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Calcitriol Promotes Keratinocyte Differentiation and Barrier-Associated Protein Expression: Potential Involvement of Cav1-Related Calcium Signaling

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Background: Skin barrier dysfunction is closely associated with impaired keratinocyte differentiation. Calcitriol helps maintain the stable state of the epidermis, but the role of caveolin-1 (Cav1)-associated calcium signaling in calcitriol-induced skin barrier-associated responses remains unclear.

Methods: HaCaT human keratinocytes were treated with different concentrations of calcitriol, with or without methyl-β-cyclodextrin, to explore Cav1's role in D3-induced differentiation. Cell metabolic activity, apoptosis, intracellular Ca^{2+} levels, and migration were assessed using Cell Counting Kit-8 (CCK-8), Hoechst staining, Fluo-4AM staining, and scratch assays, respectively. The gene and protein levels of differentiation- and barrier-related markers, as well as calcium channel proteins (Cav1, p-Cav1, TRPV6 and Orai1), were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting.

Results: Following calcitriol treatment, the differentiation of HaCaT cells was significantly promoted, the expression of differentiation- and barrier-related proteins was increased, and cell migration was significantly enhanced ($p < 0.05$). Calcitriol increased Cav1 expression and phosphorylation, accompanied by elevated intracellular Ca^{2+} levels and increased expression of the calcium-related proteins TRPV6 and Orai1 ($p < 0.05$). After disruption of Cav1-associated membrane signaling by methyl-β-cyclodextrin (MβCD), the calcitriol-induced increases in differentiation markers, barrier-associated proteins, and intracellular Ca^{2+} levels were significantly attenuated ($p < 0.05$), suggesting that Cav1-associated calcium signaling may be involved in calcitriol-induced keratinocyte differentiation and the expression of barrier-associated proteins.

Conclusion: Calcitriol increased intracellular Ca^{2+} levels and was associated with increased expression of Cav1-related calcium signaling components, accompanied by enhanced keratinocyte differentiation and expression of barrier-associated proteins. Cav1-associated signaling may therefore participate in calcitriol-induced keratinocyte differentiation and epidermal barrier-associated responses. These findings provide mechanistic insight into calcitriol-mediated keratinocyte differentiation and highlight the potential involvement of Cav1-associated signaling in calcitriol-induced regulation of keratinocyte differentiation and barrier-associated protein expression.

Keywords: calcitriol; Cav1; keratinocytes; calcium signaling; epidermal barrier function

Introduction

The skin, as the largest organ of the human body, serves as the primary barrier against external harmful factors and plays a pivotal role in maintaining barrier function [1]. This barrier function is mainly achieved through the stratum corneum, formed by the differentiation of epidermal keratinocytes [2]. Atopic dermatitis (AD) is a common chronic inflammatory skin disease characterized by complex interactions between barrier disruption and immune dysregulation [3,4]. Patients with AD are more susceptible to skin infections and may develop complications such as *Staphylococcus aureus* sepsis [5]. Although most studies have focused on filaggrin mutations, the physical barrier also plays a crucial role in the pathogenesis of AD [6]. Within the physical barrier, the stratum corneum and tight junctions are of central importance. When the epidermal barrier is damaged, keratinocytes initiate regenerative programs involving proliferation and redifferentiation to restore the impaired structure [7,8]. This process encompasses multiple regulatory events, including cell cycle control, calcium signaling pathways, tight junction protein expression, and the synthesis of cornified envelope proteins [9]. A calcium ion gradient is considered a key regulator of keratinocyte differentiation: extracellular Ca^{2+} concentrations progressively increase toward the upper epidermal layers, thereby activating differentiation programs [10]. In addition, the expression of tight junction proteins represents

a crucial molecular determinant of barrier integrity and is often co-upregulated with differentiation markers [11].

Calcitriol, a fat-soluble vitamin with multiple biological activities, is well recognized for its classical role in calcium and phosphorus metabolism. Recent studies have highlighted its importance in keratinocyte differentiation, immune regulation, and wound healing [12,13]. Previous findings have indicated that calcitriol can induce keratinocyte differentiation, enhance the expression of differentiation-related genes, and increase intracellular Ca^{2+} levels by modulating calcium channels, thereby activating downstream signaling pathways. However, whether calcitriol-mediated calcium signaling depends on the activation of specific calcium channels, and how this subsequently influences differentiation and barrier-associated molecular responses, remains largely unexplored.

Caveolin-1 (Cav1), a structural protein enriched in caveolae of the plasma membrane, has been implicated in the regulation of membrane-associated signaling and intracellular calcium homeostasis [14,15]. In keratinocytes, Cav1 has been implicated in cellular signaling processes associated with proliferation, differentiation, and barrier-associated responses [16,17]. While the functions of Cav1 have been extensively investigated in cancer and cardiovascular biology [18], its role in keratinocyte differentiation and epidermal barrier regulation remains poorly understood.

Therefore, HaCaT human keratinocytes were used as an *in vitro* model and treated with varying concentrations of calcitriol (1α,25-dihydroxyvitamin D3) to evaluate its effects on cell metabolic activity, apoptosis, intracellular calcium concentration, cell cycle gene expression, and differentiation- and barrier-related protein expression. Furthermore, methyl-β-cyclodextrin (MβCD) was used to disrupt cholesterol-dependent membrane signaling associated with Cav1-enriched domains, allowing us to explore whether Cav1-associated signaling may be involved in calcitriol-induced keratinocyte differentiation and the expression of barrier-associated proteins. This work provides further insight into the association between calcitriol-induced keratinocyte differentiation and Cav1-related calcium signaling and may provide a basis for future studies of Cav1-associated mechanisms in epidermal homeostasis.

Materials and Methods

Cell Line and Culture Conditions

Human keratinocyte cell line HaCaT (Cat No. GNHu64) was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai). Cell identity was verified by Short Tandem Repeat (STR) analysis and confirmed to match the reference profile. Mycoplasma contamination was assessed using a PCR detection kit according to standard procedures, and all cultures used in this study tested negative. The cells were cultured in DMEM (12491015, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (10099141C, Gibco, CA, USA) and 1% penicillin-streptomycin (PB180120, Procell, Wuhan, China). During the induction of differentiation, 1.8 mmol/L calcium chloride (from HY-Y0406E, Mackey, NJ, USA) was added to the medium. All cells were cultured at 37 °C in a humidified incubator containing a carbon dioxide concentration of 5%. Passaging was performed when the cell confluence reached 70–80%, and the medium was changed every 2 days. Calcitriol (1α,25-dihydroxyvitamin D3; HY-10002, MCE, NJ, USA) was dissolved in anhydrous DMSO (HY-Y0320C, MCE, NJ, USA) to prepare a stock solution with a concentration of 10 mM, which was stored at 4 °C in the dark and diluted to the required concentration before use. The control group received an equal volume of DMSO to ensure a consistent final solvent concentration across all groups.

