome proteins by two serine proteases of the kallikrein family, SCTE/KLK5/hK5 and SCCE/ KLK7/hK7. *J Invest Dermatol* 122: 1235–1244. doi: [10.1111/j.0022-202X.2004.22512.x](https://doi.org/10.1111/j.0022-202X.2004.22512.x) PMID: [15140227](https://pubmed.ncbi.nlm.nih.gov/15140227/)
20. Furio L, Pampalakis G, Michael IP, Nagy A, Sotiropoulou G, Hovnanian A (2015) KLK5 Inactivation Reverses Cutaneous Hallmarks of Netherton Syndrome. *PLoS Genet* 11: e1005389. doi: [10.1371/journal.pgen.1005389](https://doi.org/10.1371/journal.pgen.1005389) PMID: [26390218](https://pubmed.ncbi.nlm.nih.gov/26390218/)
21. Kelsell DP, Norgett EE, Unsworth H, Teh MT, Cullup T, Mein CA, et al. (2005) Mutations in ABCA12 underlie the severe congenital skin disease harlequin ichthyosis. *Am J Hum Genet* 76: 794–803. doi: [10.1086/429844](https://doi.org/10.1086/429844) PMID: [15756637](https://pubmed.ncbi.nlm.nih.gov/15756637/)
22. Akiyama M (2014) The roles of ABCA12 in epidermal lipid barrier formation and keratinocyte differentiation. *Biochim Biophys Acta* 1841: 435–440. doi: [10.1016/j.bbaliip.2013.08.009](https://doi.org/10.1016/j.bbaliip.2013.08.009) PMID: [23954554](https://pubmed.ncbi.nlm.nih.gov/23954554/)
23. Akiyama M, Sugiyama-Nakagiri Y, Sakai K, McMillan JR, Goto M, Arita K, et al. (2005) Mutations in lipid transporter ABCA12 in harlequin ichthyosis and functional recovery by corrective gene transfer. *J Clin Invest* 115: 1777–1784. doi: [10.1172/JCI24834](https://doi.org/10.1172/JCI24834) PMID: [16007253](https://pubmed.ncbi.nlm.nih.gov/16007253/)

24. Mattiuzzo NR, Toulza E, Jonca N, Serre G, Guerrin M (2011) A large-scale multi-technique approach identifies forty-nine new players of keratinocyte terminal differentiation in human epidermis. *Exp Dermatol* 20: 113–118. doi: [10.1111/j.1600-0625.2010.01188.x](https://doi.org/10.1111/j.1600-0625.2010.01188.x) PMID: [21255089](https://pubmed.ncbi.nlm.nih.gov/21255089/)
25. Kume T, Deng KY, Winfrey V, Gould DB, Walter MA, Hogan BL (1998) The forkhead/winged helix gene *Mf1* is disrupted in the pleiotropic mouse mutation congenital hydrocephalus. *Cell* 93: 985–996. PMID: [9635428](https://pubmed.ncbi.nlm.nih.gov/9635428/)
26. Cui CY, Ishii R, Campbell DP, Michel M, Piao Y, Kume T, et al. (2016) *Foxc1* Ablated Mice Are Anhidrotic and Recapitulate Features of Human Miliaria Sweat Retention Disorder. *J Invest Dermatol*.
27. Tkocz D, Crawford NT, Buckley NE, Berry FB, Kennedy RD, Gorski JJ, et al. (2012) BRCA1 and GATA3 corepress FOXC1 to inhibit the pathogenesis of basal-like breast cancers. *Oncogene* 31: 3667–3678. doi: [10.1038/onc.2011.531](https://doi.org/10.1038/onc.2011.531) PMID: [22120723](https://pubmed.ncbi.nlm.nih.gov/22120723/)
28. Somerville TD, Wiseman DH, Spencer GJ, Huang X, Lynch JT, Leong HS, et al. (2015) Frequent Derepression of the Mesenchymal Transcription Factor Gene FOXC1 in Acute Myeloid Leukemia. *Cancer Cell* 28: 329–342. doi: [10.1016/j.ccell.2015.07.017](https://doi.org/10.1016/j.ccell.2015.07.017) PMID: [26373280](https://pubmed.ncbi.nlm.nih.gov/26373280/)
29. Xia L, Huang W, Tian D, Zhu H, Qi X, Chen Z, et al. (2013) Overexpression of forkhead box C1 promotes tumor metastasis and indicates poor prognosis in hepatocellular carcinoma. *Hepatology* 57: 610–624. doi: [10.1002/hep.26029](https://doi.org/10.1002/hep.26029) PMID: [22911555](https://pubmed.ncbi.nlm.nih.gov/22911555/)
30. Zhou Y, Kato H, Asanoma K, Kondo H, Arima T, Kato K, et al. (2002) Identification of FOXC1 as a TGF-beta1 responsive gene and its involvement in negative regulation of cell growth. *Genomics* 80: 465–472. PMID: [12408963](https://pubmed.ncbi.nlm.nih.gov/12408963/)
31. Koster MI, Roop DR (2004) The role of p63 in development and differentiation of the epidermis. *J Dermatol Sci* 34: 3–9. PMID: [14757276](https://pubmed.ncbi.nlm.nih.gov/14757276/)
32. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, et al. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 28: 511–515. doi: [10.1038/nbt.1621](https://doi.org/10.1038/nbt.1621) PMID: [20436464](https://pubmed.ncbi.nlm.nih.gov/20436464/)
33. Xing Y, Yu T, Wu YN, Roy M, Kim J, Lee C (2006) An expectation-maximization algorithm for probabilistic reconstructions of full-length isoforms from splice graphs. *Nucleic Acids Res* 34: 3150–3160. doi: [10.1093/nar/gkl396](https://doi.org/10.1093/nar/gkl396) PMID: [16757580](https://pubmed.ncbi.nlm.nih.gov/16757580/)