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Original Article

Reduced hydration-induced decreased caveolin-1 expression causes epithelial-to-mesenchymal transition

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Abstract: The reduced hydration environment induced by disruption of epithelial barrier function after injury results in excessive scarring, but the underlying mechanisms are poorly understood. We demonstrated that exposing keratinocytes to a reduced hydration environment causes epithelial-to-mesenchymal transition (EMT) and induces caveolin-1-dependent downregulation of E-cadherin. Reduced caveolin-1 expression and increased Snail expression are associated with low expression levels of E-cadherin. Caveolin-1 downregulation increases the transcriptional activity of β-catenin-TCF/LEF-1, and overexpression of caveolin-1 inhibits EMT that results from reduced hydration. Our findings suggest a role for caveolin-1 downregulation in linking aberrant EMT to the reduced hydration environment: findings that may lead to new developments in the prevention and treatment of hypertrophic scar.

Keywords: Caveolin-1, EMT, reduced hydration

Introduction

As a fundamental biological process, the repair of skin wounds is important to the continuity of life. The ideal outcome for wound repair is functional and scarless healing, which rarely occurs because any disturbances in the wound microenvironment, such as a perturbed barrier, mechanical stimulation [1], matrix stiffness and chemical stimuli [2, 3], have considerable impacts on the regulation of cell behaviors, inevitably giving rise to hypertrophic scars. Among the numerous factors influencing wound healing, the hydration status of the environment is one of the most important [4-7]. The main reason why mucosal wounds exhibit faster healing and minimal scarring than skin wounds is that a fully hydrated environment is present in mucosal wounds, which can reduce the excessive inflammatory response and dermal collagen hypertrophy [8, 9]. The important role of epithelial hydration environment in wound healing has been highlighted in many researches. Studies have found that silicone gels can reduce scar formation and have established the mechanism firmly to be due to proving an appropriate hydration environment with resulting decreased in proinflammatory cytokine expression. However, reduced hydration environment can led to the up-regulation of proinflammatory cytokine that are involved in cutaneous wound repair and scarring [10, 11]. An abnormal proliferation and differentiation of the epidermis and a fibroproliferative disorder of the dermis are the major histological features of hypertrophic scars, and these pathological structures are the leading causes for the clinical manifestation of scarring, such as abnormal sensations, contractures, erosion of the skeletal structure, and even lifelong disability [12]. However, little is known about how the reduced hydration environment affects the formation of the pathological structures that are observed in hypertrophic scars.

Epithelial-mesenchymal transition (EMT), which is characterized by the loss of epithelial features and gain of a mesenchymal-like phenotype, is an intricate biological process of embryonic development, tissue repair, and diseas-

es [13]. Growing evidence has revealed the widespread relevance of EMT in chronic diseases such as cancer progression and fibrosis [14]. The common features among these diseases are that the transformed epithelial cells detach, produce matrix-degrading proteolytic enzymes, and exhibit fibroblast-like characteristics [15]. When fibrosis occurs in the kidney, liver and lung, myofibroblasts arise from the conversion of epithelial cells through the EMT process and promote fibrosis [14, 16, 17]. In addition, EMT also contributes to skin fibrotic diseases, such as systemic sclerosis and psoriasis [18, 19], and recent studies reported that EMT-related genes were elevated and EMT-like features could be observed in hypertrophic scars, indicating the involvement of EMT in the occurrence of scarring [20, 21]. Cellular reprogramming during EMT is initiated by diverse extracellular stimuli, and we speculated that a reduced hydration environment may act as the "trigger point" for EMT and thus result in hypertrophic scar formation.

Caveolin-1 is a major structural protein of caveolae and functions as a signaling platform for processes such as membrane trafficking, lipid metabolism and signal transduction [22]. Accumulated evidence indicates that caveolin-1 negatively regulates organ fibrosis, including lung, heart, liver and skin fibrosis [23-25]. Recent findings revealed that caveolin-1 downregulation may result in an aberrant response to mechanical stimuli from the local environment, resulting in collagen accumulation and scar formation [26]. Thus, as a scaffold for a variety of signaling molecules, caveolin-1 transmits extracellular stimulus into cells and converts them into biological information, and the absence of caveolin-1 may lead to the progression of various fibrotic disorders [27]. However, whether caveolin-1 responds to hydration status changes in the environment to modulate cell function and hypertrophic scar formation, is still unknown.

In this study, a reduced hydration environment was examined as a potential trigger for EMT. We showed that reduced hydration leads to negative regulation of caveolin-1, which results in the downregulation of E-cadherin and increased transcriptional activity of β-catenin-TCF/LEF-1, enhancing the EMT process. Our results implicate caveolin-1 as an important modulator of EMT and confirm caveolin-1 as a promising antifibrotic target in fibrotic diseases, such as excessive scarring.

Materials and methods

Cell culture and treatment

Primary keratinocytes were isolated from human skin specimens that were obtained by skin grafting. Dispase (Life Technologies) was used to separate the epidermis from the dermis, and the epidermal layer was digested with trypsin (Corning). Cells were harvested by filtering through a 100 mm cell filter (BD Biosciences, Bedford, MA, USA) and were then pelleted and cultured in Defined Keratinocyte Serum-Free Medium (Gibco, Grand Island, NY, USA). The cells were used at passages 2-4.

Different hydration conditions were created by controlling the sodium concentration of the culture environment. Previous studies have shown that an increase in sodium flux and alterations in the expression of downstream genes that are involved in cutaneous wound healing and hypertrophic scar formation were caused by the reduced hydration status [6]. Thus, the reduced hydration status was simulated by increasing the extracellular sodium concentration by 10% in the keratinocyte monolayer culture model, which induced sodium flux and alterations of downstream gene expression (**Figure 1A**). A 10% increase in sodium corresponds to a physiological level reached in the extracellular fluid in a water-deprived thirsty animal.

RNA interference

Cells were plated and grown to approximately 80% confluency. SMARTpool: siGENOME snail siRNA (Dharmacon, Lafayette, CO, USA) was used to knock down the expression of snail in keratinocytes. siGENOME Non-Targeting siRNA Pools (Dharmacon) that were composed of four siRNAs designed to target no known genes in humans were used as negative controls for siRNA. The DharmaFECT 1 siRNA Transfection Reagent (Dharmacon) was used to transfect keratinocytes with caveolin-1, snail or scrambled siRNA oligonucleotides. The efficiencies of RNA interference were confirmed by Western blotting analysis.

Plasmids

The plasmid pUbC-GFP expressed GFP from the ubiquitin C (UbC) promoter (Addgene, Cambridge, MA). pCMV-Cav1 expressed GFP-tagged caveolin-1 from the CMV promoter (Origene, Rockville, MD), and pUbC-Cav1 expressed GFP-tagged caveolin-1 from the ubiquitin promoter cloned from the pUbC-GFP and pCMV-Cav1 plasmids. All plasmids were purified using Qiagen Giga-prep kits (Qiagen, Chatsworth, CA).