Experimental Groups and Model Establishment

This study consisted of two experimental parts. In both, HaCaT cells were cultured for 6 consecutive days in medium containing 1.8 mmol calcium chloride to induce differentiation.

The first part investigated the effects of different calcitriol concentrations on keratinocyte differentiation. HaCaT cells were divided into four groups: the first was the control group without calcitriol, the second was the low-dose group with 10 nM of calcitriol, the third was the medium-dose group with 100 nM of calcitriol, and the fourth was the high-dose group with 1000 nM of calcitriol. calcitriol was dissolved in DMSO, diluted with culture medium to the desired concentrations, and applied directly to the cells for 6 days under standard culture conditions (37 °C, 5% CO_2).

The second part evaluated the role of Cav1-associated signaling in calcitriol-induced keratinocyte differentiation. HaCaT cells were divided into three groups: Control group, calcitriol group, and calcitriol + MβCD group. For the calcitriol + MβCD group, cells were pre-treated with 5 mM MβCD (HY-101461, MCE, NJ, USA) for 2 h to disrupt Cav1-associated membrane signaling, followed by treatment with 100 nM calcitriol. Cells were then cultured under differentiation-inducing conditions for 6 days. The culture medium was replaced every 2 days with fresh medium containing the corresponding concentrations of calcitriol and/or MβCD to maintain consistent treatment conditions.

Assessment of Cell Metabolic Activity Using the CCK - 8 Assay

HaCaT cells were seeded into 96-well plates at a density of 5×10^3 cells per well, and then treated with different concentrations of calcitriol. Cell metabolic activity was assessed at days 0, 2, 4, and 6 using the Cell Counting Kit-8 (CCK-8) assay (C0038, Beyotime, Shanghai, China) according to the manufacturer's instructions. 10 μL of the

reagent was added to each well and incubated at 37 °C for 2 hours. OD value was measured at 450 nm with a microplate reader (AMR-100, Allsheng, Hangzhou, China). Three replicate wells were set for each group, and the mean value was taken for statistical analysis.

Hoechst Staining for Apoptosis Detection

HaCaT cells were seeded into 6-well plates before treatment and cultured under the indicated experimental conditions. After 6 days of calcitriol and/or MβCD treatment, cells were fixed with 4% paraformaldehyde for 10 minutes and washed three times with PBS. Hoechst 33258 dye (40729ES10, Yeasen, Shanghai) was added and incubated in the dark for 30 minutes. The cells were observed using a fluorescence microscope (CKX53, OLYMPUS, Shenzhen) with an excitation wavelength of 340 nm. Five random regions were selected for each group, and the apoptotic cells were quantified using ImageJ software (version 1.8.0, Tanon Science & Technology Co., Ltd., Shanghai, China). The apoptosis rate was calculated as the number of apoptotic cells divided by the total number of cells multiplied by 100%.

Measurement of Intracellular Ca^{2+} Concentration

On day 6, the intracellular free calcium ion^{+} content was determined using the Fluo-4AM fluorescent probe (S1060, Beyotime, Shanghai). Cells were incubated with 5 μM Fluo-4AM at 37 °C for 30 minutes, washed three times with HBSS (C0219, Beyotime, Shanghai), and then incubated for another 30 minutes to achieve deesterification. Fluorescence images were acquired using a fluorescence microscope (FITC channel, excitation at 488 nm). Five random fields per group were analyzed, and the mean fluorescence intensity was quantified using ImageJ (version 1.8.0, Tanon Science & Technology Co., Ltd., Shanghai, China).

Scratch Wound Assay

Cells were seeded in 6-well plates and cultured to confluence. A sterile 200- microliter pipette tip was used to create a straight scratch in the center of each well, and detached cells were gently removed with phosphate buffer. Fresh culture medium containing calcitriol and/or MβCD was then added according to the assigned groups. Images of the same wound area were captured at 0, 12, and 24 hours. Scratch width was measured using ImageJ (version 1.8.0, Tanon Science & Technology Co., Ltd., Shanghai, China) to calculate the cell migration rate.

RNA Extraction and qRT-PCR

Total ribonucleic acid was extracted using TRIzol reagent (15596018, Thermo Fisher Scientific, MA, USA), and then treated with DNase I (11284932001, Sigma-Aldrich, MiI, USA). cDNA was synthesized from 1 microgram of RNA using a High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific, Waltham, MA, USA). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) amplification was performed using PowerUp™ SYBR™ Green Master Mix (11732088, Thermo Fisher Scientific, MA, USA). Relative mRNA expression was normalized to *GAPDH* using the $2^{-\Delta\Delta Ct}$ method. Primer sequences are listed in Table 1.

Western Blotting

Cells were lysed with RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) to extract total protein, and protein concentration was determined using a BCA Protein Assay Kit (P0012, Beyotime, Shanghai, China). Equal amounts of protein (20 μg) were separated by polyacrylamide gel electrophoresis (PAGE) and transferred onto PVDF membranes (IPFL00010, Merck, MA, USA). Membranes were blocked with 5% non-fat milk for 1 h and incubated overnight at 4 °C with primary antibodies (Table 2). After incubation with secondary antibodies (goat anti-rabbit IgG-HRP, ab6721, Abcam, 1:2000; goat anti-mouse IgG-HRP, ab205719, Abcam, 1:2000) at room temperature for 1 hour, protein bands were visualized using the Bio-Rad ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA), and relative expression levels were quantified using ImageJ software (version 1.8.0, Tanon Science & Technology Co., Ltd., Shanghai, China).

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). The normality of data distribution was assessed using the Shapiro-Wilk test before statistical analysis. For comparisons among multiple independent groups, one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test was used. For scratch wound assays, in which the same areas were continuously monitored at 0, 12, and 24 h, repeated-measures two-way ANOVA followed by Tukey's multiple comparison test was performed. A p-value < 0.05 was considered statistically significant.

