Original Article

Long non-coding RNA HULC regulates TLR4 expression by acting as ceRNA to attract miR-663b in skin fibroblasts of pediatric burns

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Abstract: Objective: The study aims to elucidate the impact of LncRNA HULC in human skin fibroblasts (HSF) after burns in children. HULC might act as endogenous sponges for miR-663b to regulate the gene expression of TLR4. Methods: This study included 46 children with deep second-degree burns. On the 5th day after the injury, eligible samples from all patients were collected. HSF cells were selected to establish a thermal-injured model. qRT-PCR was applied to detect the expression of HULC, miR-663b, and TLR4 mRNA in burn wound and normal skin tissue. The dual-luciferase reporter and RIP assay were performed to explore a targeted binding relationship between HULC and miR-663b, or miR-663b and TLR4. Cell proliferation and invasion were evaluated through the assay of CCK-8 and transwell assay. The expression levels of α-SMA, Collagen I, MMP-1, and TIMP-1, which are associated with extracellular matrix (ECM) production, were examined by western blot. Results: HULC and TLR4 mRNA expression were reduced on the 5th day after thermal injury in burn wounds, while miR-663b expression increased significantly (P<0.05), when compared to expression in the normal tissue. HULC and TLR4 mRNA concentration in HSF cells showed a transient increase after thermal injury, and a gradual decline with time was observed subsequently when compared to the control group. An inverse expression of miR-663b with the expression of HULC and TLR4 mRNA was observed simultaneously (P<0.05). A deficiency of HULC promotes the proliferation, invasion, and ECM synthesis of HSF cells with thermal injury; HULC functions as a ceRNA of miR-663b. Inhibitors of miR-663b partially rescued the effects on thermal-injured HSF cells induced by HULC deficiency (P<0.05). TLR4 is a target gene of miR-663b. The up-regulation of TLR4 also partially reversed the effect on the thermal-injury of HSF cells resulting from HULC deficiency (P<0.05). Conclusion: LncRNA HULC may function as a molecular sponge to regulate the expression of the miR-663b/TLR4, and thereby inhibit the proliferation, invasion, and ECM synthesis of thermal-injured HSF cells. HULC knockdown might significantly promote wound healing in children after burns.

Keywords: HULC, miR-663b, TLR4, pediatric burns, fibroblasts

Introduction

Local skin burns are divided into I-IV degrees according to the burn wound depth. A deep second-degree burn refers to the thermal injury extending to the deep dermis, which makes treatment more complicated and sensitive to infection. The wound scar formation and dysfunction after a deep wound healing also bring huge challenges for treatment [1-3].

The dermis performs wound repair by regulating the function of human skin fibroblasts (HSF) and the synthesis of extracellular matrix (ECM), thereby determining the quality of scar formation [4, 5]. Once the thermal damage happens, HSF cells are activated to recover the integrity of the tissue. ECM metabolism plays a key role in the process of wound healing by determining the quality and prognosis of wound repair [6, 7]. However, the molecular mechanism of healing is not understood completely.

Long non-coding RNAs (LncRNAs) play an important regulatory role in the progression of many diseases [8]. Also, LncRNAs regulate the expression of mRNAs by acting as sponges of microRNAs (miRNAs) or competitive endogenous RNAs (ceRNAs) [8, 9]. Moreover, an increasing number of LncRNAs have been recog-
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1. Introduction

LncRNA HULC is known to play a crucial role in wound healing after thermal injury. For example, LncRNA XIST promotes cell proliferation and inhibits fibroblast apoptosis, resulting in burn wound healing [10]. Additionally, LncRNA AC067945.2 was identified to inhibit collagen expression and ECM synthesis in skin fibroblasts [11]. According to current reports, LncRNA HULC aggravates the damage of skin fibroblasts induced by ultraviolet rays, involving an up-regulation of the expression of BNIP3 and activation of the JAK/STAT3 signaling pathway [12]. However, the underlying mechanism of LncRNA HULC in skin burns (thermal injury) has not yet been elucidated, and further research is required.

2. Materials and methods

2.1. Samples

46 children with deep second-degree burns in our hospital were included in this study. The inclusion criteria were: (1) flame burns or hydrothermal ablation; (2) hospitalization in 3 hours after injury; (3) burns of multiple sites; (4) the total body surface area involved is less than 20%, and no obvious third-degree burn wounds are observed. Exclusion criteria excluded pediatric burn patients with (1) drug treatment after injury; (2) with age ≥5 years old; (3) with injury exceeding 3 hours. According to the previous literature, scabs from all patients were taken on the 5th day after burns [4]. The normal skin tissues around the burn area were selected as a control. The skin tissues were embedded as blocks and subjected to a cryostat, then stored at -80°C for further use. This study was approved by the Medical Committee of our hospital and all patients signed an informed consent form.

