Original Research

Long non-coding RNA HULC affects the proliferation, apoptosis, migration, and invasion of mesenchymal stem cells

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Impact statement

Exploring the molecular mechanisms of growth and function in MSCs is the key to improve their clinical therapeutic effects. Currently, more and more evidence show that the long non-coding RNA (lncRNA) plays an important role in the growth, stemness and function of MSCs.Both HULC and MALAT1 are the earliest discovered LNCRNAs, which are closely related to tumor growth. All of them can promote the growth of liver cancer stem cells. Previously, we have studied the effects of MALAT1 on the growth and function of MSCs. In this study, we focused on the effects of HULC on MSCs. We elucidated the effects of HULC on the growth and differentiation of MSCs, and explored the relationship between inflammatory stimuli and HULC expression in MSCs. Our findings provide a new molecular target for the growth and clinical application of MSCs.

Abstract

Further studies on the molecular mechanisms of mesenchymal stem cells in the maintenance of growth and function are essential for their clinical application. Growing evidence has shown that long non-coding RNAs (lncRNAs) play an important role in the regulation of mesenchymal stem cells. Recently, it is reported that highly upregulated in liver cancer (HULC), with another lncRNA MALAT-1, accelerated liver cancer stem cell growth. The regulating role of MALAT-1 in mesenchymal stem cells has been investigated. However, the effects of HULC on the mesenchymal stem cells are unknown. In this study, we overexpressed HULC in mesenchymal stem cells derived from umbilical cord and analyzed the cell phenotypes, proliferation, apoptosis, migration, invasion and differentiation of mesenchymal stem cells. We found that overexpression of HULC significantly promotes cell proliferation through promoting cell division and inhibits cell apoptosis. HULC-overexpressed mesenchymal stem cells migrate and invade faster than control mesenchymal stem cells. HULC has no effect on phenotypes and differentiation of mesenchymal stem cells. Furthermore, we found that the expression of HULC in mesenchymal stem cells could be reduced by several inflammatory factors, including TNF-α, TGF-β1, and R848. Taken together, our data demonstrated that HULC has a vital role in the growth and function maintenance of mesenchymal stem cells without affecting differentiation.

Keywords: Mesenchymal stem cells, highly upregulated in liver cancer, cell proliferation, cell apoptosis, inflammation

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Introduction

Mesenchymal stem cells (MSCs) have been highly regarded as an effective therapeutic modality in regenerative medicine,[1] due to their self-renewal ability, easy expansion and multiple differentiation potential.[2,3] However, in order to develop a more effective stem cell-based clinical treatment strategy, deeper understanding of the molecular mechanisms of stem cells in the maintenance of growth and function is required. Many molecules are involved in the maintenance of stem cell growth and function and one of those are the long non-coding RNAs (lncRNAs).[4,5]

Previous studies have shown that miRNA plays an important role in the growth and differentiation of MSCs.[6–8] However, recent studies have found that lncRNA plays an equally regulatory role in MSCs.[9] LncRNA highly up-regulated in liver cancer (HULC) is one of the earliest discovered tumor-related lncRNA and is a predictive molecular marker for liver cancer.[10,11] HULC was able to promote the growth, apoptosis, invasion and angiogenesis of cancer cells.[12–15] A more recent study demonstrated that HULC, with another lncRNA MALAT-1 HULC,[14] accelerated liver cancer stem cell growth.[16]

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Such stemness and proliferation regulating role of MALAT-1 are reported in non-cancer stem cells such as MSCs as well.[17] However, the effects of HULC on MSCs are unclear.

The purpose of this study is to explore the possible functions of the HULC in MSCs. We upregulated the expression of HULC in MSCs and tested the phenotype, proliferation, apoptosis, migration, invasion and differentiation. The results showed that upregulating HULC could promote cell proliferation, migration and invasion and inhibit cell apoptosis without affecting the phenotype and differentiation ability.

Material and methods

Umbilical cord collection

Umbilical cord tissues were collected from normal pregnancies that underwent caesarean section in the Affiliated Drum Tower Hospital of the Nanjing University Medical School from September 2015 to April 2016. This research plan was discussed and approved by the Nanjing Drum Tower Hospital Ethics-Committee, and all the pregnant women gave written consent. Multiple gestations, maternal hypertension like chronic hypertension and preeclampsia, gestational diabetes mellitus, hepatic disease, the HELLP syndrome, in vitro fertilization and embryo transfer (IVF-ET), chronic nephritis or other infectious and fetal congenital defect were excluded.