Quantitative real-time PCR assay

Total RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), after which RNA was reverse transcribed with a thermocycler (S1000, Bio-Rad) and the First Strand cDNA Synthesis Kit (Invitrogen). Real-time RT-PCR was performed using the SYBR qPCR mix (Toyobo) and a Real-Time PCR Detection System (Bio-Rad iQ5). β-Actin was used as a normalization control, and the relative gene expression was analyzed using the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: Snail forward: 5'-ATG CCG CGC TCC TTC CTG GTC AGG-3', reverse, 5'-TCA GCG AGG GCC TCC GGA GCA GCC-3'; E-cadherin forward: 5'-CGA GAG CTA CAC GTT CAC GG-3', reverse, 5'-GGG TGT CGA GGG AAA AAT AGG-3'; and β-actin forward: 5'-GTG GTG GTG AAG CTG TAG CC-3', reverse, 5'-GAC GAG GCC CAG AGC AAG AGA GG-3'.

Western blotting

Total cell proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer (Invitrogen). Nuclear and cytosolic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein were separated by SDS-PAGE (polyacrylamide gel electrophoresis), transferred onto nitrocellulose membranes (Amersham, Chalfont, UK) for immunoblotting, and incubated with 5% skim milk for blocking. Mouse anti-human E-cadherin (Abcam), mouse anti-human vimentin (Abcam), mouse anti-human caveolin-1 (Abcam), mouse anti-human snail (Abcam), mouse anti-human fibronectin (Abcam), mouse anti-human twist (Abcam), mouse anti-human Lamin B1 (Proteintech, Rosemont, IL, USA), mouse anti-human α-tubulin (Sigma) and mouse anti-human β-actin (Sigma) were used as primary antibodies. A horseradish peroxide (HRP)-conjugated antibody (Vector) was used as a secondary antibody. The bands were visualized using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare). Band intensity was quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Coimmunoprecipitation assay

Cells were lysed with precooled RIPA buffer containing protease inhibitors (Roche, Indianapolis, IN) and precleared by incubation with protein A cross-linked to agarose (Sigma). The cell lysates were then incubated with an anti-E-cadherin antibody or anti-caveolin-1 antibody. The immunocomplexes were collected by centrifugation and then incubated with a specific antibody against E-cadherin or caveolin-1. HRP-conjugated antibody was used as a secondary antibody, and signals were detected using an ECL detection kit (GE Healthcare).

Luciferase reporter assays

To measure the transcriptional activity of TCF/LEF-1, cells were transiently transfected with a TCF/LEF-1 reporter (pTOP-FLASH) or an empty plasmid (pFOP-FLASH). Then, the cells were lysed in Passive Lysis Buffer (Promega). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) with a GloMax Luminometer (Promega) according to the manufacturer's instructions. In addition, pA3Luc-caveolin-1, pGL-E-cadherin vector, or pLuc-snail was transfected as described above to measure the promoter activity of caveolin-1, E-cadherin and snail, respectively.

Immunofluorescence staining

Cells were grown on glass coverslips and then fixed in 4% formaldehyde, permeabilized in PBS containing 0.2% Triton X-100, blocked with 1% BSA, and incubated with anti-E-cadherin, anti-vimentin, and anti-caveolin-1 primary antibodies at 4°C overnight. After washing, the cells were further incubated with Alexa Fluor 594-conjugated secondary antibody or Fluor 488-conjugated secondary antibody (Abcam). Cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). After the final washes and mounting, cells were visualized and imaged using a fluorescence microscope (BX51 WI Olympus).

Statistical analysis

The data in this study are expressed as the mean ± standard deviation (SD). The number of independent replicates of every experiment is represented using the letter 'n' in the figure legend. The differences between experimental groups were compared using paired Student's t-test and ANOVA test. All statistical analyses were performed using SPSS 18.0 software (SPSS, Chicago, IL, USA), with $P < 0.05$ considered to be statistically significant.

Results

Epithelial cells that are cultured under reduced hydration undergo EMT

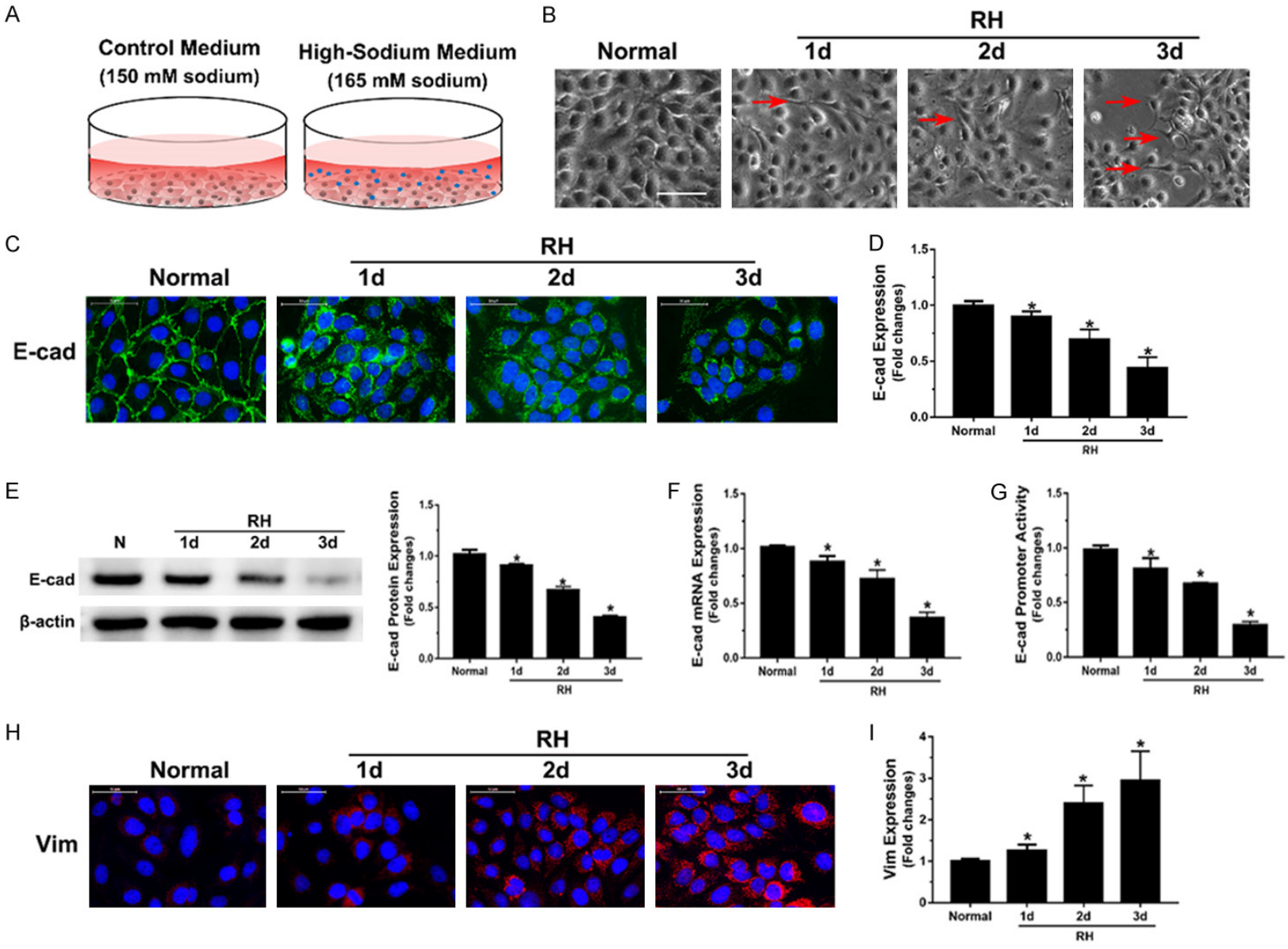
The reduced hydration status was simulated by increasing the extracellular sodium concentration by 10% in the keratinocyte monolayer culture model, which induced sodium flux and alterations of downstream gene expression (**Figure 1A**). The plasticity of keratinocytes in mesenchymal morphology could be observed after 1 day of treatment with high sodium medium and became much more evident with prolonged culture time. As shown by optical microscopy, in contrast to the cells cultured under normal conditions, which were flat and formed tight cell-cell contacts, cells cultured in high sodium medium lost cell-cell adhesions and exhibited spindle-shaped morphology and "long-armed" intercellular connections, demonstrating the morphological plasticity of keratinocytes after high sodium treatment (**Figure 1B**). Immunofluorescence staining showed that E-cadherin was evenly distributed in a continuous pattern near the apical surface at cell-cell contacts under normal conditions, while 1 day of high sodium treatment resulted in internalization of E-cadherin protein from the cell-cell contacts, which became more obvious with prolonged culture time (**Figure 1C**). Moreover, the expression of E-cadherin protein decreased gradually over time after high sodium treatment (**Figure 1D**, **1E**). Additionally, high sodium treatment suppressed the mRNA expression and promoter activity of E-cadherin in a time-dependent manner (**Figure 1F**, **1G**), suggesting that high sodium treatment causes downregulation of E-cadherin expression at the transcriptional level. Vimentin fibers were observed in the perinuclear regions of keratinocytes treated with high sodium medium for 1 day, and the expression of vimentin gradually increased with prolonged treatment with high sodium (**Figure 1H**, **1I**). Western blot analysis also showed increased expression of vimentin in a time-dependent manner when keratinocytes were cultured under high sodium conditions compared with normal conditions (**Figure 1J**). Meanwhile, the mRNA expression of IL-1β, IL-6 and TNF-α increased gradually over time after high sodium treatment (**Figure 1K-M**).