2.2. Cell culture

Human skin fibroblasts (HSF) were purchased from ATCC in the United States. Cells were cultured with RPMI-1640 medium (31800, Beijing Solarbio Technology Co., Ltd., China) and 10% fetal bovine serum (S9030, Solarbio, China) in an incubator with 5% CO$_2$ atmosphere at 37°C.

According to the established method published in a previous study to trigger cellular thermal injury [22], the HSF cells were challenged with 52°C water for 30 s, while cells as controls were incubated with 37°C water simultaneously. Subsequently, two groups of cells were cultured in a normal incubator.

2.3. Cell transfection and group division

After an intervention against HULC, miR-663b, and TLR4 expression respectively, group division was arranged as follows: Blank group (transfected with blank plasmid), si-NC group (transfected with si-NC), miR-NC group (transfected with miR-NC), si-HULC group (transfected with si-HULC), miR-663b mimic group (transfected with miR-663b mimic), si-HULC + miR-663b group. All transfections were performed using Lipofectamine 2000 (Invitrogen, Life Technologies). The transfection efficiency was evaluated by qRT-PCR.

3. Results

The protein encoded by the TLR4 gene is a member of the Toll-like receptor (TLR) family and plays an important role in pathogen recognition and the activation of innate immunity [18]. TLR4 functions as one of the important components to avoid excessive inflammation and to trigger tissue repair after infection or injury [19]. For example, Chmerin activates the NF-kB signaling pathway through TLR4 which further induces the release of inflammatory mediators ECM degradation, and cell senescence [20]. Moreover, TLR4 has been verified to promote a microvascular inflammatory response and barrier dysfunction after burns, which could be rescued significantly by siRNA intervention [21]. Therefore, we posited that TLR4 plays an indispensable role in the regulation of healing after burns.

The present study further investigated the effect of LncRNA HULC in burns. Moreover, this study suggested that, by acting as a sponge for miR-633b, LncRNA HULC up-regulated TLR4, inhibited proliferation of skin HSCs, and exacerbated skin injury. Reducing HULC expression might be used for recovery after burns.

4. Conclusion

In summary, LncRNA HULC is involved in the regulation of wound healing after thermal injury. Further studies are needed to elucidate the potential therapeutic role of miRNAs in skin burns.
NC group (transfected with si-HULC and miR-NC), si-HULC + miR-inhibitor group (transfected with si-HULC and miR-663b inhibitor), si-HULC + TLR4 group (transfected with si-HULC and TLR4 overexpression vector), and si-HULC + vector group (transfected with si-HULC and blank vector). According to the manufacturer’s manual, the LipofectamineTM 3000 kit (L3000015, Thermo Fisher, USA) was used for transfection.

**qRT-PCR**

Trizol reagent (R0016, Beyotime Biotechnology Co., Ltd., Shanghai, China) was used to extract total RNA from collected tissues and cells, and an ultraviolet spectrophotometer (Alpha1500, Shanghai Bioon Instrument Co., Ltd., China) was used to measure the purity and concentration of RNA. As described by the manufacturer’s instructions, a reverse transcription kit (T2240, Beijing Solarbio Technology Co., Ltd., China) was employed to transcribe RNA reversely into cDNA. More details of the PCR reaction system and conditions are described in the kit instructions (BJ001243, Thermo Fisher, USA). The 2^ΔΔCt method was used for calculating the relative expression of the gene. U6 was selected as the internal control of miR-663b, and GAPDH as the internal control of HULC and TLR4. The primer sequences are shown in Table 1.

**CCK-8**

The cells were seeded into 96-well plates at a density of 1×10^3/well, and 10 μL CCK-8 solution (C0038, Shanghai Beyotime Biotechnology Co., Ltd., China) was added to each well at 24 h, 48 h, and 72 h. Afterward, the cells were incubated at 37°C for 2 hours, and the optical density was measured at 490 nm.