Isolation of MSCs

Umbilical cord-derived MSCs were isolated as we reported before.[17] The umbilical cords were obtained and stored in 1×PBS with 1% penicillin and streptomycin. Firstly, the tissues were washed with PBS until all blood was removed, in a sterile environment. Secondly, umbilical arteries and vein were dissected with tweezers and scissors. Then, the tissues were teared up and cut into pieces. Thirdly, the tissues were digested in an enzyme cocktail for 3 h at 37°C. Then, we centrifuged the digestion mixture and washed it with PBS and DF-12 medium sequentially. Fourthly, we re-suspended the precipitate with fresh culture medium containing 87% DF-12, 12% FBS (fetal bovine serum, Gibco) and 1% antibiotics, and incubated the mixture at 37°C in a 5% CO_2 saturating humidified atmosphere in culture plates. Two days later, we removed the supernatant together with tissues not attached, washed the plates with PBS gently and then added fresh complete culture medium. Then, we detached the cells with 0.25% trypsin until they covered the whole plate and transferred them into new plates.

After the second to fourth cell passages, we tested the cell face-specific phenotypic antigens of MSCs using flow cytometry (BD, FACS Calibur) assay as reported before.[18] MSCs were presented as $CD29^+$, $CD90^+$, $CD73^+$, $CD44^+$, $CD106^-$, $CD19^-$, HLA-DR^-, $CD45^-$ $CD11b^-$ and $CD14^-$.

Transfection

HULC (H) mimic and HULC mimic control plasmid (H-NC) (GENEray Biotechnology) were transfected into MSCs by lipofectimine-3000 reagent (Invitrogen) until the cells reached 30–50% confluence. Forty-eight hours after transfection, we took pictures of MSCs under a microscope. Cells were collected for detecting surface antigens and for the following experiments.

Cell viability analysis

The effect of HULC on the cell viability of MSCs was detected using the CCK-8 kit (Sigma, USA). We first seeded the same quantity of MSCs in a 96-well plate and transfected H-NC or H after the MSCs reached 30% confluence. After 48 h, the supernatants were removed and fresh medium containing 10% CCK-8 was added. Three hours later, we measured the cell viability by a multi-detection micro plate reader (Bio-Tech, USA).

Cell density analysis

The pictures of MSCs after transfecting H or H-NC were taken and analyzed using ImageJ Launcher.

Cell cycle analysis and apoptosis analysis

Cell cycle and apoptosis were analyzed using flow cytometry. For cell cycle analysis, the cells were detached after 48 h transfection, and incubated in 70% cold ethanol overnight at 4°C. After washing the cells with PBS twice, the cells were incubated in PBS containing 50 μg/mL PI (propidium iodide, eBioscience), 20 μg/mL RNase A (Sigma) and 0.2% Triton-X100 for 30 min at room temperature (RT) in the dark. Then 2×10^4 cells were collected and analyzed using Flow Cytometry (FACS) according to the instructions. For cell apoptosis analysis, the supernatants together with cells were collected after 48 h transfection, and then the cells were centrifuged at 300*g* and washed with PBS twice. Then, the cells were incubated with Annexin V followed by PI according to the instructions. The cells were detected by FACs within 30 minutes and the results were analyzed using FlowJO-V10 (BD Bioscience).

MSCs migration assay

MSCs were scratched with the same 1 ml pipette after transfection for 48 h. After washing the cells with PBS, we added fresh complete medium for another 12 and 24 h. Then, we used a microscope to take pictures at 40× magnification and ImageJ Launcher to analyze the results.

MSCs invasion assay

Firstly, 80 μl diluted Matrigel (1:2 with DF-12) was added to the upper chamber of transwell membrane filters (8 μm size pore, Millipore) and incubated at 37°C till the Matrigel completely coagulated. Then, the MSCs transfected with H or H-NC were detached and 2×10^4 of the cells were seeded in the upper chamber for 24 h. After removing the non-invaded cells using cotton swabs, the cells were fixed with 4% PFA for 30 min at RT and then stained using crystal

violet solution for 20 min and then washed with distilled water. We randomly chose 10 fields of view per chamber and imaged. The cells' qualification was performed by blind counting.

Total RNA and real-time PCR

Total RNA was acquired using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Then 1 μg RNA was reverse-transcribed into cDNA using reverse transcriptase kit (TaKaRa). Real-time quantitative PCR (q-PCR) was performed by Step-One (Applied Bio-Systems). Relative expression of the target gene was analyzed using the ΔΔCt method.