Reduced hydration induced downregulation of caveolae and E-cadherin

To test whether there is an interaction between caveolin-1 and E-cadherin, we first detected the expression of caveolin-1, and the results showed that the expression of caveolin-1 protein in cells after high sodium treatment decreased gradually over time (**Figure 2A**). Meanwhile, the mRNA expression and promoter activity of caveolin-1 also decreased in a time-dependent manner (**Figure 2B**, **2C**). Additionally, a double immunohistochemical staining experiment was carried out and revealed that partial colocalization of E-cadherin and caveolin-1 could be observed at cell-cell contacts under normal conditions. High sodium treatment caused a substantial loss of cell surface-associated E-cadherin and caveolin-1 expression, and both proteins were in an intracellular compartment after high sodium treatment (**Figure 2D**). Consistent with this finding, coimmunoprecipitation showed that E-cadherin and caveolin-1 were associated with each other under normal conditions, and the association dissipated gradually after prolonged high sodium treatment (**Figure 2E**).

β-catenin dissociates from E-cadherin, accumulates in the nucleus and increases TCF/LEF-1 transcriptional activity under reduced hydration

Since E-cadherin associates with β-catenin at intercellular junctions, we asked whether the reduced hydration-induced disruption of cell-cell junctions and internalization of E-cadherin might lead to the redistribution of β-catenin localized at the membrane. A coimmunoprecipitation assay showed that the binding of E-cadherin and β-catenin was decreased over time in keratinocyte monolayer cultures with high sodium treatment (**Figure 3A**). Moreover, immunofluorescence staining analysis showed


A
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(150 mM sodium)
High-Sodium Medium
(165 mM sodium)
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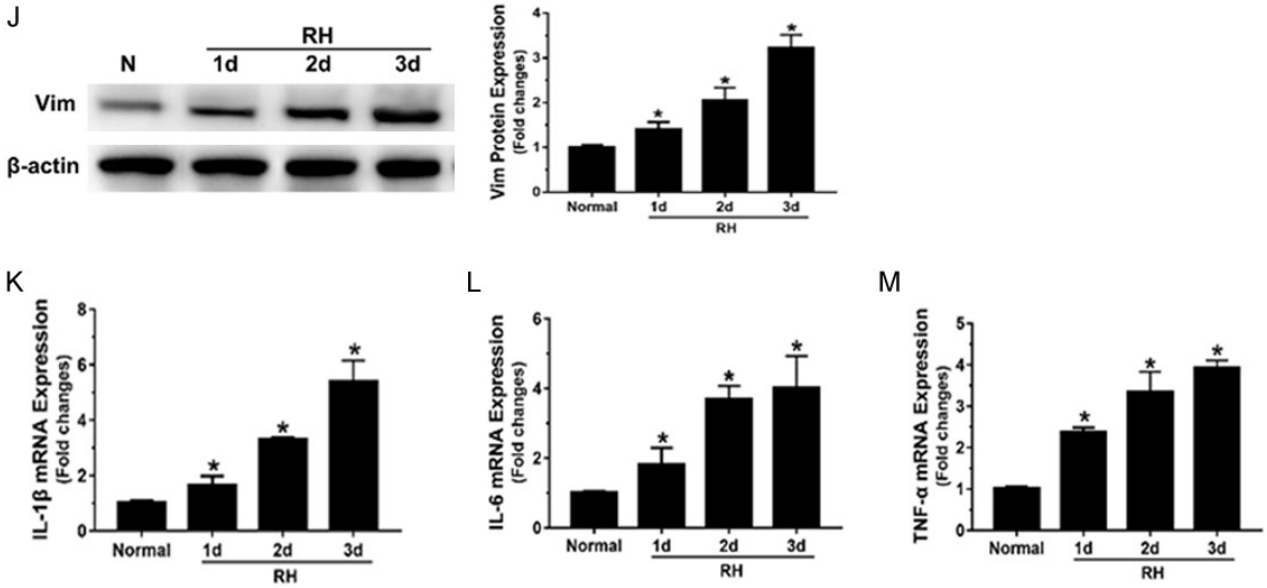


Figure 1. EMT in a keratinocyte monolayer culture model with or without high sodium treatment. A. Schematic drawing of the keratinocyte monolayer culture model with or without high sodium treatment. B. The morphological plasticity of keratinocytes with or without high sodium treatment. Scale bar = 50 mm. C, D. Immunofluorescence staining showing the expression of E-cadherin in the keratinocyte monolayer culture model with or without high sodium treatment. Scale bar = 50 mm. E. Quantification of the expression of E-cadherin by Western blot analysis (n = 4). F. The mRNA expression of E-cadherin in the keratinocyte monolayer culture model with or without high sodium treatment (n = 6). G. The promoter activity of E-cadherin in the keratinocyte monolayer culture model with or without high sodium treatment (n = 6). H, I. Immunofluorescence staining showing the expression of vimentin in the keratinocyte monolayer culture model with or without high sodium treatment. Scale bar = 50 mm. J. Quantification of the expression of vimentin by Western blot analysis (n = 4). K-M. The mRNA expression of IL-1β, IL-6 and TNF-α in the keratinocyte monolayer culture model with or without high sodium treatment (n = 6). RH = reduced hydration. $*P < 0.05$.

