

KLF4上调角质形成细胞中Keratin 17表达的分子机制

张 帅, 谈 园, 殷秀琴, 罗 宏[△]

长沙市第一医院 皮肤科(长沙 410005)

【摘要】 目的 通过探讨转录因子KLF4在调节角质蛋白17(Keratin 17, KRT17)表达中的作用, 揭示银屑病患者皮损中KRT17过度表达的分子机制。方法 以18例寻常型银屑病患者皮损组织为银屑病组, 10例健康人皮肤为对照组。采用实时荧光定量PCR和Western blot检测银屑病皮损组织及正常皮肤标本中KLF4的表达水平, 检测HaCat细胞中KLF4过表达叠加组蛋白乙酰转移酶EP300(E1A binding protein p300, EP300)干扰后KRT17的表达水平。染色质免疫共沉淀(chromatin immunoprecipitation, ChIP)检测银屑病皮损组织及正常皮肤标本中KRT17启动子区KLF4结合水平及组蛋白H3乙酰化水平, 检测HaCat细胞中KLF4过表达叠加EP300干扰后KRT17启动子区KLF4结合水平及组蛋白H3乙酰化水平。免疫共沉淀(co-immunoprecipitation, Co-IP)检测KLF4与EP300的相互作用。结果 银屑组皮损组织KLF4表达水平、KRT17启动子区KLF4结合水平及组蛋白H3乙酰化水平均高于对照组皮肤标本($P<0.01$)。与转染对照组相比, KLF4过表达组KRT17表达水平升高($P<0.01$); KLF4过表达叠加EP300干扰组KRT17表达水平低于KLF4过表达组($P<0.01$)和转染对照组($P<0.05$)。与转染对照组相比, KLF4过表达组KRT17启动子区组蛋白H3乙酰化水平升高($P<0.01$); KLF4过表达叠加EP300干扰组KRT17启动子区组蛋白H3乙酰化水平低于KLF4过表达组($P<0.01$)和对照组($P<0.01$)。Co-IP证实KLF4与EP300能形成蛋白复合物。结论 过度表达的KLF4通过协同EP300上调KRT17启动子区组蛋白H3乙酰化水平, 介导银屑病患者皮损中KRT17的过度表达。

【关键词】 银屑病 角质形成细胞 角质蛋白17 kruppel样因子4 E1A结合蛋白p300

Molecular Mechanism of KLF4 Up-regulating Keratin 17 Expression in Keratinocytes ZHANG Shuai, TAN Yuan, YIN Xiu-qin, LUO Hong[△]. Department of Dermatology, the First Hospital of Changsha, Changsha 410005, China

[△] Corresponding author, E-mail: lhdoctor@yeah.net

【Abstract】 **Objective** To explore the role of Kruppel-like factor 4 (KLF4) in the regulation of Keratin 17 (KRT17) expression, and to reveal the molecular mechanism of overexpression of KRT17 in psoriatic lesions. **Methods** The skin lesions of 18 patients with psoriasis vulgaris were taken as experimental group and 10 healthy persons as control group. Real time-PCR and Western blot were used to detect the expression of KLF4 in psoriasis and normal skin samples, and the changes of KRT17 expression in HaCat cells after transfection of KLF4 overexpression and EP300 interfering plasmid. ChIP-qPCR was used to detect KLF4 binding and histone H3 acetylation levels in the promoter region of KRT17 in psoriasis and normal skin samples, and the changes of KLF4 binding and histone H3 acetylation levels in the promoter region of KRT17 in HaCat cells after transfection of KLF4 overexpression and EP300 interfering plasmid. Co-IP detects the interaction between KLF4 and EP300. **Results** The expression level of KLF4, KLF4 binding level and histone H3 acetylation level in the promoter region of KRT17 in psoriasis group were significantly higher than those in normal group ($P<0.01$). Compared with the control group, the expression level of KRT17 was significantly higher after KLF4 overexpression ($P<0.01$). After KLF4 overexpression combined with EP300 interference, the expression level of KRT17 was significantly lower than that of KLF4 overexpression group ($P<0.01$), slightly lower than that of control group ($P<0.05$). Compared with the control group, the histone H3 acetylation level in KRT17 promoter region in KLF4 over-expression group was increased significantly ($P<0.01$). After KLF4 over-expression combined with EP300 interference, the acetylation level of histone H3 in KRT17 promoter region was significantly lower than that in KLF4 overexpression group ($P<0.01$) and control group ($P<0.01$). Co-IP confirmed that KLF4 and EP300 could form protein complexes. **Conclusion** Excessive KLF4 increases the level of histone H3 acetylation in KRT17 promoter region by synergistic EP300, and mediates the over-expression of KRT17 in psoriatic lesions.