Western blotting analysis

The protein in MSCs was extracted and the lysate was heated for 10 min at 98°C with SDS-PAGE loading buffer. Then 50 μg of the lysate was electrophoresed by SDS/PAGE (from 8 to 15%, adjusting to the molecular weight of the target protein) (Bio-Rad). The proteins were electro-blotted onto PVDF membranes (Roche) and blocked with bovine serum albumin (5%) for at least 30 min. Then, we incubated the membranes with primary antibodies against target proteins overnight at 4°C. Tubulin or GAPDH were set as internal reference. On the next day, secondary antibodies linked to horseradish peroxidase (HRP) were used to incubate with PVDF membranes. Enhanced Immobilon Western chemiluminescent HRP substrate (Millipore) was used for visualizing the blots.

Adipogenesis and osteogenesis

Adipogenesis and osteogenesis differentiation experiments were performed as previously reported.[18] In brief, MSCs were planted to six-well plates and transfected with H-NC or H as we described above. Two days post confluence, adipogenesis medium (containing DF-12, 10% FBS, 1 mM dexamethasone, 0.5 mM isobutylmethylxanthine, 1% penicillin/streptomycin, 5 mg/ml insulin and 50 mM indomethacin) was added to MSCs to induce adipogenesis. Three weeks later, MSCs were washed and fixed with 4% PFA for 30 min and stained with Oil Red for 10 min. Osteogenesis was induced using MODM (MSC Osteogenic Differentiation Medium, ScienCell) following the manufacturer's instructions. Sixteen days later, cells were washed, fixed and stained with alizarin red for 10 min.

Statistical analysis

All data were analyzed using Graphpad prism version 5.01, and the two-tailed Student's t-test was used to compare statistical significance. The data are expressed as mean ± S.E.M. $P < 0.05$ was regarded as a statistical significance.

Results

HULC has no effect on phenotypes and differentiation of MSCs

To analyze whether HULC affects the phenotypes and differentiation of umbilical cord-derived MSCs, HULC expression plasmid (H), HULC negative control (H-NC), small interfering RNAs for HULC (si-H) and small interfering RNA negative control (si-NC) were synthetized and transfected into MSCs by lipofectimine-3000.The results showed that the HULC mimics upregulated the expression of HULC significantly (Supplementary Figure S1(A)). However, all of the three interfering RNAs for HULC had no effects on the expression of HULC in MSCs (Figure S1(B)). As HULC was reported highly expressed in hepatocellular carcinoma cells, we transfected the si-NC and si-H into HepG2 (a hepatocarcinoma cell line) and checked the expression of HULC. The results showed that all the three siRNAs reduced the expression of HULC in HepG2 (Figure S1(C)). As HULC was much less expressed in MSCs compared with that in HepG2, siRNAs may hard to further decrease the expression of HULC in MSCs. Therefore, in the following studies, we focused on the effect of high expression of HULC on MSCs.

The expression of CD90, CD73, CD44, CD29, CD19, CD106, CD45, CD11b, CD14 and HLA-DR on MSCs was analyzed after transfecting H-NC or H for 48 h. The results showed that all of the surface antigens of MSCs were not changed after overexpressing HULC (Figure S2(A)). Meanwhile, the differentiation was also analyzed after overexpressing HULC in MSCs. As shown in Figure S2(B) and (C), MSCs could differentiate into bone and adipose in the specific conditions. However, overexpressing HULC did not affect the differentiation of MSCs.

HULC promotes the proliferation of MSCs

After transfection with H-NC or H for 24 h, the density of MSCs was analyzed. The results showed that the number of MSCs were more in the H group than that in the H-NC group. The statistical result showed that the cell density was 48.67 ± 0.8819 in the H-NC group and 57.67 ± 2.028 in the H group (Figure 1(a) and (b)). Then, we tested the cell viability after transfecting with H-NC or H by CCK-8 kit. The results showed that HULC could promote cell viability of MSCs (Figure 1(c)).

To figure out how HULC promoted cell density and viability, the effects of HULC on the cell cycle were analyzed. The analysis showed that overexpressing HULC could drive the cell cycle into S phase (35.943 ± 2.028 vs. 45.135 ± 1.301) and G2/M phase (13.640 ± 0.440 vs. 14.947 ± 0.325) whereas decrease G1 phase (50.412 ± 2.276 vs 39.908 ± 1.539), thus to accelerate cell division (Figure 2(a) and (b)). Besides, we also checked the expression of cell cycle-related genes, and found that CCND1, CDK6, CCNE1 were increased after overexpressing HULC in MSCs both at the mRNA (Figure 2(c)) and protein levels (Figure 2(d) and (e)).