Results

Effects of Calcitriol on the Growth of Human Keratinocytes

The effects of calcitriol on HaCaT cell growth and calcium homeostasis were investigated. Cell metabolic activity was assessed at 0, 2, 4, and 6 days under different calcitriol concentrations. Apoptosis and intracellular Ca^{2+} levels were evaluated on day 6.

CCK-8 analysis showed that the metabolic activity of cells treated with calcitriol gradually decreased over time. From day 4 onward, the OD450 values in the calcitriol group were significantly lower than those in the control group (Fig. 1A, $p < 0.05$). Hoechst 33258 staining revealed a low level of apoptotic nuclear morphology in all groups, with no obvious differences, indicating that calcitriol treat-

Table 1. Primer sequences used for RT-PCR.

Gene	Forward sequence (5′–3′)	Reverse sequence (5′–3′)
CCND1	TCTACACCGACAACTCCATCCG	TCTGGCATTTTGGAGAGGAAGTG
CCND2	GAGAAGCTGTCTCTGATCCGCA	CTTCCAGTTGCGATCATCGACG
CDKN1A	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG
CDKN1C	AGATCAGCGCCTGAGAAGTCGT	TCGGGGCTCTTTGGGCTCTAAA
GAPDH	CAGGAGGCATTGCTGATGAT	GAAGGCTGGGGCTCATTT

Table 2. Information on primary antibodies.

Antibody	Brand	Catalog number	Dilution ratio
Filaggrin	Thermo Fisher, MA, USA	PA5-115235	1:1000
Loricrin	Abcam, Cambridge, UK	ab176322	1:1000
Involucrin	Abcam, Cambridge, UK	ab181980	1:10,000
KRT10	Abcam, Cambridge, UK	ab76318	1:50,000
Claudin-1	Abcam, Cambridge, UK	ab211737	1:2000
Cav1	Abcam, Cambridge, UK	ab32577	1:1000
p-Cav1	Abcam, Cambridge, UK	ab75876	1:2000
TRPV6	Proteintech, IL, USA	13411-1-AP	1:1000
Orai1	Abcam, Cambridge, UK	ab111960	1:1000
GAPDH	Proteintech, IL, USA	60004-1-Ig	1:5000

ment did not induce marked apoptosis (Fig. 1B,C). Fluo-4AM staining further showed that intracellular calcium ion (Ca^{2+}) levels changed in a calcitriol dose-dependent manner (Fig. 1D,E, $p < 0.05$), suggesting that calcitriol may regulate cell function through calcium signaling.

Effects of Calcitriol on Cell Cycle Progression and Barrier-Related Protein Expression in Keratinocytes

Cell cycle regulation and barrier function were assessed by examining the mRNA levels of cell cycle regulatory factors and the expression of key differentiation and barrier proteins.

qRT-PCR analysis showed that calcitriol treatment altered the expression pattern of cell cycle-associated genes. Specifically, CCND1 and CCND2 expression was increased, whereas CDKN1A and CDKN1C expression was decreased in a dose-dependent manner (Fig. 2A–D). These changes indicate that calcitriol modulates cell cycle-associated transcriptional programs during keratinocyte differentiation. However, considering that CCK-8 primarily reflects cellular metabolic activity rather than proliferation capacity, these results should not be interpreted as direct evidence of enhanced cell proliferation. Instead, calcitriol may induce a coordinated metabolic and transcriptional adaptation associated with keratinocyte maturation. Western blot analysis showed that the differentiation-related proteins filaggrin, loricrin, involucrin, and KRT10 increased in a dose-dependent manner. The expression levels of these proteins were significantly higher in the high-dose group than in the control group (Fig. 2E–I, $p < 0.05$), indicating that calcitriol may effectively promote the differentiation of keratinocytes and enhance the expression of epidermal barrier- related proteins.

Effects of MβCD-Mediated Disruption of Cav1-Associated Membrane Signaling on Calcitriol-Induced Keratinocyte Responses

Based on the dose-dependent effects of calcitriol on keratinocyte differentiation markers and intracellular Ca^{2+} levels (Figs. 1,2), 100 nM calcitriol was selected for subsequent mechanistic studies. This concentration exerted robust effects on keratinocyte differentiation and calcium signaling while avoiding the use of a relatively high pharmacological concentration. Although 1000 nM calcitriol did not show obvious cytotoxic effects, 100 nM was considered more appropriate for mechanistic investigation due to its sufficient biological activity and greater relevance to commonly used experimental concentrations.

To further investigate whether Cav1-associated membrane signaling may be involved in calcitriol-induced regulation of keratinocyte responses, CCK-8, Hoechst 33258 staining, and scratch wound assays were performed in the control, calcitriol, and calcitriol + MβCD groups. CCK-8 assays showed that calcitriol significantly reduced HaCaT cell metabolic activity compared with the control group (Fig. 3A, $p < 0.05$), whereas MβCD treatment partially attenuated this effect, suggesting that membrane cholesterol-dependent signaling associated with Cav1-enriched domains may be involved in calcitriol-induced cellular responses. Hoechst 33258 staining revealed that apoptosis-related nuclear changes remained limited in the calcitriol group, with no significant difference compared with the control group. However, the calcitriol + MβCD group exhibited a significantly increased apoptosis rate compared with both the control and calcitriol groups (Fig. 3B,C, $p < 0.05$), suggesting that disruption of Cav1-associated signaling may compromise keratinocyte homeostasis and in-