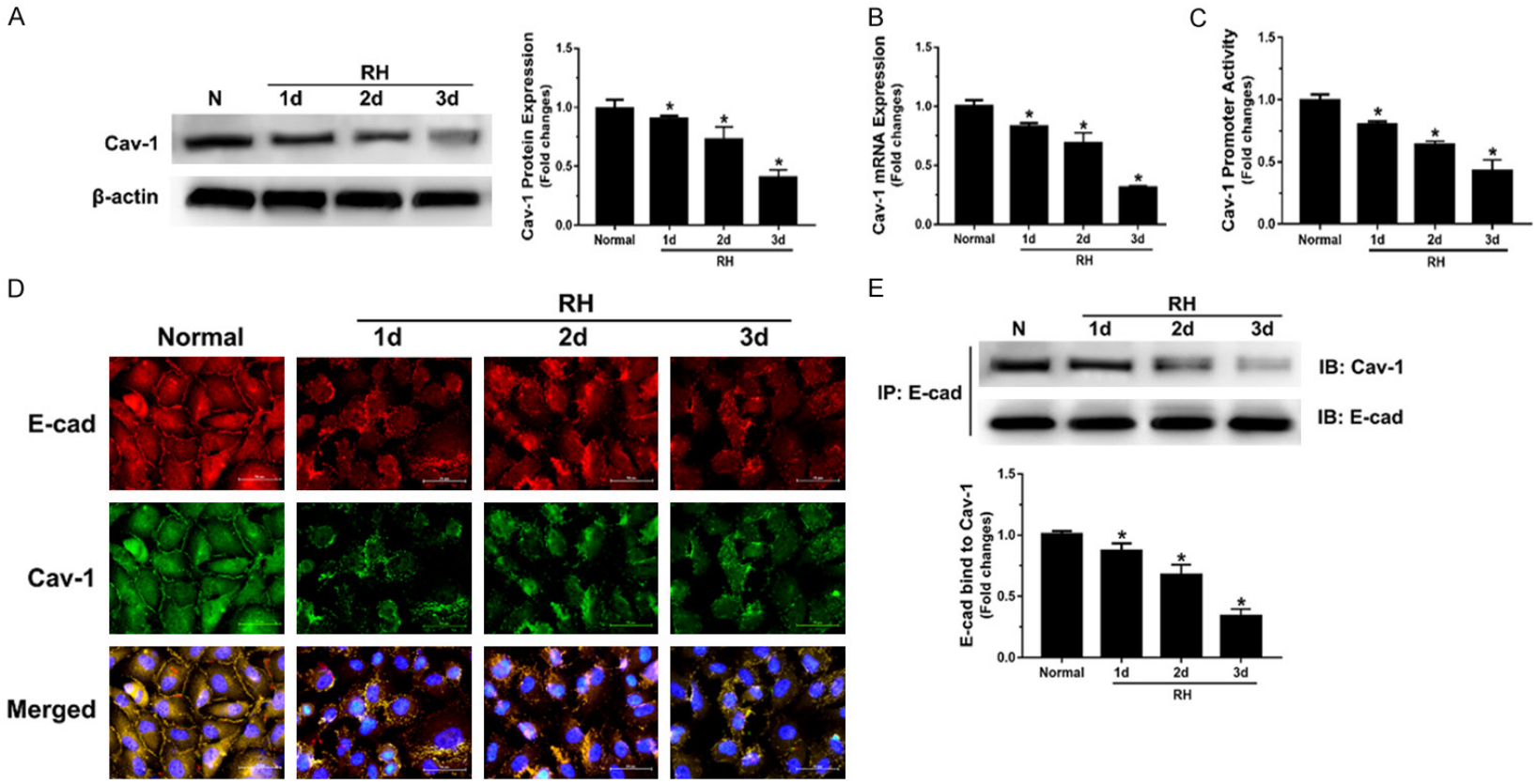


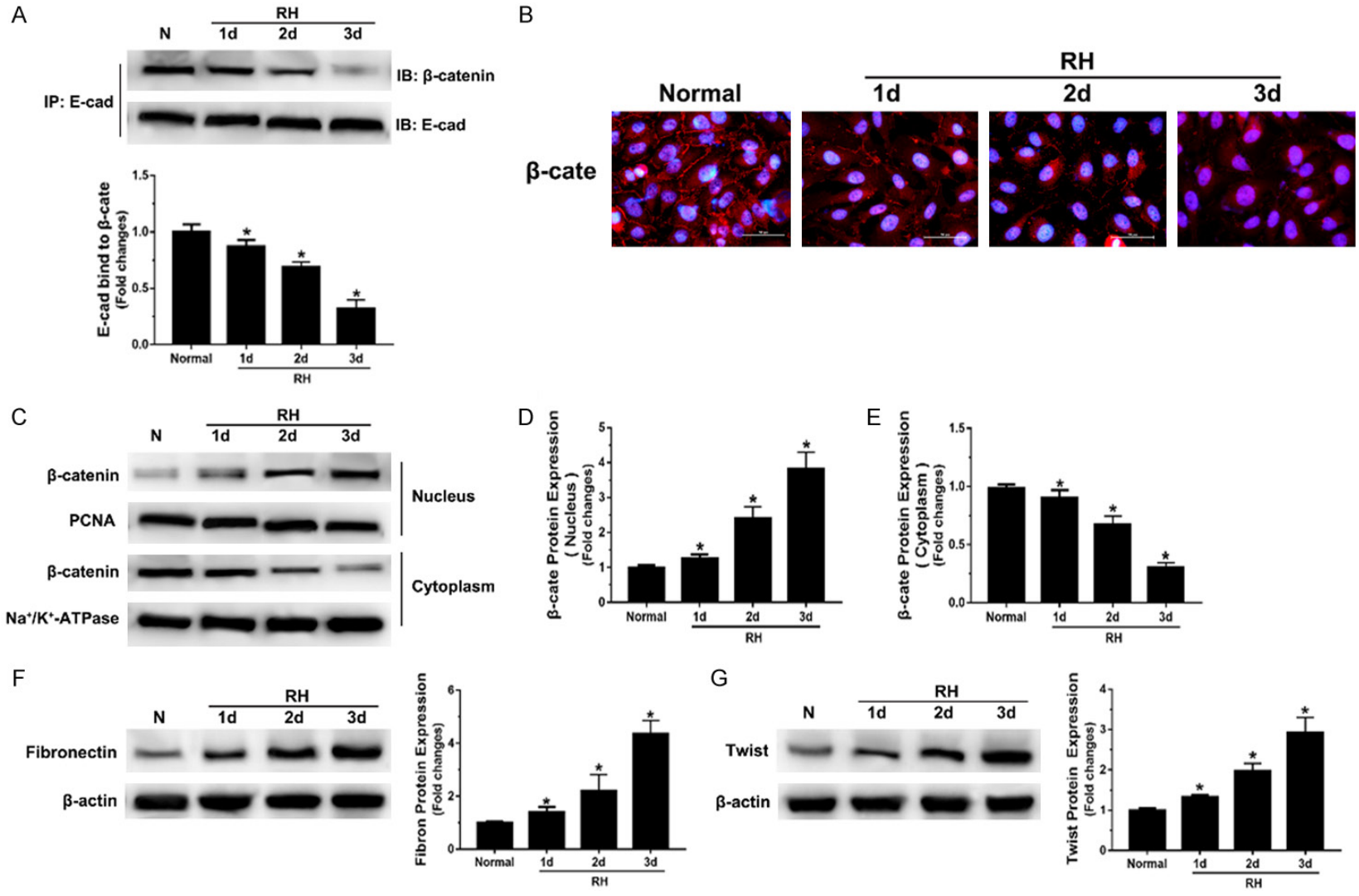
Figure 2. Reduced hydration induced downregulation of caveolae and E-cadherin. A. The protein expression of caveolin-1 in the keratinocyte monolayer culture model with or without high sodium treatment (n = 4). B. The mRNA expression of caveolin-1 in the keratinocyte monolayer culture model with or without high sodium treatment (n = 6). C. The promoter activity of caveolin-1 in the keratinocyte monolayer culture model with or without high sodium treatment (n = 6). D. Double immunofluorescence staining showing the surface-associated E-cadherin and caveolin-1 expression in the keratinocyte monolayer culture model with or without high sodium treatment. Scale bar = 50 mm. E. Quantification of the association of E-cadherin with caveolin-1 by coimmunoprecipitation (n = 4). *$P < 0.05$.