The apoptosis of MSCs was analyzed after overexpressing of HULC. The results showed that HULC inhibited cell apoptosis (10.76 ± 0.9322 in H-NC vs. 7.820 ± 0.1893 in H) (Figure 3(a) and (b)). Additionally, the expression of BCL-2

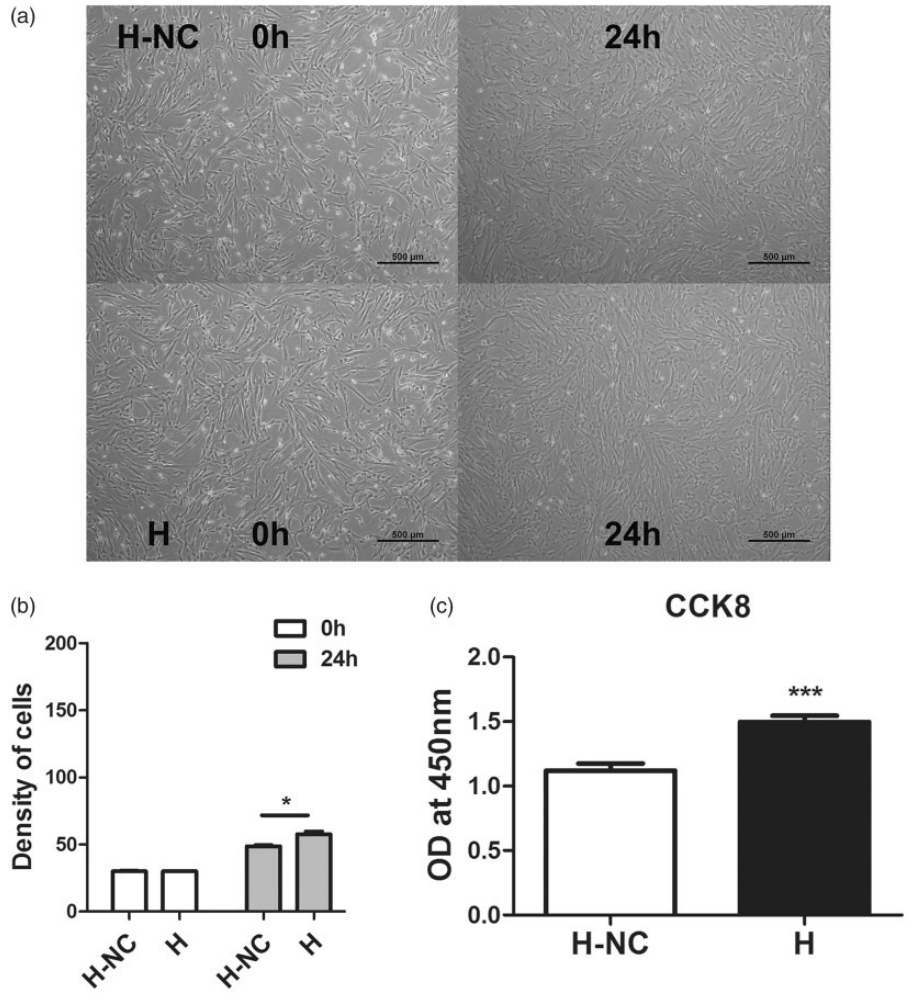

Figure 1. HULC promotes cell density and viability of MSCs. (a and b) After transfection of H-NC or H into MSCs for 0 and 24 h, cells were pictured and analyzed using Image J. (c) Cell viability was analyzed using CCK-8 kit. All of the results are from three independent experiments. Values are means ± S.E.M. $^{*}P < 0.05$, $^{***}P < 0.001$.

and caspase-3 in MSCs was also analyzed. The results supported that overexpressing HULC could inhibit cell apoptosis as BCL-2 and caspase-3 were highly expressed while cleaved-caspase-3 was decreased after overexpressing HULC (Figure 3(c) to (e)).

HULC promotes migration and invasion of MSCs

The migration of MSCs was examined through scratching with a pipette after overexpression of HULC. The results showed that HULC-overexpressed MSCs migrated faster than H-NC group (Figure 4(a) and (b)). Similarly, the HULC-overexpressed MSCs could invade better than H-NC (31.25 ± 5.573 in H-NC vs. 56.75 ± 6.290, $P < 0.05$) (Figure 4(c) and (d)). Then, we tested the expression of several migration-related genes, including MMP2,[19–21] N-cadherin,[22] and Vimentin.[23] The results showed that MMP2, N-cadherin, and Vimentin were highly expressed after overexpressing HULC (Figure 4(e) and (f)).