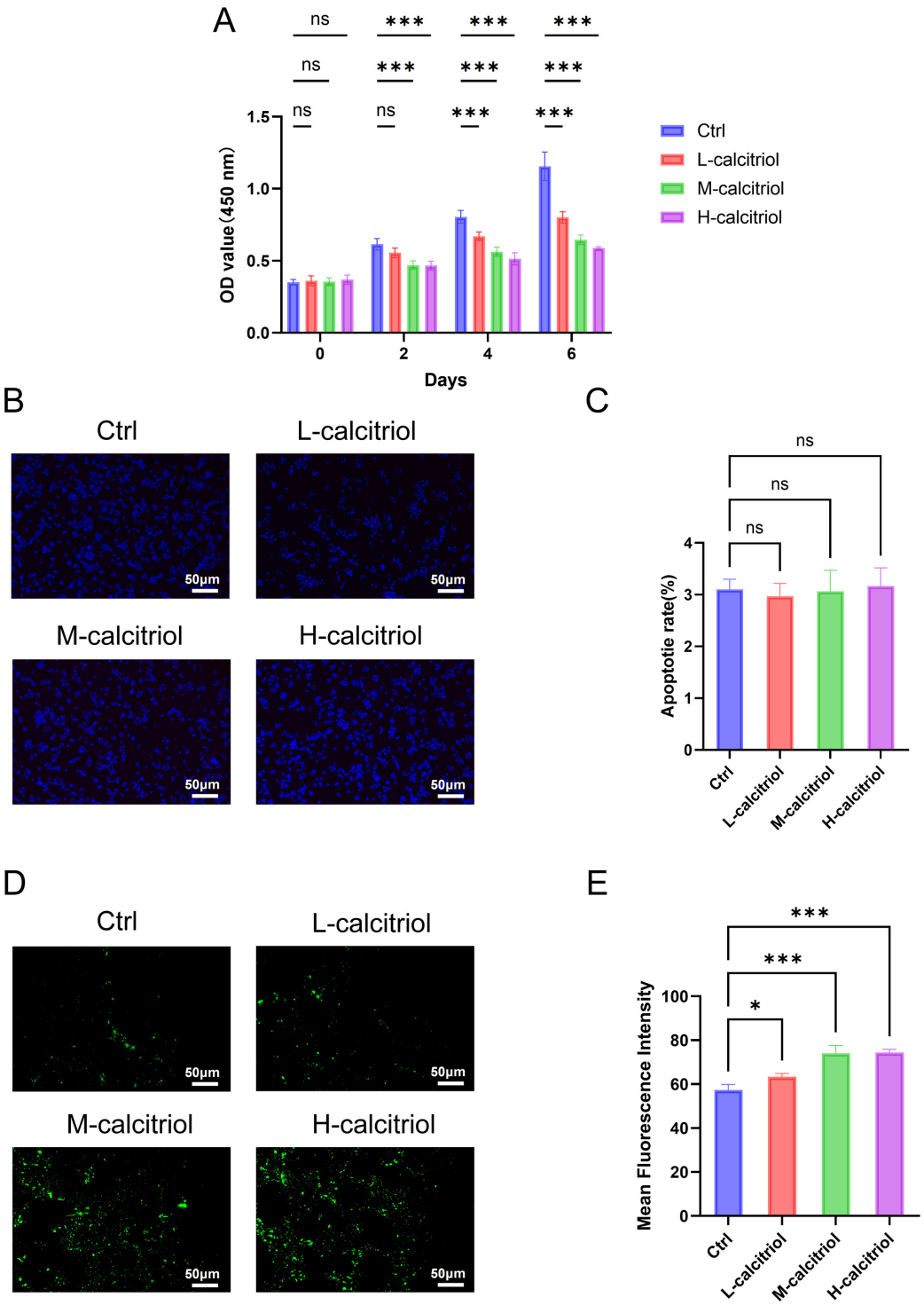

Fig. 1. Effects of calcitriol on HaCaT cell growth. (A) Cell metabolic activity at different time points after calcitriol treatment; (B,C) Hoechst 33258 staining and apoptosis analysis at day 6 (magnification, 200×); (D,E) intracellular Ca^{2+} concentration and fluorescence intensity analysis (magnification, 200×). n = 3, *$p < 0.05$, ***$p < 0.001$, ns $p > 0.05$.

crease cellular susceptibility to apoptotic stress. Scratch wound assays demonstrated that calcitriol significantly enhanced cell migration at 12 h and 24 h compared with the control group ($p < 0.05$), whereas disruption of Cav1-associated membrane signaling by MβCD markedly reduced migration capacity (Fig. 3D,E, $p < 0.05$), suggesting that cholesterol-dependent membrane signaling associated with Cav1-enriched domains may be involved in calcitriol-induced keratinocyte migration.