【Key words】 Psoriasis Keratinocyte Keratin 17 KLF4 EP300

银屑病是一种慢性炎症性增殖性皮肤病, 典型的临床表现为红斑、鳞屑, 好发于头皮、膝盖、肘部的伸侧、躯干、小腿前部和指甲。银屑病症状严重者可累及心、肝、肾、眼等系统, 还可因继发感染、电解质紊乱而危及

生命^[1]。T细胞介导的炎症反应在银屑病的发病机制中扮演重要角色。其中辅助性T细胞1(T helper cell 1, Th1)及17(Th17)细胞, 可以通过产生肿瘤坏死因子- α (tumor necrosis factor- α , TNF- α)、 γ -干扰素(interferon- γ , IFN- γ)抑制白介素(interleukin, IL)-17 A(IL-17A)、IL-17F、IL-

[△] 通信作者, E-mail: lhdoctor@yeah.net

22等细胞因子,介导炎症反应的发生^[2-4]。

大量研究证实,角蛋白17(keratin 17, KRT17)在银屑病皮损的角质形成细胞(keratinocyte, KC)中表达明显升高,且与银屑病的疾病进程密切相关^[5-8]。KRT17含有与链球菌M6蛋白相似的表位,这些表位可以刺激Th1细胞、Th17细胞产生银屑病相关细胞因子IFN- γ 、IL-17、IL-22等,这些细胞因子又能诱导KRT17的表达,从而在KRT17和T细胞之间形成一个相互促进的环路,导致炎症反应和表皮细胞周期异常等病理改变^[9-11]。然而,银屑病患者KC中KRT17表达异常的分子机制目前仍不明确。

本研究课题组预先对KRT17基因启动子DNA序列进行了转录因子结合位点预测分析,发现KRT17启动子-137~-442 bp区域包含多个转录因子KLF4(Kruppel-like factor 4)结合位点。此外,我们通过软件预测发现KLF4与组蛋白乙酰转移酶EP300(E1A binding protein p300, EP300)存在相互作用的可能性。本研究拟通过证实KLF4与EP300对KC中KRT17表达的调控,阐述银屑病患者皮损KC中KRT17表达异常的分子机制。

1 资料与方法

1.1 研究对象及标本的获取

选择2016年10月-2018年6月长沙市第一医院皮肤科

收治的18例寻常型银屑病患者为研究对象,行病理活检时获取的部分皮损组织标本作为银屑病组。其中男10例,女8例,年龄20~47(30.9 \pm 7.0)岁;另取10例来自我院眼科手术后的健康人皮肤组织标本作为对照组,其中男4例,女6例,年龄19~50(30.7 \pm 9.7)岁。本研究通过长沙市第一医院伦理委员会审批(KL-2016014),所有标本来源的患者和健康对照者均签署知情同意书。

1.2 实时荧光定量PCR

去除皮肤标本的皮下脂肪等多余组织,并剪成1~2 mm³小块放入研钵中,加入液氮并迅速研磨粉碎。粉碎后组织按每100 mg加入1 mL Trizol(Invitrogen, CA, USA),混匀后转入离心管。离心管置于冰上并用电动均浆器充分匀浆1~2 min。匀浆后混合物于12 000 r/min、4℃离心5 min,取上清后每毫升Trizol加入200 μ L氯仿,后续按常规Trizol法抽提出总RNA。使用逆转录试剂盒PrimeScriptTM RT reagent Kit (TaKaRa Bio, Japan)将总RNA逆转录成cDNA。采用SYBR Green荧光染料法进行实时荧光定量PCR(Real time-PCR)(Thermal Cycler DiceTM Real Time System II, TaKaRa Bio),反应条件为95℃预变性30s,随后40个PCR循环(95℃ 5s, 60℃ 35s)。检测基因引物序列见表1,引物合成由华大基因公司完成。以GAPDH基因为内参照,2^{- $\Delta\Delta C_t$} 法测定目的基因的相对表达量,并以健康对照组目的基因的表达量为1。