Inflammatory factors inhibit the expression of HULC

In our study, we found that overexpressing HULC could enhance cell proliferation, migration and invasion, and inhibit cell apoptosis, but how to control the expression of HULC in MSCs was unknown. Inflammatory factors were known to play important roles in many diseases.[24–26] TNF-α, TGF-β1, and R848 were used to stimulate MSCs respectively, and we found that the expression of HULC could be inhibited by all these inflammatory factors (Figure 5(a) to (c)).

Discussion

Recently, MSCs have been widely used in animal models and clinical treatment. We also showed that MSCs have an ideal therapeutic effect in the treatment of diseases such as preelampsia,[24] Asherman's syndrome,[25,26] and sepsis.[27] In order to improve the proliferation and function of MSCs, IL-1β or poly (I:C) pretreatment was proved to enhance the anti-inflammation ability of MSCs and promote the therapeutic effects in the treatment of DSS-induced colitis and sepsis.[28,29] Tian also reported that inhibiting the senescence of MSCs could increase the therapeutic potential of MSC sheets for tissue regeneration.[30] These reports suggest that deeper understanding of the molecular mechanisms of

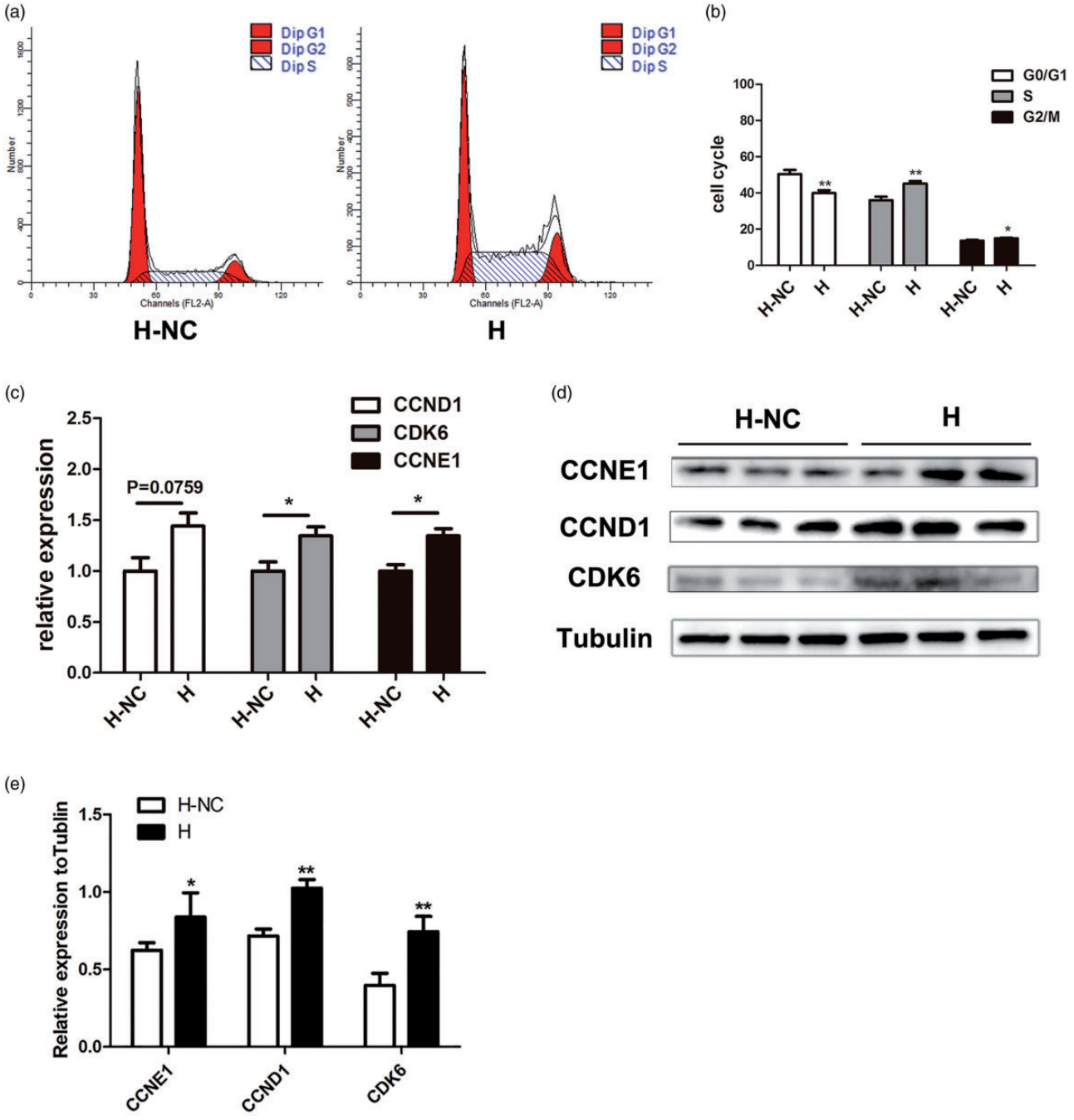

Figure 2. HULC promotes cell proliferation by enhancing cell division. After transfection with H-NC or H for 48 h, cells were subsequently assayed for DNA content using flow cytometry. Images in (a) show the cell cycle distribution, and the statistical analysis is shown in (b) for