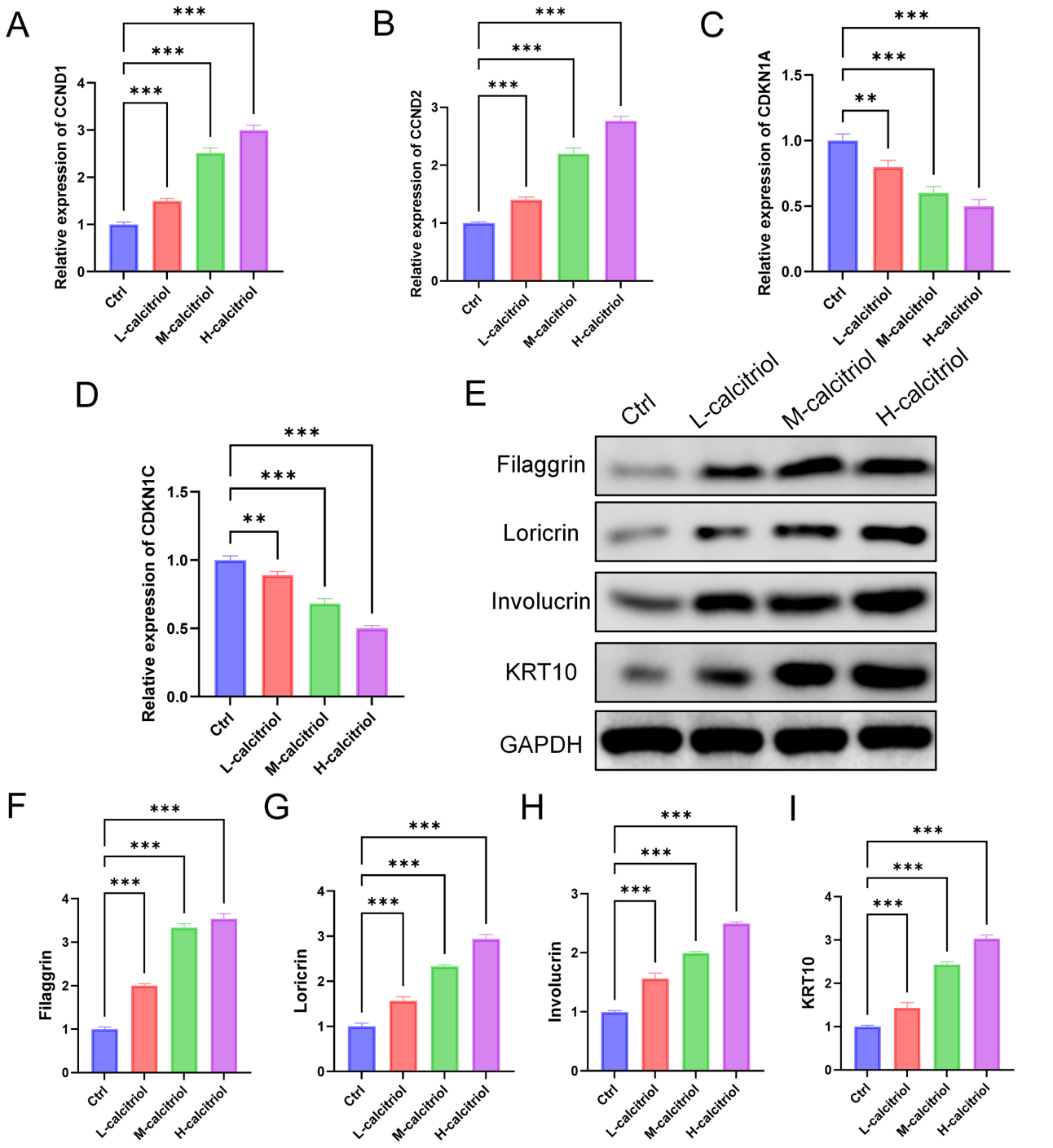

Fig. 2. Effects of calcitriol on cell cycle-associated gene expression and differentiation/barrier-related proteins in HaCaT cells. (A–D) mRNA expression of CCND1, CCND2, CDKN1A, and CDKN1C after calcitriol treatment; (E) Western blot analysis of differentiation/barrier proteins; (F–I) densitometric analysis of the relative protein expression of filaggrin, loricrin, involucrin, and KRT10 normalized to the GAPDH. n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$.

Validation of Cav1-Mediated Effects of Calcitriol on Keratinocyte Differentiation and Barrier-Associated Protein Expression

To further determine whether calcitriol promotes keratinocyte differentiation and barrier protein expression via Cav1 signaling, gene and protein expression levels were assessed. qRT-PCR analysis showed that calcitriol increased the expression of Cyclin D1 and Cyclin D2 while reducing the expression of CDKN1A and CDKN1C (Fig. 4A–D, $p < 0.05$), indicating that calcitriol regulates the expression of cell cycle-associated genes during keratinocyte differentiation. These transcriptional changes were par-

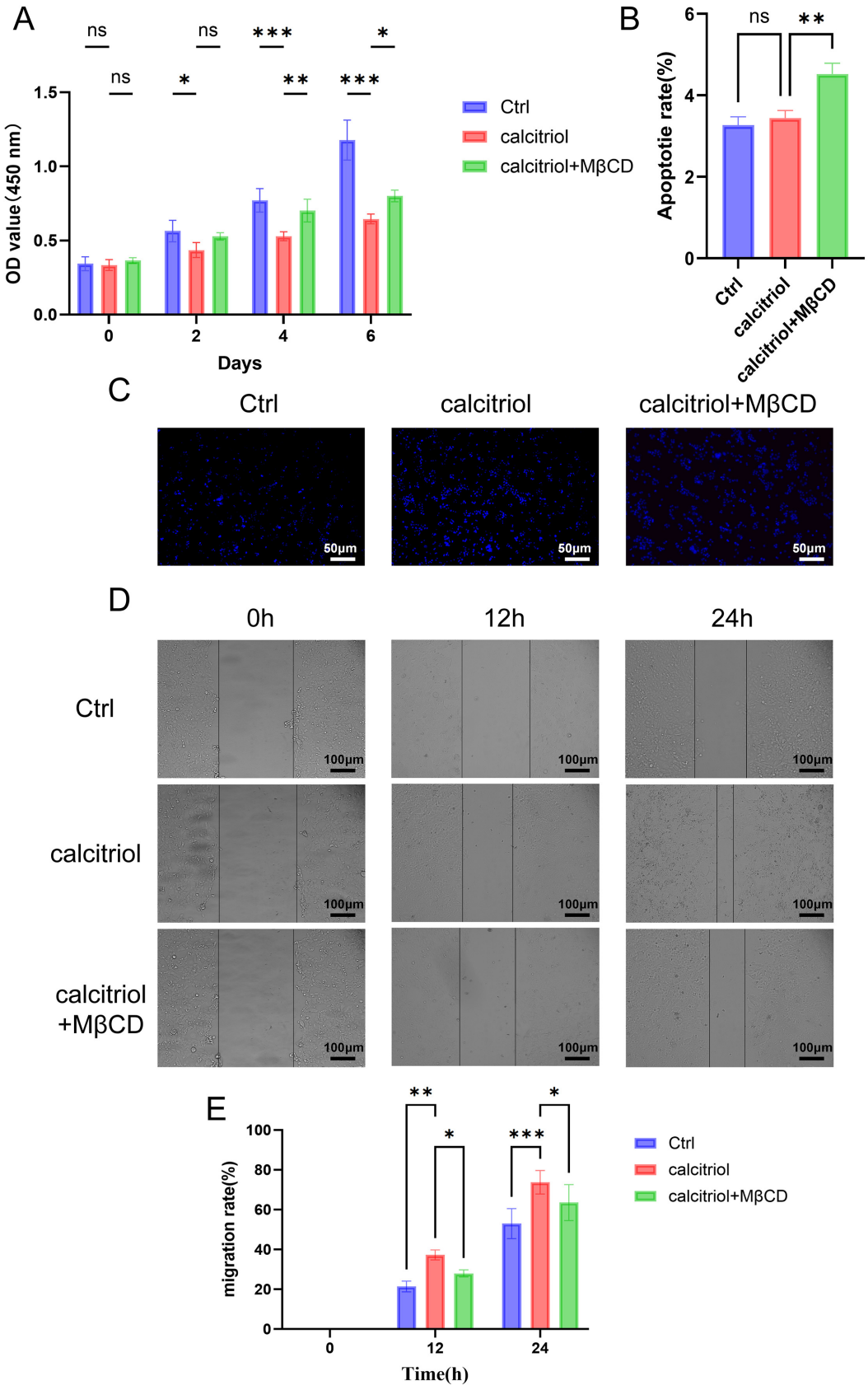

Fig. 3. Effects of Cav1-associated signaling on calcitriol-regulated cellular responses in HaCaT cells. (A) Cell metabolic activity; (B,C) apoptosis detection and analysis (magnification, 200×); (D) scratch assay showing cell migration (magnification, 100×); (E) quantification of wound closure rate. n = 3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns $p > 0.05$.

tially attenuated following disruption of Cav1-associated membrane signaling by MβCD, suggesting that Cav1-associated signaling may be involved in the cell cycle-related molecular responses induced by calcitriol. Western blot analysis further confirmed that calcitriol markedly enhanced the expression of differentiation- and barrier-related proteins compared with control, whereas disruption of Cav1-associated membrane signaling by MβCD attenuated these effects (Fig. 4E–J, $p < 0.05$), suggesting that Cav1-associated membrane signaling may participate in calcitriol-induced keratinocyte differentiation and barrier-associated protein expression.