that the membrane location of β-catenin decreased gradually after high sodium treatment compared to that in normal cells, in which β-catenin was primarily restricted to intercellular junctions (**Figure 3B**). To determine whether β-catenin that dissociates from E-cadherin can enter into the nucleus under reduced hydration conditions and associate with transcription factors of the TCF/LEF-1 family, nuclear protein and cytoplasmic proteins of SKC were isolated, and Western blot analysis revealed that the nuclear localization of β-catenin increased and the cytoplasmic localization of β-catenin decreased in a time-dependent manner when keratinocyte monolayer culture with high sodium treatment (**Figure 3C-E**). Accordingly, the downstream target genes of β-catenin transactivation, which are also EMT-related proteins, fibronectin and twist, were significantly increased over time in keratinocyte monolayer culture with high sodium treatment (**Figure 3F**, **3G**). Since β-catenin that accumulates in the nucleus will bind to and activate the transcription factors of the TCF/LEF-1 family, we determined the effect of reduced hydration on the transcriptional activity of TCF/LEF-1. A luciferase reporter assay showed that TCF/LEF-1 transcriptional activity increased over time in keratinocyte monolayer cultures with high sodium treatment (**Figure 3H**). In addition, β-Engrailed (β-Eng), in which the C-terminal transactivation domain of β-catenin is replaced with the transcriptional repression domain of Drosophila Engrailed, was used to inhibit the transcriptional activity of β-catenin. As shown in **Figure 3I**, β-Eng failed to increase TCF/LEF-1 transcriptional activity under reduced hydration conditions, indicating that reduced hydration-induced TCF/LEF-1 activation is β-catenin dependent. Therefore, the above data suggest that under reduced hydration conditions, β-catenin dissociates from cell-cell contacts and is released from E-cadherin, accumulates in the nucleus and increases TCF/LEF-1 transcriptional activity.

Decreased caveolin-1 expression is necessary for elevated snail expression and β-catenin transcriptional activity under reduced hydration

We next determined whether a direct causal relationship exists between the reduced expression of caveolin-1 and E-cadherin. Keratinocytes were transfected with a vector expressing caveolin-1, and the keratinocyte monolayer culture model exhibited stably increased expression of endogenous caveolin-1, even after high sodium treatment (**Figure 4A**). The mRNA levels of E-cadherin decreased after high sodium treatment but increased dramatically in the keratinocyte monolayer culture model with stable overexpression of caveolin-1 (**Figure 4B**), suggesting that E-cadherin downregulation caused by caveolin-1 is regulated at the transcriptional level. Moreover, the promoter activity of E-cadherin increased in the keratinocyte monolayer culture model that overexpressed caveolin-1, even after high sodium treatment (**Figure 4C**). Accordingly, TCF/LEF-1 transcriptional activity increased under reduced hydration or after high sodium treatment but significantly increased in the keratinocyte monolayer culture model that overexpressed caveolin-1 (**Figure 4D**).