表1 实时荧光定量PCR引物序列

Table 1 Real time-PCR primer sequences

Gene	Forward	Reverse	Length/bp
KLF4	5'-GCTCACCCACCTTCTTCACCC-3'	5'-AATTTCCATCCACAGCCGTCCC-3'	237
KRT17	5'-GCTTTGGGGGTGTTGATGGG-3'	5'-CGGTGGCTGTGAGGATCTTGTT-3'	234
GAPDH	5'-ATGGGGAAGGTGAAGGTCG-3'	5'-GGGTCATTGATGGCAACAATA-3'	108

1.3 蛋白免疫印迹(Western blot)

取100 mg修剪后皮肤标本,剪碎后加入2 mL玻璃匀浆器中,同时加入含1%PMSF的RIPA裂解液300 μ L,置于冰上进行匀浆。裂解30 min后,将裂解液移至1.5 mL离心管中,4℃,12 000 r/min离心5 min,上清即为总蛋白提取液。测定样本蛋白浓度后,每个样本取30 μ g总蛋白进行SDS-PAGE凝胶电泳,并电转印到PVDF膜。用5%脱脂牛奶作为封闭液室温封闭转印膜1 h,加入一抗Anti-KLF4(滴度为1:300)、Anti-KRT17(滴度为1:300)及Anti-GAPDH抗体(滴度为1:1000)(Cell Signaling Technology, MA, USA),4℃孵育过夜。洗膜后加入二抗室温孵育1h,漂洗后显影,使用ImageJ软件采集条带灰度值并分析结果。灰度值与GAPDH比值为目的蛋白的相对含量。

1.4 染色质免疫共沉淀(chromatin immunoprecipitation, ChIP)检测KRT17启动子区KLF4富集及组蛋白H3乙酰化水平

使用EpiQuik Tissue ChIP Kit(Epigentek, NY, USA)进行ChIP实验。将修剪后皮肤标本剪成1~2 mm³小块,放入终体积分数为1%甲醛室温交联20 min。加甘氨酸至终浓度为0.125 mol/L,混匀后于室温下放置5 min终止交联。将交联好的组织块放入匀浆器并加入Homogenizing Buffer进行组织解离。4℃,3 000 r/min离心5 min收集解离细胞后进行裂解并超声打断DNA。分别加入anti-KLF4(滴度为1:200)或anti-H3ac(滴度为1:200)抗体,4℃反应过夜。加入protein A琼脂糖珠,沉淀靶蛋白-DNA复合物。终浓度0.2 mol/L氯化钠65℃解交联过

夜。DNA回收试剂盒纯化回收富集的DNA, PCR或实时荧光定量PCR检测各组DNA富集量($2^{-\Delta\Delta Ct}$ 法)。*KRT17*启动子ChIP-PCR/qPCR引物序列见表2。扩增片段位置为

-42 ~ -198 bp的引物用于实时荧光定量PCR, 检测各组

表2 *KRT17*启动子ChIP-qPCR引物序列
Table 2 ChIP-PCR primers for *KRT17* promoter

Fragment position/bp	Forward	Reverse	Length/bp
-42--198	5'-GGCACTCAGCAGGAGGATTT-3'	5'-TGGGCGTAGCGATTACAACA-3'	156
-361--443	5'-GAGGGAACAAGCCCCAA-3'	5'-ACATCCCCAAGGTCCCA-3'	82
-707--778	5'-GGGTTGTGGAGAAAGGC-3'	5'-CATATTATCCGCTGATAG-3'	71

*KRT17*启动子区KLF4富集及组蛋白H3乙酰化水平。

1.5 免疫共沉淀 (co-immunoprecipitation, Co-IP) 验证KLF4与EP300相互结合

银屑病患者皮损组织提取总蛋白, 提取方法同蛋白免疫印迹。总蛋白中加入anti-KLF4抗体, 4 °C孵育过夜。加入protein A/G PLUS 琼脂糖珠, 室温孵育2 h进行免疫沉淀。离心收集琼脂糖珠, PBS洗涤两次后使用上样缓冲液洗脱蛋白复合物, 使用anti-KLF4 (滴度为1 : 200) 及anti-EP300 (滴度为1 : 200) 抗体进行Western blot (步骤同1.3), 检测蛋白富集物中是否存在KLF4及EP300蛋白, 进而证实KLF4与EP300的相互结合。实验重复3次。