the percentage of cells in different phases of cell cycle. We also examined the cell cycle-related genes and found that CCND1, CDK6, and CCNE1 were all increased after overexpressing HULC in MSCs both at mRNA (c) and protein levels (d). (e) The gray density of protein bands was measured by Image J. All the results are from three independent experiments. Values are means ± S.E. M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

proliferation and function of MSCs is required for developing an effective stem cell based therapeutic strategy.

LncRNAs (more than 200 nucleotides in length) are recently discovered to constitute a large proportion of the whole transcriptome.[31,32] LncRNA HULC was first reported to be highly expressed in hepatocellular carcinoma cell lines in 2007 by Katrin.[33] HULC was reported to be related with many kinds of cancers, such as hepatocellular carcinoma,[14] cervical cancer,[34] osteosarcoma,[35] glioma,[36] and so on. Thus, HULC has been a biomarker of many diseases and is closely related with the prognosis and metastasis of many cancers.[37] Interestingly, the excessive HULC plus MALAT1 could significantly promote the growth of liver cancer stem cells.[16] As we reported that MALAT1 could not only affect the proliferation, migration, and angiogenesis, but could also affect the function of MSCs to modulate the polarization of macrophages,[17] we wonder whether HULC can affect MSCs.

In our study, we firstly found that HULC could not only promote the proliferation of MSCs by enhancing the cell