To explore the mechanism of the reduced hydration-induced decrease in the transcription of E-cadherin, Snail expression was detected. As a transcription factor, Snail has been shown to bind to the E boxes in the E-cadherin promoter, repress transcription of E-cadherin, and induce EMT. The protein expression of Snail increased over time in the keratinocyte monolayer model treated with high sodium (**Figure 4E**). Moreover, the mRNA level and promoter activity of Snail also increased in a time-dependent manner in both models in response to high sodium treatment (**Figure 4F**, **4G**). However, overexpression of caveolin-1 reversed the increased expression and promoter activity of Snail after high sodium treatment (**Figure 4H-J**), indicating that the expression of Snail is regulated by caveolin-1 under reduced hydration conditions. To determine whether Snail was involved in E-cadherin expression, Snail was inhibited by siRNA (**Figure 4K**), and the results showed that the protein and mRNA expression, as well as the promoter activity of E-cadherin, which were reduced under reduced hydration or after high sodium treatment, were increased after Snail inhibition (**Figure 4L-N**). In addition, the increased TCF/LEF-1 transcriptional activity induced by high sodium treatment was markedly decreased after Snail inhibition (**Figure 4O**).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E-cad bind to β-cate (Fold changes)
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Nucleus
β-catenin
Na+/K+-ATPase
Cytoplasm
D
β-cate Protein Expression (Nucleus) (Fold changes)
E
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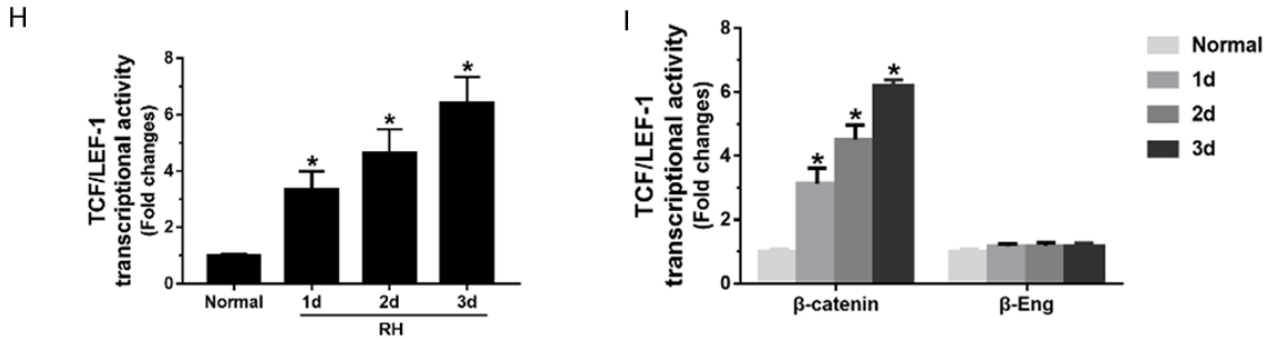
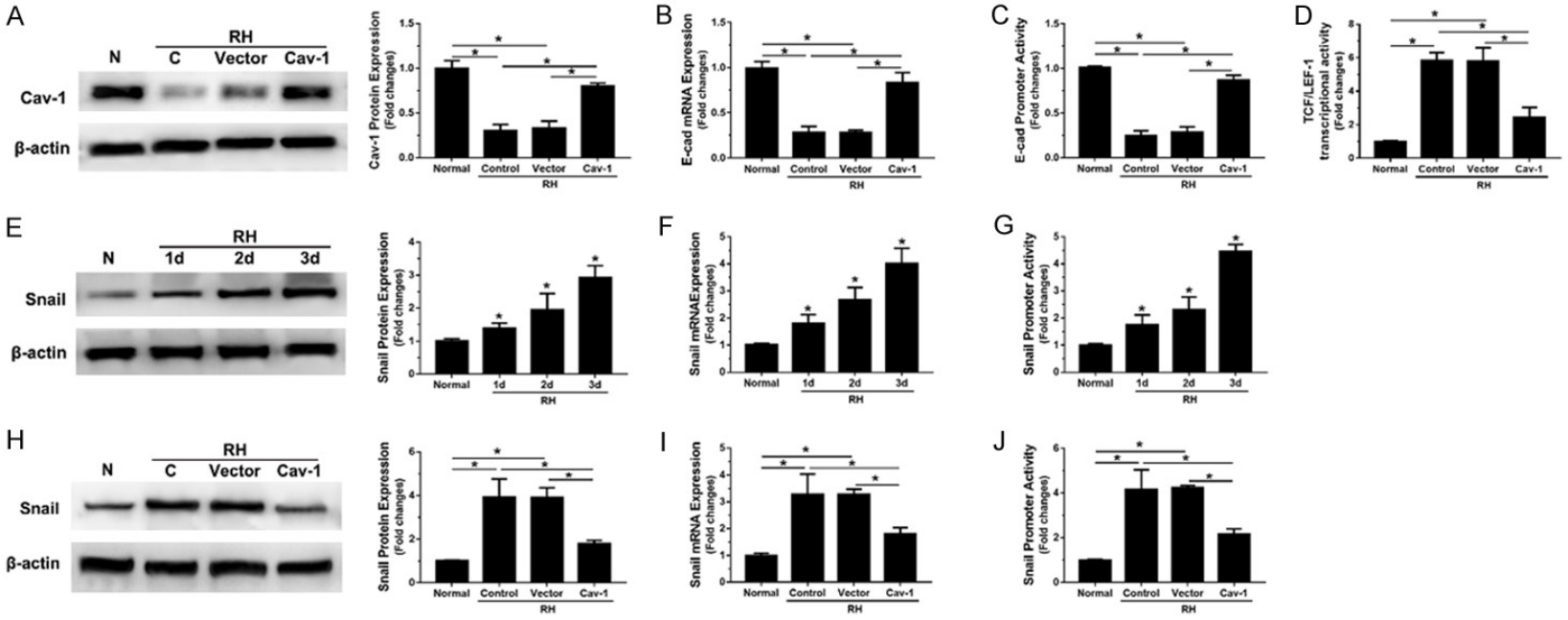


Figure 3. β-Catenin that dissociates from E-cadherin increases TCF/LEF-1 transcriptional activity in the keratinocyte monolayer culture model with high sodium treatment. A. Quantification of the association of E-cadherin with caveolin-1 in the keratinocyte monolayer culture model with high sodium treatment (n = 4). B. Immunofluorescence staining showing surface-associated E-cadherin expression in the keratinocyte monolayer culture model with or without high sodium treatment. Scale bar = 50 mm. C-E. Western blot analysis showing the cytoplasmic and nuclear localization of β-catenin in the keratinocyte monolayer culture model with high sodium treatment (n = 4). F. The protein expression of fibronectin in the keratinocyte monolayer culture model with high sodium treatment (n = 4). G. The protein expression of twist in the keratinocyte monolayer culture model with high sodium treatment (n = 4). H, I. The promoter activity of TCF/LEF-1 in the keratinocyte monolayer culture model with high sodium treatment (n = 6). *$P < 0.05$.



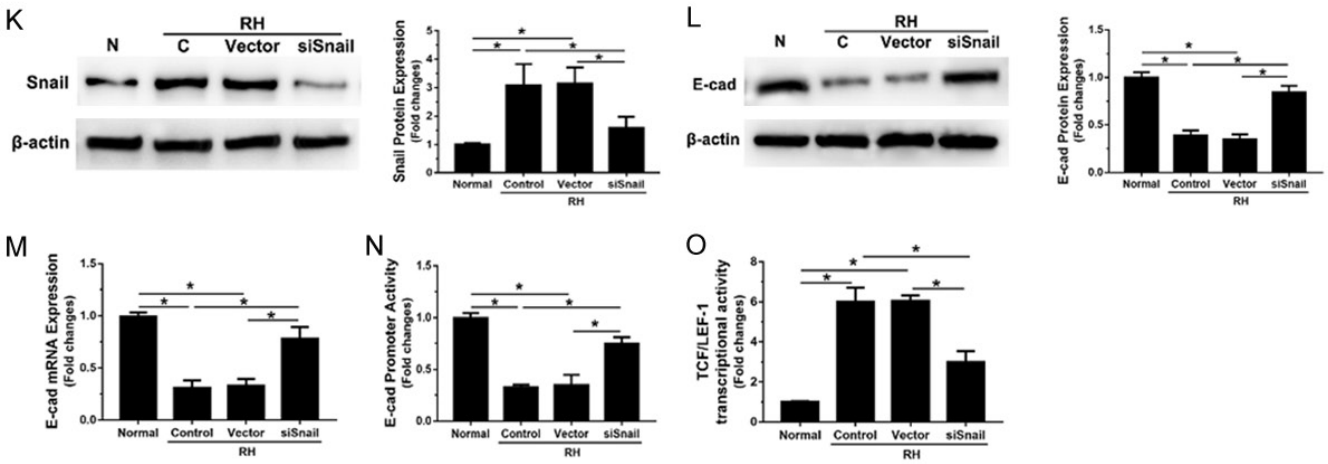


Figure 4. Decreased caveolin-1 induced elevated snail expression and β-catenin transcriptional activity in the keratinocyte monolayer culture model with high sodium treatment. A. The efficiency of overexpression of caveolin-1 in the keratinocyte monolayer culture model was confirmed by Western blot analysis (n = 4). B. The mRNA expression of E-cadherin after overexpression of caveolin-1 in the keratinocyte monolayer culture model with high sodium treatment. (n = 6). C. The promoter activity of E-cadherin after overexpression of caveolin-1 in the keratinocyte monolayer culture model with high sodium treatment (n = 6). D. The promoter activity of TCF/LEF-1 after overexpression of caveolin-1 in the keratinocyte monolayer culture model with high sodium treatment. (n = 6). E. The protein expression of Snail in the keratinocyte monolayer culture model with high sodium treatment (n = 4). F. The mRNA expression of Snail in the keratinocyte monolayer culture model with high sodium treatment (n = 6). G. The promoter activity of Snail in the keratinocyte monolayer culture model with high sodium treatment (n = 6). H. The protein expression of Snail in the keratinocyte monolayer culture model overexpressing caveolin-1 (n = 4). I. The mRNA expression of Snail in the keratinocyte monolayer culture model overexpressing caveolin-1 (n = 6). J. The promoter activity of Snail in the SKC model overexpressing caveolin-1 (n = 6). K. The efficiency of the inhibition of Snail in the keratinocyte monolayer culture model was confirmed by Western blot analysis (n = 4). L. The protein expression of E-cadherin in the keratinocyte monolayer culture model after inhibition of Snail (n = 4). M. The mRNA expression of E-cadherin in the keratinocyte monolayer culture model after inhibition of Snail (n = 6). N. The promoter activity of E-cadherin in the keratinocyte monolayer culture model after inhibition of Snail (n = 4). O. The promoter activity of TCF/LEF-1 in the keratinocyte monolayer culture model after inhibition of Snail (n = 6). *$P < 0.05$.