1.6 人永生化角质形成细胞HaCat的培养、转染、检测

HaCat细胞置于DMEM培养基中(其中含青霉素100 U/mL, 链霉素100 μg/mL, 10%FBS), 在37 °C、体积分数5%CO₂的细胞培养箱中培养。收获对数生长期细胞, 并将细胞悬液接种于6孔细胞培养板中继续培养。接种细胞分为3组, 即对照组、*KLF4*过表达组、*KLF4*过表达叠加EP300干扰组, 每组包含3个平行孔。细胞生长达50% ~ 60%融合度时, 使用Lipofectamine™ 2000 Transfection Reagent(Thermo Fisher Scientific, MA, USA)进行质粒转染。对照组转染pCMV6质粒+pRS质粒、*KLF4*过表达组转染pCMV6-*KLF4*质粒、*KLF4*过表达叠加EP300干扰组转染pCMV6-*KLF4*质粒+pRS-EP300质粒, 所有质粒均购自OriGene公司。转染72 h后收集细胞, 分别抽提总RNA及总蛋白, 按1.2和1.3方法进行实时荧光定量PCR和Western blot, 检测各组EP300及*KRT17*的表达水平。按1.4方法进行ChIP-qPCR, 检测各组*KRT17*启动子区组蛋白H3乙酰化水平。实验重复3次。

1.7 统计学方法

正态分布计量资料以 $\bar{x} \pm s$ 表示。采用独立样本 t 检验比较成组设计的两样本均数, 多组数据使用one-way ANOVA进行单因素方差分析, 并使用LSD检验进行两两比较, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 银屑病及正常皮肤样本中KLF4的表达

实时荧光定量PCR结果(图1A)显示, 与正常组皮肤标本相比, 银屑组皮损组织*KLF4* mRNA表达水平升高($P < 0.01$)。Western blot验证了这一结果($P < 0.01$)。见图1B、1C。

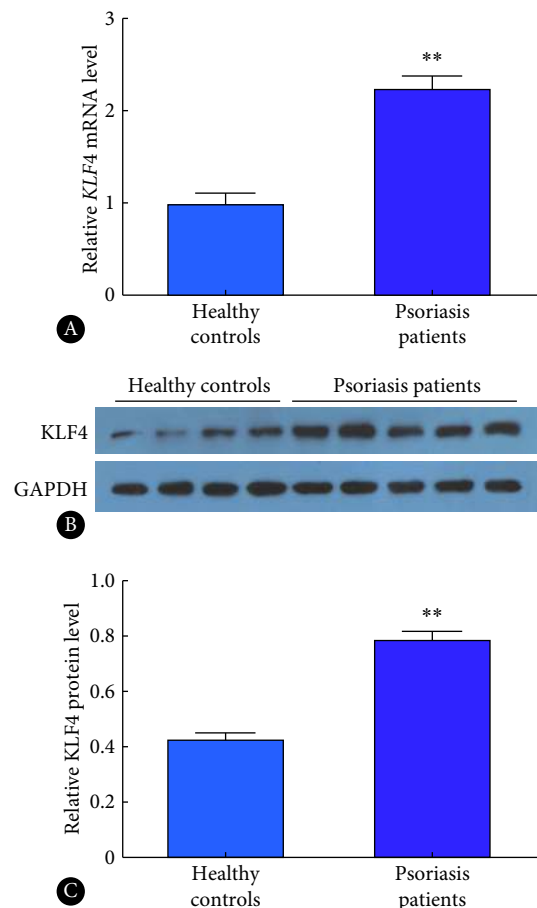


图1 银屑组 ($n=18$) 和正常组 ($n=10$) 皮肤样本中KLF4表达水平差异

Fig 1 The difference of KLF4 expression in skin samples between the psoriasis patients group ($n=18$) and healthy control group ($n=10$)

A: Real time-PCR; B: Western blot; C: Quantitative analysis of the band intensities for KLF4 in Western blot. ** $P < 0.01$, vs. healthy controls.