Effects of Calcitriol on Ca^{2+} Transport Capacity in Keratinocytes

To further examine the association between calcitriol-induced calcium responses and Cav1-related signaling, the expression of Cav1, p-Cav1, TRPV6, and Orai1 proteins and intracellular Ca^{2+} levels were examined. Western blot analysis showed that calcitriol significantly increased the expression of these proteins compared with the control group, whereas their expression levels were lower in the calcitriol + MβCD group. Notably, Cav1 phosphorylation was also markedly reduced following MβCD treatment (Fig. 5A–D, $p < 0.05$). Intracellular Ca^{2+} fluorescence assays further demonstrated that calcitriol-treated cells exhibited the highest fluorescence intensity, whereas MβCD-mediated disruption of Cav1-associated membrane signaling markedly reduced this signal (Fig. 5E,F, $p < 0.05$). These findings indicate an association between calcitriol-induced calcium responses and changes in Cav1-related signaling components. However, whether Cav1 directly regulates these calcium responses or acts upstream of TRPV6 and Orai1 remains to be determined.

Discussion

This study systematically evaluated the biological effects of calcitriol in HaCaT cells, with a focus on its role in promoting cell differentiation and barrier-related protein expression through intracellular Ca^{2+}. Combined functional and molecular analyses demonstrated that calcitriol markedly promotes keratinocyte differentiation and enhances barrier function, suggesting an important role in the formation and maintenance of the epidermal barrier.

Effects of Calcitriol on Keratinocyte Growth and Calcium Homeostasis

Our study systematically evaluated the biological effects of calcitriol in HaCaT cells, focusing on its role in regulating intracellular Ca^{2+} homeostasis, promoting cell differentiation, and enhancing barrier-associated protein expression. The results showed that calcitriol treatment moderately reduced cell metabolic activity without inducing obvious apoptosis, while increasing intracellular free Ca^{2+} levels. These findings suggest that the calcitriol-induced increase in intracellular Ca^{2+} may be associated with changes in keratinocyte differentiation. This is consistent with previous studies showing that calcitriol has been reported to affect calcium channel-related processes involving TRPV6 and Orai1, which may contribute to changes in intracellular Ca^{2+} concentrations and regulating the function of skin cells [19]. Calcium signaling is a key regulator of keratinocyte differentiation, and elevated Ca^{2+} concentration promotes the activation of differentiation-related pathways [10]. Therefore, our functional analyses indicate that the calcitriol-induced regulation of calcium homeostasis plays an important role in HaCaT cell physiology.

Calcitriol Promotes Keratinocyte Differentiation and Barrier-Associated Molecular Responses Through Cell Cycle Regulation

Gene expression analysis of cell cycle regulators revealed that calcitriol increased the expression of CCND1 and CCND2 while reducing CDKN1A and CDKN1C expression. These results suggest that calcitriol modulates cell cycle-associated transcriptional programs during keratinocyte differentiation. However, changes in the expression of CCND1, CCND2, CDKN1A, and CDKN1C do not necessarily indicate enhanced or suppressed cell proliferation, as keratinocyte differentiation involves complex alterations in cell cycle-associated gene expression. Therefore, these molecular changes may represent transcriptional remodeling during differentiation rather than a direct promotion of cell cycle progression. Although cell cycle regulation is closely associated with keratinocyte maturation, further studies are required to clarify the precise relationship between calcitriol-induced cell cycle changes and differentiation processes. In addition, calcitriol significantly increased the expression of differentiation- and barrier-related proteins, including filaggrin, loricrin, involucrin, and KRT10, further supporting its positive role in epidermal barrier repair and reconstruction. These results align with recent studies showing that calcitriol activates VDR-mediated transcriptional networks, thereby suppressing proliferation and promoting terminal differentiation [20,21,22]. In particular, calcitriol treatment has been reported to improve epidermal barrier integrity by regulating barrier-associated proteins and tight junction components in atopic dermatitis models, supporting its potential role in epidermal regulation of barrier-associated proteins and homeostasis [13,23].

Preliminary Investigation of Cav1-Associated Signaling Involvement in Calcitriol-Mediated Keratinocyte Differentiation

To clarify the role of Cav1 in calcitriol-mediated regulation, Cav1-associated membrane signaling was disrupted using MβCD. This intervention altered the biological effects of calcitriol, increasing cell proliferation while reduc-