**Western blot**

Total protein was extracted from cultured cells. After SDS/PAGE electrophoresis, the protein was transferred to a polyvinylidene fluoride (PVDF) membrane. α-SMA (1:1,000, ab108531, ABCAM, UK), Collagen I (1:1,000, ab34710, ABCAM, UK), MMP-1 (1:1,000, ab134184, ABCAM, UK), TIMP-1 (1:1,000, ab211926, ABCAM, UK) antibodies were applied for incubation as the primary antibody, followed by incubation with the secondary antibody goat rabbit anti-IgG (1:1,000, ab133470, ABCAM, UK). To observe protein expression, ECL (electrochemical luminescence) kit (35055, Thermo Fisher Scientific, Inc. USA) was used, and Image J software was used for analyzing the band density.

**Transwell assay**

Pre-coated Matrigel (base glue; 354234, Beijing Solarbio Technology Co., Ltd., China) was in the upper chamber of the Transwell chamber. In a serum-free medium, 1×10^4 thermal-injured cells were transplanted into the upper chamber, and the lower chamber was added with 500 μL of RPMI1640 medium (31800, Beijing Solarbio Technology Co., Ltd., China) containing 10% fetal bovine serum. After incubation for 24 h at 37°C and 5% CO₂, the cells without successful invasion in the upper chamber were removed. Then, after fixation with 4% paraformaldehyde (P0099, Beyotime Biotechnology Co., Ltd., Shanghai, China) and staining, the cells were counted under an optical microscope (DSX100, OLYMPUS, Japan).

**RIP**

EZ-Magna RIP kit (17-700, Shanghai Haoran Biotechnology Co., Ltd., China) was utilized for RIP experiments according to the manufacturer’s instruction. After transfection for 24 hours, the cells were collected with the Ago2 antibody for the RIP assay, and IgG was used as a negative control. Then quantitative RT-PCR was employed to detect co-precipitated RNA.

**Luciferase report analysis**

The full-length sequences of HULC and TLR4 3’-UTR with or without mutant miR-663b binding sites were cloned into the pmir-Reporter

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**Table 1. Sequence of primers used for qRT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
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<tbody>
<tr>
<td>HULC</td>
<td>Forward: ATCTGCAAGCCAGGAAAGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTTGTTGATGCTTTGGTCGTG-</td>
</tr>
<tr>
<td>miR-663b</td>
<td>Forward: CUAUAAUGAUCUACCACAUAAA</td>
</tr>
<tr>
<td></td>
<td>Reverse: UAUUGGUUAGAUACUAUA GUU</td>
</tr>
<tr>
<td>TLR4</td>
<td>Forward: TATCATCAGTGTCTCGGTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAGTCTCATTCGCTGCTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: ACCACAGTCATGCCCATCAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCCACCACCT GTT GCTGTA</td>
</tr>
<tr>
<td>U6</td>
<td>Forward: GCUUCGCGACGCAUAUAACUAA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CGCUUCACGAUJUUGCGGUCAU</td>
</tr>
</tbody>
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plasmid, and the wild-type and mutant reporter plasmids were transformed into HSF cells with miR-663b mimic and control vectors. After transfection for 48 hours, the luciferase activity in heat-damaged HSF cells was analyzed by using a dual-luciferase reporter gene detection kit (DOO10, Beijing Solarbio Technology Co., Ltd., China).

**Statistical analysis**

SPSS 20.0 was used to analyze the data, and each experiment was repeated at least three times. The results are shown as mean ± standard deviation (X ± sd). The t-test was used for data analysis between two groups, one-way analysis of variance (ANOVA) for data analysis among multiple groups, and LSD test for pairwise comparison. P<0.05 was considered significant.

**Results**

The expression of HULC declined significantly in burn wound tissue and HSF cells after thermal injury

Compared to normal tissues (n=46), the expression of HULC of burn wounds after thermal injury for 5 days was significantly reduced (P<0.0001; Figure 1A). Besides, compared with the control group, after a short-term increase of the HULC level in thermal-injured HSF cells, a progressive decrease of HULC was observed at 24 and 36 hours (Figure 1B), suggesting that HULC dysregulation might participate in the wound healing process due to burns.

HULC knockdown promotes cell proliferation and invasion of thermal-injured HSF cells, as well as ECM synthesis

In order to explore the biological role of HULC in the process of wound healing, si-NC and si-HULC were transfected into thermal-injured HSF cells. The transfection efficiency of si-HULC was first to be validated (Figure 2A). Subsequently, the CCK-8 method showed that the knockdown of HULC significantly enhanced cell proliferation (Figure 2B), when compared to the si-NC group. As shown in Figure 2C, 2D, the ability of cell invasion in the si-HULC group was also significantly increased. Moreover, HULC downregulation induced a significant increase of ECM markers in thermal-injured HSF cells, e.g. α-SMA, Collagen I, and TIMP-1, while a decrease of MMP-1 was observed (Figure 2E, 2F). These results indicate that the inhibition of HULC can promote the proliferation and invasion of HSF cells with thermal-injury and ECM synthesis.