2.2 银屑病及正常皮肤样本中KRT17启动子区KLF4结合水平及组蛋白H3乙酰化水平比较

ChIP-PCR结果证实, KLF4能结合于KRT17启动子-42 ~ -443 bp区域。ChIP-qPCR结果显示, 与正常组相比, 银屑组中KRT17启动子区KLF4结合水平升高

($P < 0.01$); KRT17启动子区组蛋白H3乙酰化水平增加($P < 0.01$)。见图2。

2.3 KLF4与EP300相互作用的验证

Co-IP结果显示, KLF4能与EP300相互结合, 形成蛋白复合体。见图3。

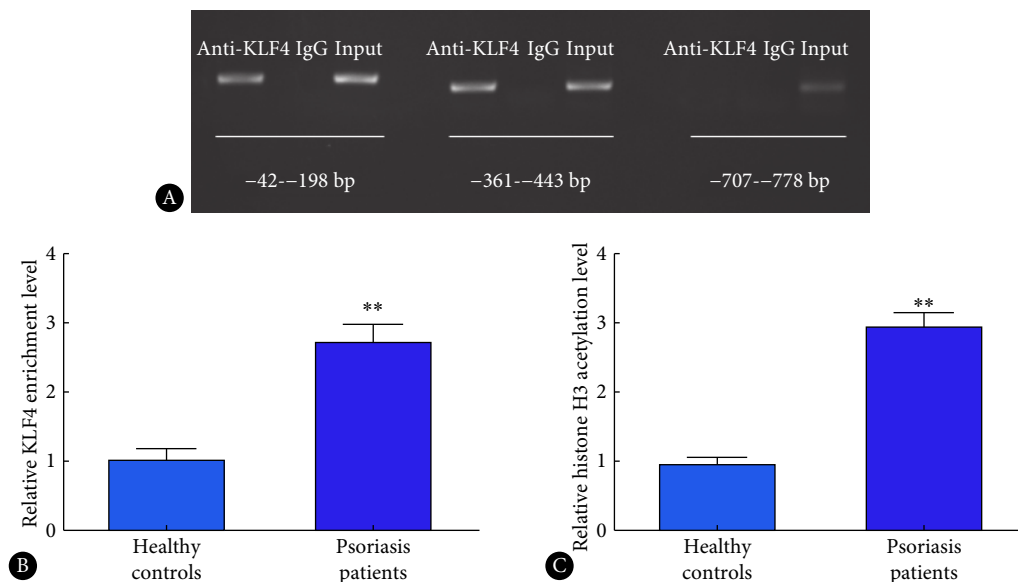


图 2 两组皮肤样本中KRT17启动子区KLF4结合水平及组蛋白H3乙酰化水平差异

Fig 2 Differences of KLF4 binding level and histone H3 acetylation level in KRT17 promoter region between two groups

A: ChIP-PCR results; B-C: The results of ChIP-qPCR (B: KLF4 binding level in KRT17 promoter region; C: Histone H3 acetylation level in KRT17 promoter region), $n=6$. ** $P < 0.01$.

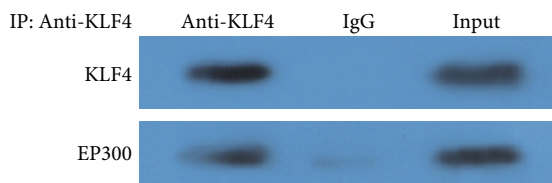


图 3 Co-IP检测KLF4与EP300的相互作用

Fig 3 Detection of the interaction of KLF4 and EP300 by Co-IP

2.4 过表达KLF4及干扰EP300后对KRT17表达的影响

实时荧光定量PCR结果显示, 与转染对照质粒组相比, KLF4过表达组KRT17表达水平升高(1.05 ± 0.17 vs. 6.94 ± 1.16 , $P < 0.01$); KLF4过表达叠加EP300干扰后, KRT17表达水平低于KLF4过表达组(0.75 ± 0.11 vs. 6.94 ± 1.16 , $P < 0.01$)和对照组(0.75 ± 0.11 vs. 1.05 ± 0.17 , $P < 0.05$)。见图4。