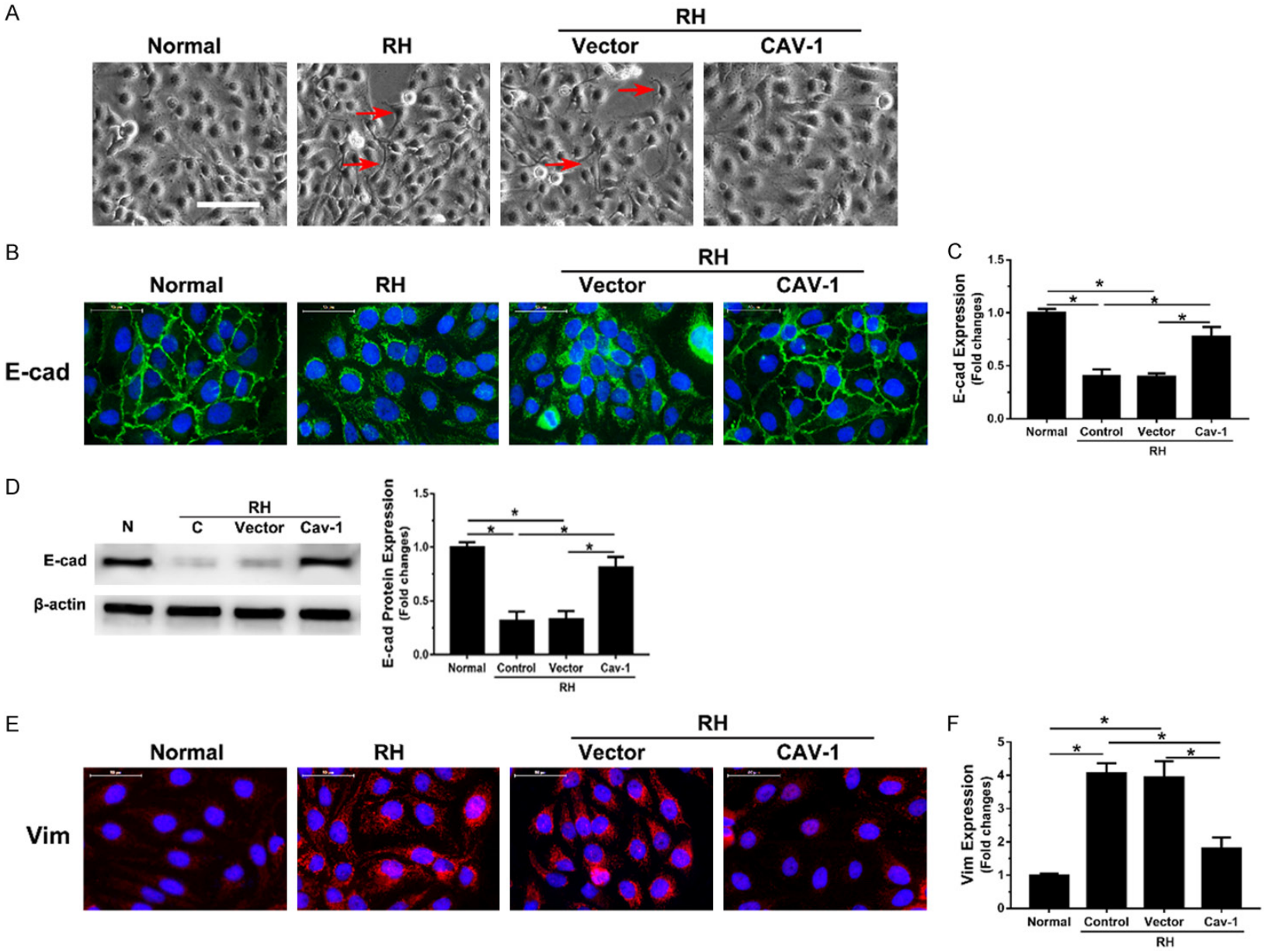
Decreased caveolin-1 expression is necessary for reduced hydration-induced EMT

We next examined whether decreased caveolin-1 expression plays a role in reduced hydration-induced EMT. As shown in **Figure 5A**, the keratinocytes that exhibited spindle-shaped morphology and "long-armed" intercellular connections after high sodium treatment, were restored to flat morphology and formed tight cell-cell contacts after overexpression of caveolin-1. The high sodium-induced internalization of E-cadherin protein was reversed by overexpression of caveolin-1, and E-cadherin was once again distributed in a continuous pattern at cell-cell contacts (**Figure 5B**). Moreover, the high sodium-induced decreased expression of E-cadherin protein increased after overexpression of caveolin-1 (**Figure 5C**, **5D**). The vimentin fibers, which could be observed in keratinocytes treated with high sodium medium, disappeared after overexpression of caveolin-1 (**Figure 5E**), and the high sodium-induced increased expression of vimentin was markedly reduced after overexpression of caveolin-1 (**Figure 5F**, **5G**). In addition, the mRNA expression of IL-1β, IL-6 and TNF-α, which increased in keratinocytes treated with high sodium medium, decreased after overexpression of caveolin-1 (**Figure 5H-J**).

Discussion

The hydration status is one of the numerous factors that influence wound healing, and maintenance of a fully hydrated epithelial microenvironment can prevent hypertrophic scar formation and remodel existing scars [10, 11]. Our previous studies have demonstrated that reduced hydration increased the release of proinflammatory cytokines, such as S100A8, S100A9, S100A12 and HMGB1, leading to collagen hypertrophy and scar formation [7, 28, 29]. EMT plays important roles not only in morphogenesis but also in cancer progression, wound repair, and tissue fibrosis [30]. There are many factors that influence and promote EMT, such as signaling molecules, transcription factors, microRNAs, and epigenetic factors [31]. In this study, we found that reduced hydration acts as a potential trigger for EMT, which is involved in hypertrophic scar formation. Previous studies have demonstrated that Nax, an atypical sodium channel, acts a sodium-sensing molecule in the central nervous system, playing an important role in maintaining sodium homeostasis in mammals [32]. Recent researches have shown that during the wound healing, Nax in the epidermis responds to the changes in sodium concentration, mediating proinflammatory cytokines expression, such as IL-1β, IL-6 and TNF-α and maintaining epithelial barrier function homeostasis [6]. Therefore, we hypothesize that under the reduced hydration, Nax sensors the high sodium environment, mediating the proinflammatory cytokines expression (IL-1β, IL-6 and TNF-α), regulating the expression of Caveolin-1 and the following EMT process.