HULC attracts miR-663b

An elevation of miR-663b expression was observed on the 5th day after thermal injury in burn wound tissue, while in thermal-injured HSF cells, its expression declined temporarily, and, subsequently, a gradual increase over time was observed (Figure 3A, 3B). The alteration of miR-663b was negatively correlated with the expression of HULC (Figure 3C). The bioinformatics analysis website also reported existing binding sites of HULC and miR-663b (Figure 3D). The data from the dual-luciferase reporter experiment further showed that miR-663b transfection significantly reduced the luciferase activity of 293 T cells transfected with HULC-wt, whereas it had no influence on the luciferase activity of 293 T cells transfected with HULC-mut (Figure 3E). The RIP experiment showed that both HULC and miR-663b showed a significant enrichment in the anti-Ago2 group (Figure 3F). The above results suggest HULC functions as a ceRNA of miR-663b.
HULC knockdown partially reversed the effect on thermal injury of HSF cells by downregulation of miR-663b

Compared to the si-NC group, improved proliferation and invasion of thermal-injured HSF cells were observed in the si-HULC group, while an inhibited proliferation and invasion of thermal-injured HSF cells in the si-HULC + miR-inhibitor group when compared with the si-HULC + miR-NC group (both P<0.05, Figure 4A-C). Western blot also showed that the expression of α-SMA, Collagen I, and TIMP-1 protein in the si-HULC group were increased corresponding to the si-NC group, while the expression of MMP1 protein was inhibited. In converse with the si-HULC + miR-NC group, the expression of α-SMA, Collagen I, and TIMP-1 in the si-HULC + miR-inhibitor group decreased, while the expression of MMP-1 increased (Fig-
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The above results indicated that the promoting effects of HULC inhibition on thermal-injured HSF cells were partially reversed through the downregulation of miR-663b.

HULC activated TLR4 by regulating miR-663b

The present study found that TLR4 was expressed at a low level in burn wound tissues (Figure 5A). Compared to the control group, the TLR4 concentration in thermal-injured HSF cells elevated in a short time, but gradually decreased with time (Figure 5B). In thermally-injured HSF cells, silencing HULC induced suppression of TLR4 expression, with a similar effect resulting from miR-663b overexpression (Figure 5C, 5D). Correlation analysis revealed that the expression of TLR4 in thermally-injured HSF cells was positively correlated with HULC (Figure 5E), but negatively associated with miR-663b (Figure 5F). Several binding sites of miR-663b and TLR4 (Figure 5G) were identified through bioinformatic analysis. The dual-luciferase reporter further found that miR-663b significantly reduced the luciferase activity of wild-type 3'UTR vector with TLR, but had no significant effect on the mutant 3'UTR vector, suggesting a targeted binding exists between TLR and miR-663b (Figure 5H). The RIP also showed that both TLR4 and miR-663b were significantly enriched in the anti-Ago2 group (Figure 5I). The above results indicated that TLR4 might be a downstream target of miR-663b, and HULC promoted the expression of TLR4 through upregulation of miR-663b.

The advancing effect of silencing HULC on thermally-injured HSF cells was partially inhibited by TLR4 activation

The present study found that the promoted cell proliferation in the si-HULC group was inhibited by simultaneously up-regulating TLR4 (Figure 6A). The invasion of cells in the TLR4 + si-HULC group was significantly lower than that in the si-HULC + vector group (Figure 6B, 6C). Western blot disclosed that the expression of α-SMA, Collagen I, and TIMP-1 in the TLR4 + si-HULC group decreased significantly when compared with the si-HULC + vector group, while an increased expression of MMP-1 was observed (Figure 6D). The above results indicated that...
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Discussion

Extracellular matrix (ECM) synthesis and human skin fibroblast (HSF) cell function both play an important role in wound healing after thermal injury [23, 24]. Burns are a common accident in children, with a higher difficulty of management than adults’ burns. The incidence of burn wound contamination and complications after burns also plays a crucial role in the progression of burns. Therefore, an exploration of the underlying molecular mechanisms is required to expand the current clinical data for the treatment of pediatric burns. In the current study, we found that the expression of HULC and TLR4 were decreased in burn tissues, while the expression of miR-633b was increased. After establishment of a cellular model of thermal injury by using HSF, we found that HULC inhibited the ECM synthesis and suppressed the proliferation and invasion of HSF. Also, this study explored the interaction among HULC, miR-633b, and TLR4 for the first time.