Western blot结果显示, 与转染对照质粒组相比, KLF4过表达组KRT17表达水平升高(0.69 ± 0.05 vs. 0.97 ± 0.07 , $P < 0.01$); KLF4过表达叠加EP300干扰后, KRT17表达水平低于KLF4过表达组(0.52 ± 0.06 vs. 0.97 ± 0.07 , $P < 0.01$)和对照组(0.52 ± 0.06 vs. 0.69 ± 0.05 , $P < 0.05$)。见图4。

2.5 过表达KLF4及干扰EP300后对KRT17启动子区组蛋白H3乙酰化水平的影响

ChIP-qPCR结果显示, 与转染对照质粒组相比, KLF4过表达组KRT17启动子区组蛋白H3乙酰化水平升高(1.06 ± 0.11 vs. 2.19 ± 0.18 , $P < 0.01$)。KLF4过表达叠加EP300干扰后, KRT17启动子区组蛋白H3乙酰化水平低于KLF4过表达组(0.39 ± 0.06 vs. 2.19 ± 0.18 , $P < 0.01$)和对照组(0.39 ± 0.06 vs. 1.06 ± 0.11 , $P < 0.01$)。见图5。

3 讨论

银屑病患者皮损中活化的角质形成细胞表达大量角蛋白KRT17, 过多的KRT17可诱导表皮增生和Th1/Th17细胞介导的炎症反应。可见KRT17在银屑病的发生发展过程中发挥着重要作用^[2, 7, 9]。但银屑病患者皮损中KRT17过度表达的原因目前还不明确。本研究通过实验证实, 与正常皮肤相比, 转录因子KLF4在银屑病皮损中表达显著上调, 且其在KRT17启动子区域的结合也明显增多; 在HaCat细胞中过表达KLF4能上调KRT17的表达。这些结果说明, 过量的KLF4可能是导致银屑病患者皮损中KRT17表达过度的重要原因。

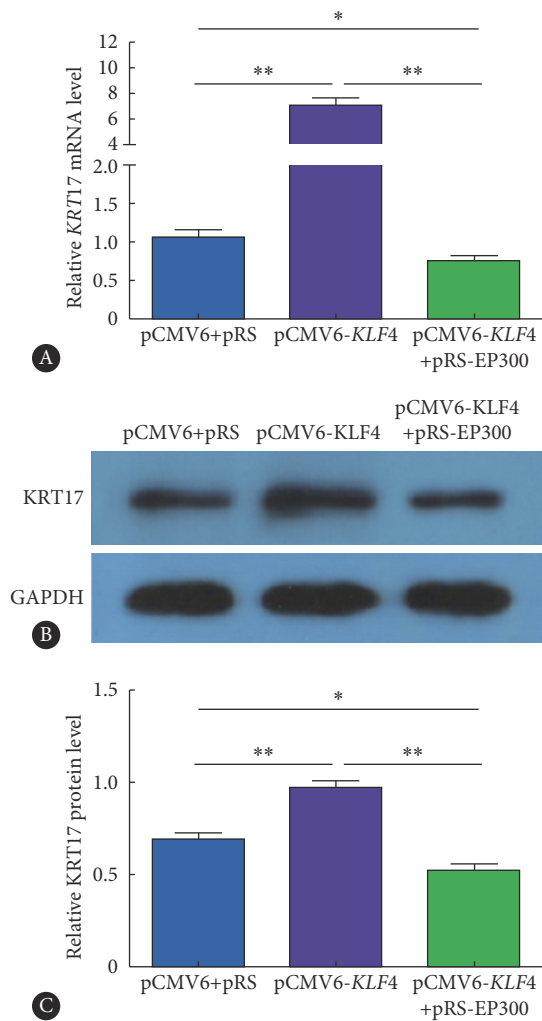


图 4 过表达 KLF4 及干扰 EP300 后对 KRT17 表达的影响

Fig 4 Effect of overexpression of KLF4 and interference with EP300 on KRT17 expression

A: Real time-PCR; B: Western blot; C: Quantitative analysis of Western blot. pCMV6+pRS: Control group; pCMV6-KLF4: KLF4 over-expression group; pCMV6-KLF4+pRS-EP300: KLF4 over-expression combined with EP300 interference group. n=3. * P<0.05, ** P<0.01.