During the process of EMT, the first and most important stage that epithelial cells undergo is cytoskeletal rearrangement and intercellular junction disruption, followed by apical-basal polarity and the acquisition of mesenchymal characteristics [31, 33]. In this study, we found that reduced hydration caused reduced expression of E-cadherin accompanied by a dramatic morphological change in epithelial cells. As an important component of epithelial adherens junctions, E-cadherin directly interacts with β-catenin and plakoglobin, regulating cell-cell adhesion via homophilic interactions with adjacent cells. Regulation of cell-cell adhesions by E-cadherin affects the dynamics of the formation and maintenance of intercellular junctions [34]. Internalization of E-cadherin is accompanied by the disruption of cell-cell adhesions [35], and reduced expression of E-cadherin has been found in many cancers, where it functions as a causal factor for tumor progression [36]. In addition, the loss of E-cadherin was involved in the EMT process by increasing the transcriptional activity of β-catenin. β-Catenin functions not only as an important component of adherens junctions but also as an essential component of Wnt signaling, which regulates the development, proliferation, and differentiation of cells [37] and plays a central role in wound repair and scar formation [34]. In this study, the E-cadherin-β-catenin adherens junctions were disrupted under reduced hydration, releasing β-catenin from the complex and making it available for nuclear translocation. By interacting with TCF/LEF-1 transcription factors, β-catenin activates EMT-related genes, such as fibronectin and twist [38].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β-actin
E-cad Protein Expression
(Fold changes)
Normal
Control
Vector
Cav-1
RH
E
Vim
Normal
RH
RH
Vector
CAV-1
F
Vim Expression
(Fold changes)
Normal
Control
Vector
Cav-1
RH

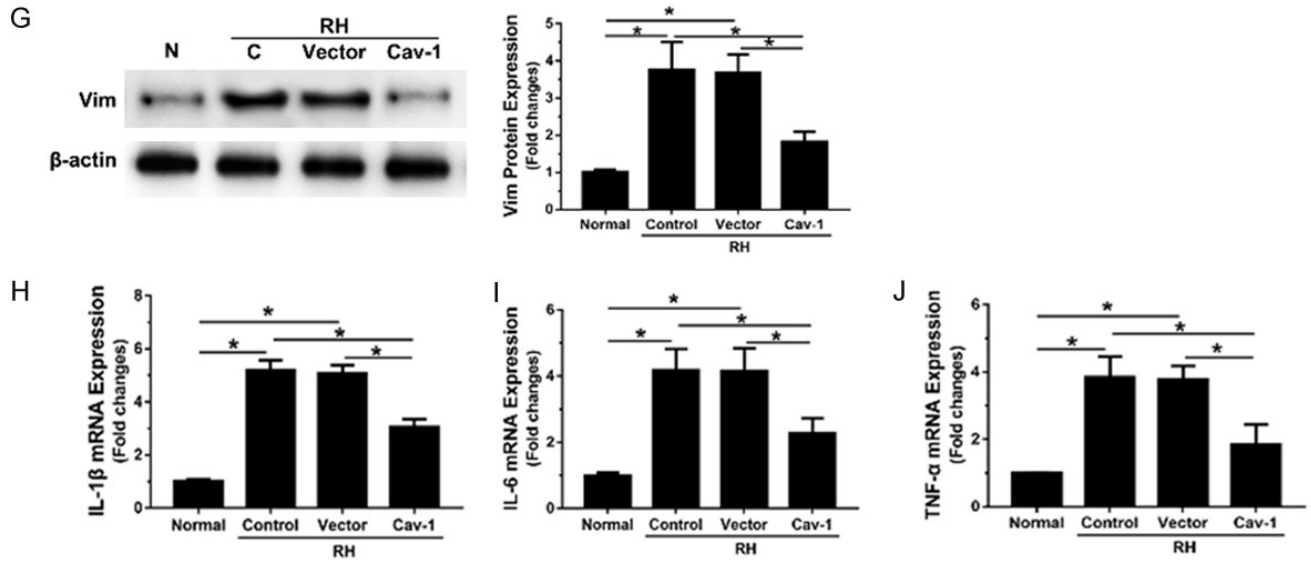

Figure 5. The role of caveolin-1 in reduced hydration-induced EMT. A. The morphological plasticity of keratinocytes overexpressing caveolin-1 with high sodium treatment. Scale bar = 50 mm. B, C. Immunofluorescence staining showing the expression of E-cadherin in the keratinocyte monolayer culture model overexpressing caveolin-1. Scale bar = 50 mm. D. Quantification of the expression of E-cadherin. E, F. Immunofluorescence staining showing the expression of vimentin in the keratinocyte monolayer culture model overexpressing caveolin-1. Scale bar = 50 mm. G. Quantification of the expression of vimentin (n = 6). H-J. The mRNA expression of IL-1β, IL-6 and TNF-α in the keratinocyte monolayer culture model with or without high sodium treatment (n = 6). *$P < 0.05$.

In this study, a reduced hydration condition was simulated by increasing extracellular sodium concentration by 10%. Since a 10% increase in sodium is a physiological level reached in the extracellular fluid in a water-deprived thirsty animal [39, 40], a reduced hydration was mimicked by higher sodium medium treatment [6]. We found that under reduced hydration, intercellular connections were disrupted, and the expression of E-cadherin decreased, accompanied by a dramatic morphological change of keratinocytes and activated EMT process. However, the exact mechanism underlying how caveolin-1 regulates E-cadherin expression is not clear. It was demonstrated that under certain conditions, E-cadherin is tyrosine phosphorylated by Src, recruiting Hakai, a c-Cbl-like ubiquitin E3 ligase, which in turn ubiquitylates E-cadherin and induces E-cadherin endocytosis [41]. In addition, we also found that the expression of Snail increased upon reduced hydration. As a transcriptional regulator, Snail directly inhibits E-cadherin promoter activity and activates the EMT process [42]. In this study, the reduced hydration-induced increase in Snail transcription was dependent on the downregulation of caveolin-1 expression.

Overall, our study demonstrates that downregulation of caveolin-1 increases the transcriptional activity of β-catenin-TCF/LEF-1, promoting the transcriptional activity of β-catenin-TCF/LEF-1, thus enhancing the EMT process and resulting in hypertrophic scar formation. Our findings indicate the potential therapeutic benefits of caveolin-1 in hypertrophic scars.

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Disclosure of conflict of interest

None.

Abbreviations

EMT, epithelial-to-mesenchymal transition; SKC, stratified human keratinocyte culture; RH, reduced hydration; DMEM, dulbecco's modified eagle medium; EGF, epidermal growth factor; UbC, ubiquitin C; Cav1, caveolin-1; CMV, cytomegalovirus; GFP, green fluorescent proteins; RT-PCR, reverse transcription-polymerase chain reaction; ECL, enhanced chemiluminescence; RIPA, radioimmunoprecipitation assay buffer; HRP, horseradish peroxidase; PBS, phosphate buffered saline; BSA, bovine serum albumin; DAPI, 4,6-diamidino-2-phenylindole; CSD, CAV-1 scaffolding domain; H&E, hematoxylin and eosin; SD, standard deviation; SEI, scar elevation index.

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