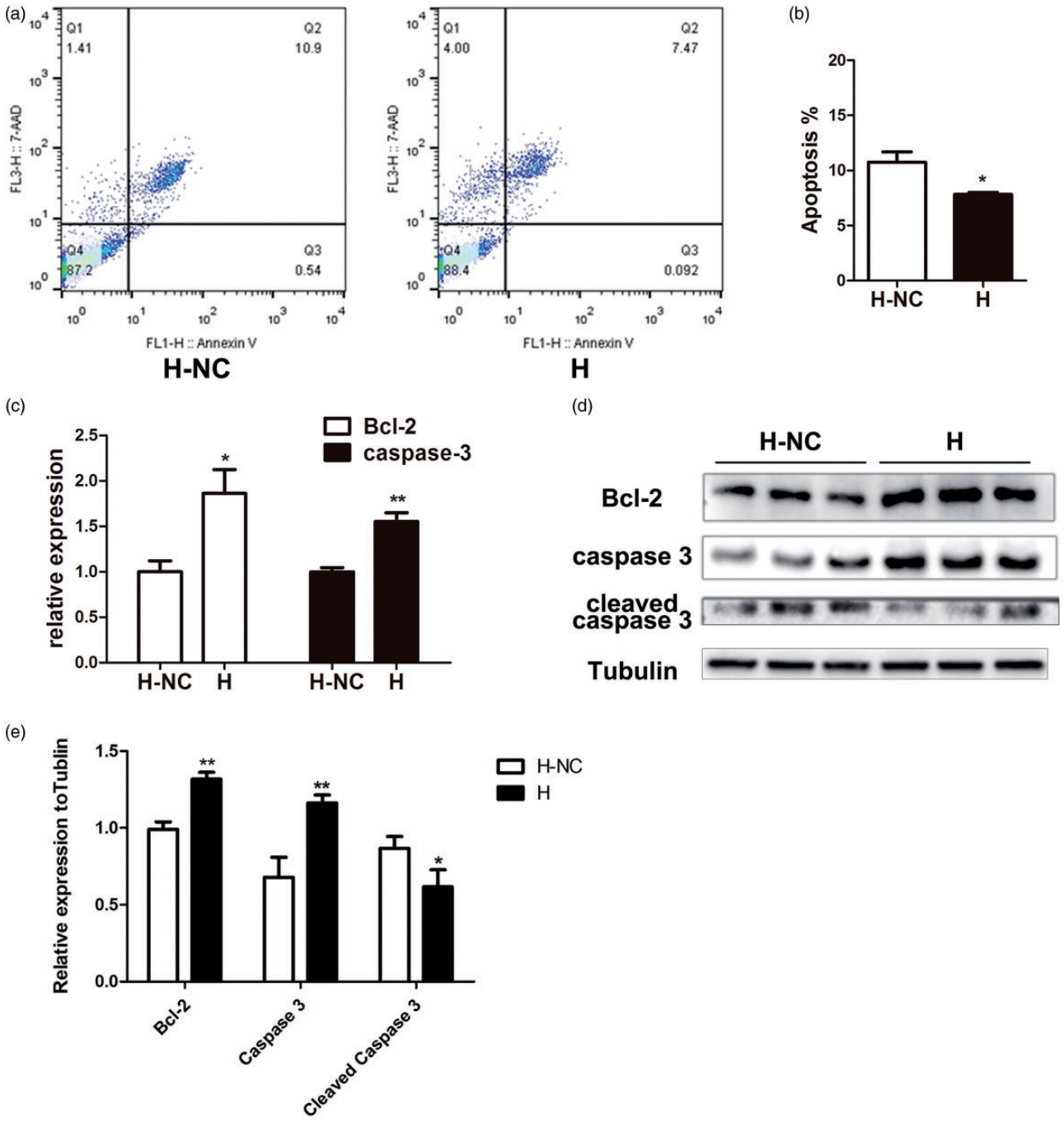

Figure 3. HULC inhibits cell apoptosis. (a and b) Cell apoptosis was analyzed after transfecting H or H-NC into MSCs. (c) qPCR analysis of BCL-2 and caspase-3. (d) Western blotting analysis of BCL-2 and caspase-3. (e) The gray density of protein bands was measured by Image J. All the results are from three independent experiments. Values are means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

division and inhibiting cell apoptosis, but could also promote the migration and invasion through upregulating the expression of N-cadherin, Vimentin and MMP2 without affecting the differentiation ability. Besides, we found that the expression of HULC was affected by some inflammatory factors such as TNF-α, TGF-β1 and R848, which reminds us that appropriate control of inflammation may enhance the therapeutic effects of MSCs.

The limitation of this study is that we failed to build a HULC-downregulating MSCs model to verify the results and the mechanisms of HULC affecting MSCs. Besides, HULC may also affect the angiogenesis and immunoregulation abilities of MSCs, which also need to be studied in the future.

In conclusion, our findings suggest that HULC could promote proliferation, migration and invasion of MSCs, inhibit apoptosis of MSCs, and HULC could be affected by several immune factors. Upregulating HULC and adequate inflammatory control may enhance the therapeutic effects of MSCs.