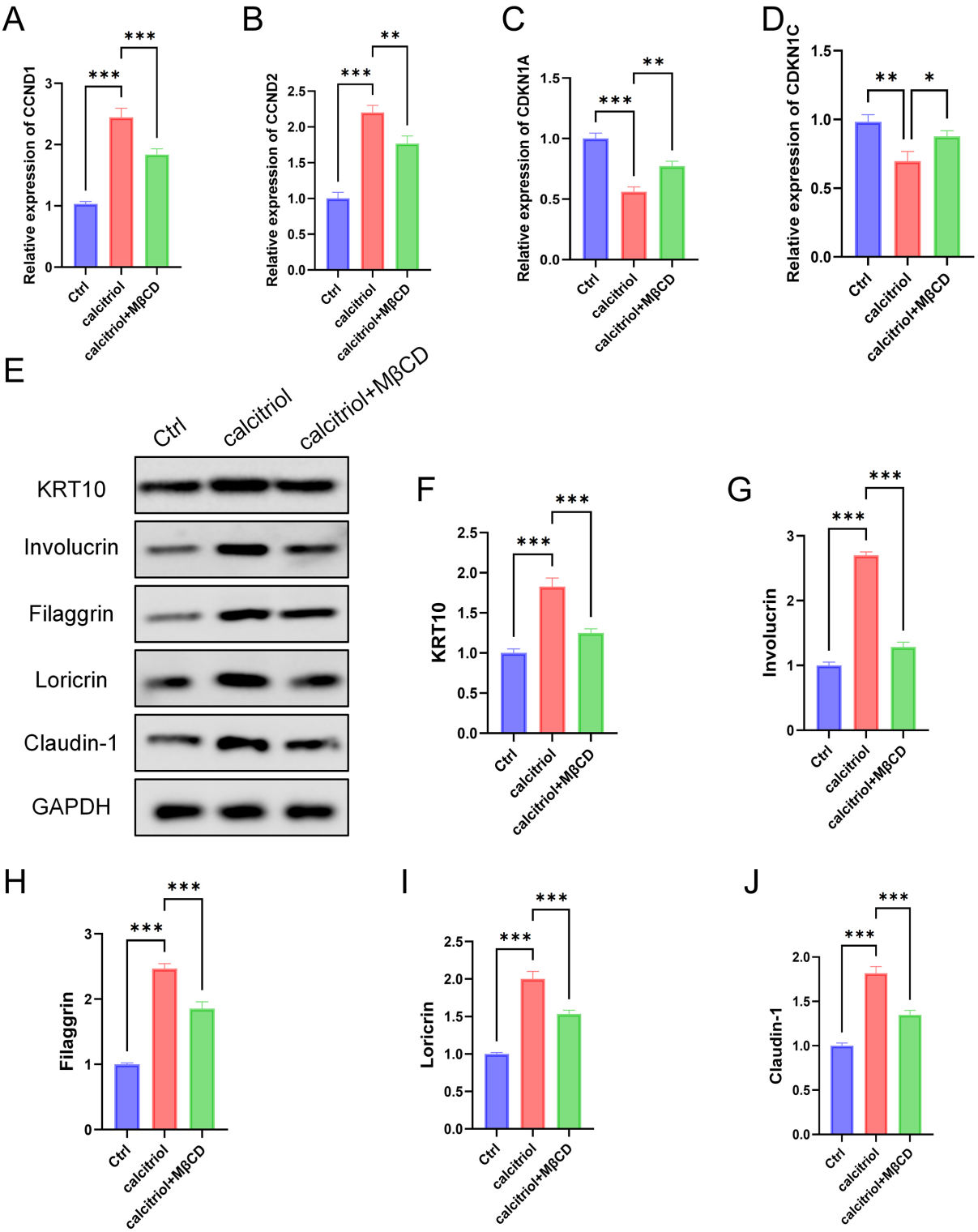

Fig. 4. Effects of MβCD-mediated disruption of Cav1-associated signaling on calcitriol-induced keratinocyte differentiation and barrier protein expression. (A–D) mRNA expression of CCND1, CCND2, CDKN1A, and CDKN1C; (E) Western blot analysis of differentiation/barrier proteins; (F–J) densitometric analysis of the relative protein expression of KRT10, involucrin, filaggrin, loricrin, and claudin-1 normalized to the GAPDH. n = 3, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

ing migration and resistance to apoptosis, suggesting that Cav1-associated membrane signaling may be involved in the cellular responses induced by calcitriol. Recent research has identified Cav1 as a key membrane signaling component involved in calcium channel regulation and epidermal differentiation [24]. Consistent with these find-

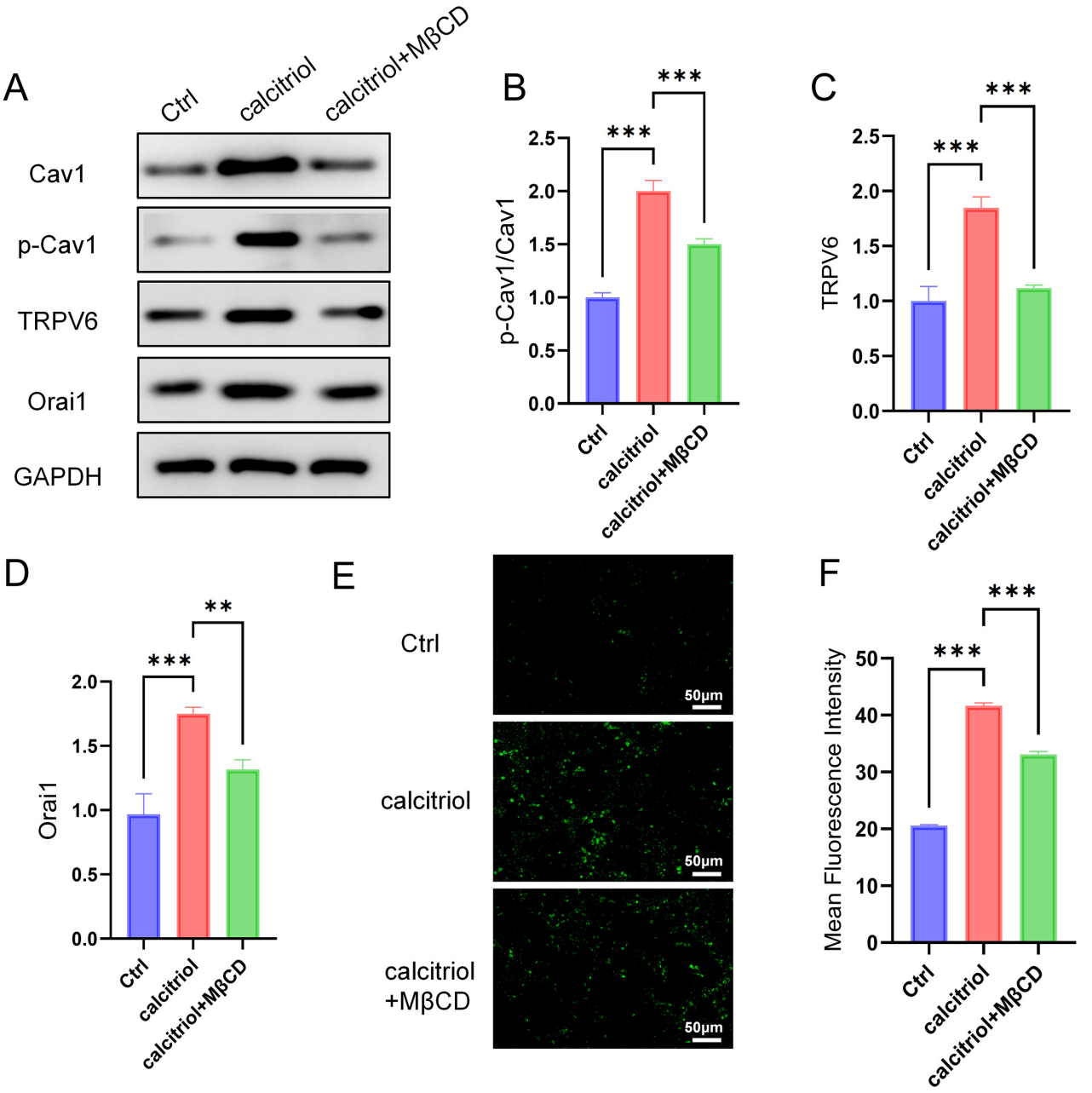

Fig. 5. Involvement of Cav1-associated calcium signaling in calcitriol-induced calcium responses in HaCaT cells. (A–D) Expression and densitometric analysis of the relative protein expression of p-Cav1/Cav1, TRPV6, and Orai1 proteins; (E) intracellular Ca^{2+} fluorescence images (magnification, 200×); (F) quantitative fluorescence intensity analysis. n = 3, $^{**}p < 0.01$, $^{***}p < 0.001$.