KLF4是一种在肠道和上皮组织中广泛表达的锌指蛋白类转录因子,参与免疫调节、炎症反应、调控细胞增殖分化、胚胎发育等重要生命过程^[12-14]。大量研究证实,KLF4与炎症反应关系密切。TNF- α 、IFN- γ 等炎症因子能明显促进巨噬细胞中KLF4的表达,从而活化巨噬细胞,加重炎症反应^[15]。IL-1 β 可通过PI3K/AKT信号通路诱导小胶质细胞中KLF4的表达,进而上调内源性IL-1 β 及其他促炎因子如环氧化酶-2(cyclooxygenase-2, COX-2)、单核细胞趋化蛋白-1(monocyte chemotactic protein 1, MCP-1)和IL-6的产生^[16]。皮炎患者外周血单个核细胞中KLF4的表达增加,导致Th17细胞过度分化,加剧了患者的炎性损伤^[17]。KLF4通过上调IL-1 β 、IL-6、基质金属蛋白酶-

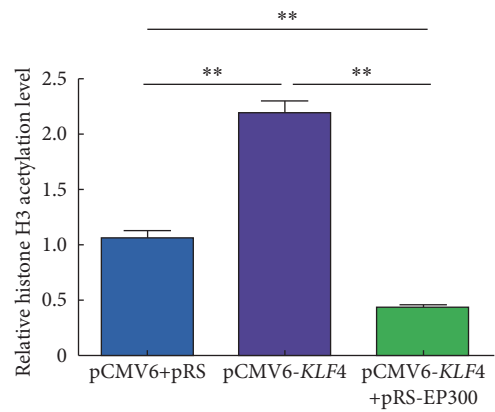


图 5 ChIP-qPCR检测 KLF4 过表达及 EP300 干扰后 KRT17 启动子区组蛋白 H3 乙酰化水平

Fig 5 Histone H3 acetylation in KRT17 promoter region was detected by ChIP-qPCR after overexpression of KLF4 and interference of EP300

**P<0.01.

13(matrix metalloproteinase, MMP-13)在滑膜中的表达,参与类风湿性关节炎的疾病进程^[18]。目前,KLF4与银屑病的相关研究十分稀缺,因而他们之间的关系并不明确。仅有郭坤等^[19]的研究发现KLF4在银屑病患者皮损中表达高于正常皮肤,这一结果与本研究结果一致。

组蛋白H3的乙酰化修饰与基因表达的活跃密切相关。组蛋白H3的N端赖氨酸残基上被乙酰转移酶引入乙酰基团,使DNA与组蛋白间的静电引力和空间位阻增大,两者间的相互作用减弱,DNA易于解聚,利于转录因子与DNA模板相结合,进而激活转录^[20-23]。EP300属于组蛋白乙酰转移酶,在调节组蛋白乙酰化过程中发挥重要作用^[24-26]。本课题证实了KLF4能与EP300形成蛋白复合体,且在HaCat细胞中过表达KLF4后,KRT17启动子区组蛋白H3乙酰化水平显著升高,同时KRT17表达也升高;KLF4过表达叠加EP300干扰后,KRT17启动子区组蛋白H3乙酰化水平显著降低,相应的KRT17表达也低于单纯KLF4过表达组及对照组。同时,我们也证实银屑病患者皮损中KRT17启动子区组蛋白H3乙酰化水平显著高于正常皮肤。综上所述,银屑病患者皮损中过量的KLF4协同且依赖于EP300,通过上调KRT17启动子区组蛋白H3乙酰化水平,促使KRT17的大量表达。表观遗传学机制十分丰富,处理组蛋白乙酰化修饰,还有组蛋白甲基化修饰。相对于组蛋白乙酰化修饰,组蛋白甲基化修饰更为复杂,不同位点的甲基化修饰可以导致基因转录的开放或关闭。KLF4是否可以通过调节组蛋白甲基化修饰,调控KRT17的表达目前还未知,这也是本研究未解释的问题,也是本研究的不足之处,后续研究中,我们将进一步探讨KLF4

与KRT17组蛋白甲基化的关系,争取完善银屑病患者皮损中KLF4调控KRT17表达的组蛋白修饰图谱。

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