ECM production after an injury is one of the primary biomarkers to predict the outcome of healing [25, 26]. During the process of wound healing, HSF cells express and secrete α-SMA protein with a contraction effect. The production of Collagen I and α-SMA are two primary markers of ECM synthesis [27, 28]. The degradation of ECM mainly depends on the MMPs secreted by tissue cells. As the crucial enzymes to regulate collagen I degradation, the

Figure 4. The effects of HULC knockdown on HSF cell thermal injury was partially reversed by a miR-663b inhibitor. A: Cell proliferation measured by CCK-8; B and C: Cell invasion measured by the Transwell assay (200×); D: Western blotting results of ECM-related proteins; E: Quantification of proteins related to ECM production. ECM: extracellular matrix. Compared with the si-NC group, *P<0.05. Compared with the si-HULC + miR-NC group, #P<0.05.
activity of MMPs depends on TIMP-1, which inhibits the degradation of ECM. Also, HSF is one of the cell types for repair during wound healing, and its activation has a benefit for recovering tissue integrity [29-31]. Previous studies have shown that the up-regulation of HULC exacerbates HSF cell damage [12]. Our research also revealed that the HULC gene deficiency improves wound healing after thermal injury by promoting ECM synthesis, HSF proliferation, and invasion. However, the underlying mechanism is still unclear. As reported, lnc-RNAs can act as ceRNAs to inhibit the expression of miRNAs in a variety of cancers or diseases [32]. To further explore the mechanism of HULC involving in wound healing after burns, this study aimed to investigate molecular regulatory networks interacted with HULC through the ceRNA network.

We identified and verified that HULC is the ceRNA of miR-663b supported by the evidence...
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Figure 6. TLR4 partially reversed the effect of silencing HULC on HSF cell thermal injury. A: Cell proliferation measured by CCK-8; B, C: Transwell invasion assay (200×); D: Western blotting results of ECM-related proteins; E: Quantification of ECM-related protein expression using western blot. ECM: extracellular matrix. Compared with the si-NC group, *P<0.05. Compared with the si-HULC + vector group, #P<0.05.

from the bioinformatic website, dual-luciferase reporter, and RIP assay. The expression of HULC and miR-663b are negatively correlated in HSF cells treated by heat. Expression of miR-663b increased in burn wound tissues, in which was observed a progressive elevation of miR-663b after a short-term reduction in HSF cells with heat treatment. This is consistent with previous studies [17]. Additionally, we found that silencing HULC and down-regulation of miR-663b partly suppressed the proliferation and invasion of HSF, as well as the ECM synthesis, suggesting that HULC can attenuate the progression of burns healing by suppression of miR-663b as sponges.

To further explore the mechanism of HULC and miR-663b associated with burn healing, TLR4 was further identified as a downstream target gene of miR-663b through bioinformatic analysis. Previous studies also verified the target relationship between miRNA-633b and TLR4 through the experiments with the dual-luciferase reporter experiment and RIP [21]. Our study observed that the expression of TLR4 negatively correlated with the change in miR-633b, while it was positively correlated to the alteration of HULC in burn wound tissues after thermal injury. Functional analysis showed that HULC regulates the expression level of TLR4 mediated by miR-633b. Up-regulation of TLR4 can partially reverse the effect of HULC knockdown in HSF cells with thermal injury, including ECM synthesis, proliferation, and invasion of HSF cells, which is similar to the performance of downregulated miR-663b. The above results indicate that HULC promotes TLR4 gene expression as a sponge for miR-663b to inhibit HSF cell proliferation, invasion, and ECM synthesis induced by thermal injury. Suppression
of HULC or elevated expression of miR-663b can effectively improve cell proliferation, invasion, and ECM synthesis in the thermally injured HSF cells, with benefit for burn healing progress.

Notably, the limited number of samples included in this study might affect the credibility of the data. Increased samples are required to validate the results of the present study. The establishment of animal models may also promote future investigation to elucidate further the implications of HULC and miR-663b in the progression of burn healing.

In summary, this study was first to explore the role of HULC in pediatric burn healing. The mechanistic research revealed that HULC acts as ceRNA to attract miR-663b like sponges, resulting in TLR4 upregulation, which inhibited the growth of skin fibroblasts and burn healing. This study elucidated a mechanism of the HULC/miR-663b/TLR4 molecular axis involved in burn healing and provided a novel molecular target for pediatric burn treatment. A mechanistic diagram of this study is shown in Figure 7.

Disclosure of conflict of interest

None.

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