ings, our results suggest that calcitriol-induced changes in intracellular Ca^{2+} levels are associated with alterations in Cav1-related signaling and calcium transport proteins. The increase in intracellular Ca^{2+} may be associated with the enhanced expression of keratinocyte differentiation and barrier-associated proteins observed following calcitriol treatment.

Mechanistic Analysis of Calcitriol-Cav1 Signaling in Barrier-Associated Differentiation Responses

To clarify the role of Cav1 in calcitriol-mediated barrier regulation, the expression of barrier-related genes and proteins was evaluated. calcitriol treatment significantly increased the expression of KRT10 and other barrier-associated markers, whereas disruption of Cav1-associated signaling reduced their expression to varying degrees. These findings suggest that calcitriol promotes the expression of epidermal barrier-associated proteins, which may be

associated with Cav1-related signaling, consistent with previous reports implicating Cav1 in regulating epidermal tight junctions [25]. Calcitriol signaling may potentially interact with Cav1-associated membrane signaling and calcium-regulatory pathways [26], although the molecular relationship between VDR signaling and Cav1 remains to be established. Notably, claudin-1 is a key tight junction protein essential for barrier integrity. Previous studies have shown that calcitriol regulates tight junction-associated proteins and improves epidermal barrier function in human keratinocytes, supporting its potential involvement in epidermal barrier-associated molecular responses [27,28].

Perspectives on Calcitriol-Mediated Regulation of Cav1-Related Calcium Transport

The regulation of calcium homeostasis by calcitriol is considered an important component of keratinocyte differentiation and epidermal homeostasis. Previous studies have shown that vitamin D signaling regulates epidermal differentiation and calcium-dependent processes, thereby promoting keratinocyte maturation and maintaining epidermal barrier integrity [29,30]. In this study, calcitriol treatment increased Cav1 expression and phosphorylation and was accompanied by increased expression of TRPV6 and Orai1 and elevated intracellular Ca^{2+} levels. These findings suggest that calcitriol-induced calcium responses are associated with changes in Cav1-related signaling and calcium transport proteins. TRPV6, a highly calcium-permeable channel, plays an important role in epidermal calcium transport and keratinocyte differentiation [31]. calcitriol may influence intracellular Ca^{2+} homeostasis in association with altered TRPV6 expression. In addition, Orai1, a pore-forming component of the store-operated calcium entry (SOCE) pathway, is a key regulator of intracellular Ca^{2+} homeostasis and contributes to keratinocyte differentiation and epidermal homeostasis [32]. Therefore, the coordinated changes in Cav1, TRPV6, Orai1, and intracellular Ca^{2+} observed following calcitriol treatment suggest that these calcium-regulatory components may be associated with calcitriol-induced keratinocyte differentiation and barrier-associated protein expression.

Furthermore, cholesterol-dependent caveolae-associated signaling by MβCD attenuated intracellular Ca^{2+} elevation, differentiation marker expression, and barrier-associated protein production, suggesting that Cav1-associated membrane signaling may be involved in calcitriol-induced keratinocyte differentiation and barrier-associated responses. Previous studies have identified Cav1 as an important regulator of membrane signaling platforms that coordinate ion channel activity and epithelial cell functions [33,34]. Collectively, our findings identify an association between calcitriol-induced keratinocyte differentiation, intracellular Ca^{2+} responses, and changes in Cav1-related calcium signaling components. The attenuation of these responses following MβCD treatment further suggests that cholesterol-dependent membrane signaling associated with Cav1-enriched domains may be involved. However, the present findings do not establish a direct causal relationship between Cav1 and downstream calcium-regulatory proteins, and further genetic and functional studies are required to clarify this potential mechanism.

Several limitations should be acknowledged in this study. First, the use of HaCaT cells as an *in vitro* model cannot fully reproduce the complex structure and physiological characteristics of human epidermis. Future studies using reconstructed epidermal models or functional barrier assays are required to validate the effects of calcitriol on skin barrier regulation. Second, keratinocyte differentiation was induced under high-calcium conditions, which may partially overlap with calcium-induced differentiation signals; therefore, calcium-controlled models are needed to further clarify the specific contribution of calcitriol. Third, although MβCD was used to disrupt cholesterol-dependent membrane signaling associated with Cav1-enriched domains, it is not a specific Cav1 inhibitor and may affect multiple membrane-related pathways. Thus, the current findings support the involvement of Cav1-associated signaling but do not establish a direct causal role for Cav1. Further validation using genetic approaches, such as Cav1 knockdown or overexpression, is required. Finally, CCK-8 assays and cell cycle-associated gene analyses do not directly reflect cell proliferation or cell cycle distribution, and additional approaches are needed to further define the relationship between calcitriol-induced differentiation and cell cycle regulation.

Conclusion

This study demonstrates that calcitriol promotes keratinocyte differentiation and enhances the expression of epidermal barrier-associated proteins. calcitriol treatment increased intracellular Ca^{2+} levels and was accompanied by enhanced expression of Cav1-associated calcium signaling components, suggesting that Cav1-related calcium signaling may contribute to calcitriol-mediated regulation of keratinocyte differentiation and barrier-associated protein expression. Although further genetic validation is required to define the specific role of Cav1, these findings provide new insights into the mechanisms underlying calcitriol-mediated epidermal homeostasis and suggest that the association between calcitriol signaling, Cav1-related calcium responses, and keratinocyte differentiation warrants further investigation.

Availability of Data and Materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

CC and ZFW designed the research study. CC performed the research and collected the data. ZFW supervised the study and contributed to data interpretation. CC drafted the manuscript. ZFW critically revised the manuscript for important intellectual content. Both authors gave final approval of the version to be published. Both authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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