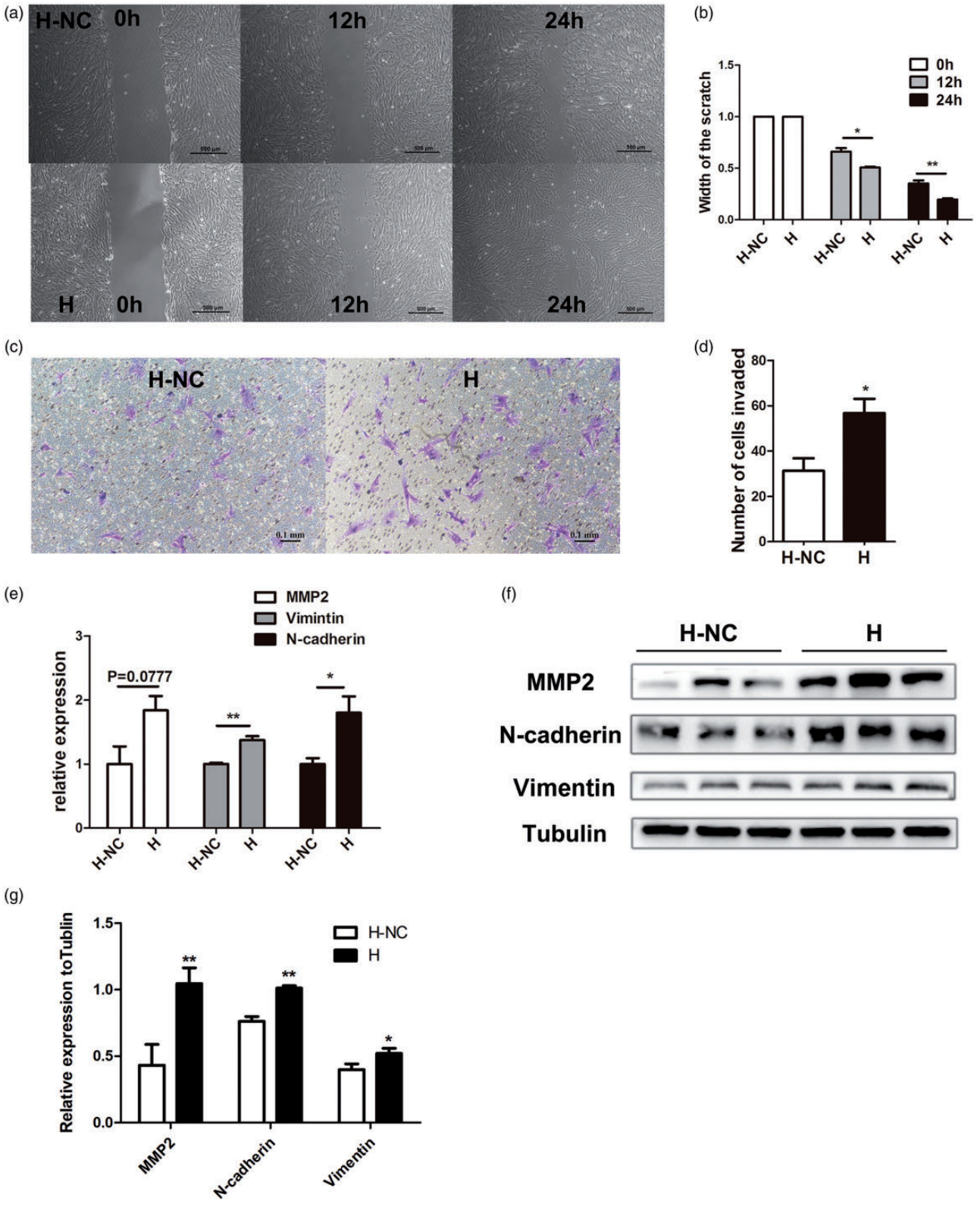

Figure 4. HULC promotes the migration and invasion of MSCs. (a) 48 h after transfection of H-NC or H into MSCs, a scratch was performed using a 1 ml pipette and pictures were taken using a microscope at 40× magnification after 0 h, 12 h, and 24 h. (b) A statistical analysis of the migration experiment was performed. (c) Analysis of migrated MSC cells transfected with H-NC or H. The number of migrated cells was quantified using a microscope at 100 magnifications after 24 h. (d) A statistical analysis of the migration experiments is shown. MMP2, N-cadherin, and Vimentin were highly expressed after overexpressing HULC both at mRNA level (e) and protein level (f). (g) The gray density of protein bands was measured by Image J. All results are from three independent experiments. Values are means ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

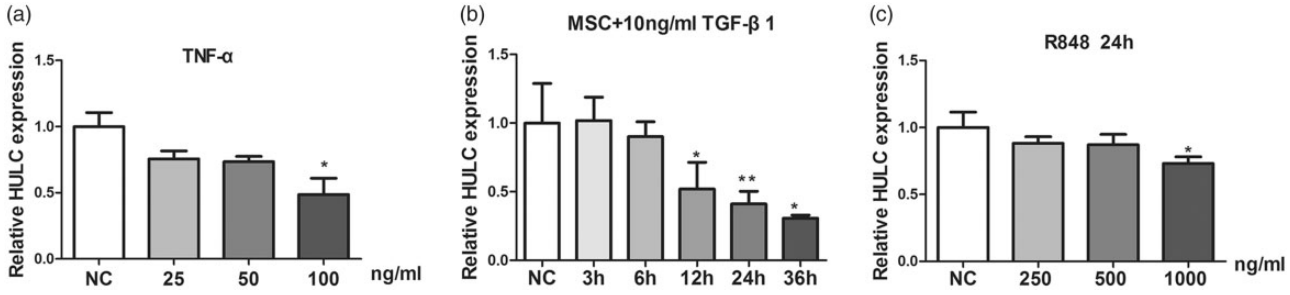

Figure 5. Inflammatory factors promote the expression of HULC. After treatment with TNF-α (a), TGF-β1 (b) and R848 (c), the expression of HULC was checked by qPCR in MSCs. All results are from three independent experiments. Values are means ± S.E.M. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Authors' contributions: XL, JW, YP, and YX performed the experiments and analyzed the data. DL participated in critical review and discussion of the article. YH and GZ supervised the project, analyzed data, and wrote the manuscript.

DECLARATION OF CONFLICTING INTERESTS

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