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Original Research

LncRNA H19 contributes to Rh2-mediated MC3T3-E1cell proliferation by regulation of osteopontin

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Abstract: 20(R)-ginsenoside Rh2 (Rh2) is one of the most active components of red ginseng, possessing the beneficial effects in cancer prevention and metabolic diseases. However, the detailed mechanisms that contribute to Rh2-mediated bone formation remain largely unknown. In this study, we assessed the expression of 17 long non-coding RNAs (lncRNAs) in cultured MC3T3-E1 cells under Rh2 treatments. We found that lncRNA H19 was significantly increased in Rh2-treated MC3T3-E1 cells. Expression of lncRNA H19 was promoted in a dose- and time-dependent manner after Rh2 treatments. Increased expression of lncRNA H19 resulted in osteopontin (OPN) overexpression in Rh2-treated MC3T3-E1 cells. Furthermore, knockdown of lncRNA H19 by specific siRNAs significantly abolished the Rh2-mediated cell proliferation effects. Knockdown of lncRNA H19 also decreased both mRNA and protein levels of OPN in the Rh2-treated cells, which was accomplished by inhibiting histones H3 and H4 acetylation of OPN promoter. Importantly, OPN knockdown fully blocked Rh2 induced MC3T3-E1 cell proliferation. Our results suggest that lncRNA H19 is an important contributor to Rh2-mediated MC3T3-E1 proliferation by regulation of OPN.

Key words: 20(R)-ginsenoside Rh2; LncRNA H19; Osteopontin; Acetylation; MC3T3-E1; Proliferation.

Introduction

The traditional Chinese medicine Ginseng, derived from the root of Panax ginseng CA Meyer (Araliaceae), has been widely used for over two thousand years in Asian countries. Ginseng and its active ingredients show widely beneficial effects in various aspects, including preventing cancer(1), improving immune system(2), inhibiting adipocyte differentiation(3), and even enhancing sexual function(4). The major active components of Ginseng are ginsenosides. Of particular interest, the 20(R)-ginsenoside Rh2 belongs to the proto-panaxadiol family and has been indicated as an important mediator in the cancer prevention due to its potential tumor-inhibitory effects(5-7). Recently, it has been documented that the Rh2(R) significantly depresses osteoclast formation and shows a stronger inhibitory effect on osteoclast formation(8, 9). Robustly, Rh2 stimulates osteoblastic differentiation and mineralization(10), and is considered to possess potent anti-osteporotic effects(11, 12), just as did the ginsenoside Rh1(13). All these studies indicate that Rh2 is critically involved in the bone formation process. However, the detailed mechanisms that contribute to Rh2-mediated bone formation remain to be elucidated.

Recent discoveries of long non-coding RNA (lncRNA) have greatly broadened our knowledge of disease initiation and progression. RNA biology study, for example, has helped identify multiple lncRNAs, such as Xist (14) and H19(15), which hold as milestones in lncRNA biology(16). In fact, lncRNAs have emerged as an essential regulator in almost all physiological and pathological aspects at chromatin remodeling, transcription, and post-transcriptional levels(17). In the present study, we aimed to investigate the possible roles of lncRNAs in the Rh2-mediated osteogenic cells proliferation. A total of 17 lncRNAs were assessed in MC3T3-E1 cells at the presence of Rh2 treatments. And a siRNA knowdown technique was employed to investigate the role of the most upregulated lncRNA-H19 in Rh2-mediated cell proliferation. Moreover, the downstream molecule underlying Rh2/lncRNA H19 pathway was further probed in MC3T3-E1 cells.

Materials and Methods

Cells and cell culture

The clonal osteoblastic cell line MC3T3-E1 was purchase from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (CAS, Shanghai, China). Cells were cultured in the dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 humidity-controlled incubator.

Reagents

20(R)-ginsenoside Rh2, Trizol solution and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay kit were obtained from the Sigma-Aldrich Co. (St Louis, MO, USA), and dissolved in 0.1% dimethylsulfoxide (DMSO, Sigma Co.) to generate a stock solution (100μg/mL), which was stored at -20°C. Stock solution was further diluted with culture media to yield the desired working concentrations (1

and 10μg/mL). The primary antibody against osteopontin (OPN) was purchased from R & D Systems Inc. (Minneapolis, MN, USA, Cat#AF808), and anti-β-actin antidoby was purchased from Sigma Co (Cat# A5316).. Chromatin immunoprecipitation (ChIP) assays were performed with antibodies recognizing acetylated histone H3 and H4 (Upstate Biotechnology, Inc., Lake Placid, NY, USA, Cat# 06-599, and Cat#06-866). siRNAs awere chemically synthesized by GenePharma (Shanghai, China).H19 siRNA 5'–CAUUCAUCCCGGUUACUUUTT–3'

Scramble siRNA 5'–TCATACTATATGACAG–3'. OPN siRNA (sense, 5′-GCCAUGACCACAUGGACGAdTdT-3′; antisense, 5′-dTdTCGGUACUGGUGUACCUGCU-3′), scramble siRNA as control for OPN experiment: (sense, 5′-CGGUAACAACGCGUACACGdTdT-3; antisense, 5′-dTdTCGTGTACGCGTTGTTACCG-3′).siRNAs were transfected with the Lipofectamine 2000 in accordance with the manufacturer's instruction.

Cell viability assay

MC3T3-E1 cells with distinct treatments were assayed to cell viability measurement using the MTT assay. Briefly, cells with Rh2 treatment were seeded into a 96-well plate at a density of 5×10^3 cells/well and cultured in the DMEM media prior to test. Control cells were treated with DMSO. For Rh2-treated MC3T3-E1 cells, extra treatment groups were included by transfection cells with the two specific siRNAs against lncRNA H19 (siRNA1+Rh2 group, and iRNA2+Rh2 group) and scramble RNA (scramble+Rh2 group). Scramble siRNA, siRNA 1, or siRNA2 transfected MC3T3-E1 cells were also included as controls.; For testing OPN's function in MTT assay, a group of Rh2-treated MC3T3-E1 cells were transfected with siRNA against OPN(siRNA OPN+Rh2 group), another group of Rh2-treated cells were transfected with scramble siRNA as contorl. DMSO treated, scramble siRNA, siRNA OPN transfected MC3T3-E1 cells were also included as controls in this assay.Cells with distinct treatments were synchronously cultured in the media for a consecutive four days. On each test day, each well was added with a total of 10μl MTT solution (5mg/mL) and subjected to further 2-4h incubation at 37°C. Thereafter, the absorbance for each well at 490nm was then measured through a microplate reader (Bio-Tek, USA). Results were obtained from triple tests.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from MC3T3-E1 cells using Trizol Reagent. For quantification of mRNA levels, total RNA (1μg) was reverse-transcribed using 200 U SuperScript II Reverse Transcriptase (Invitrogen; Carlsbad, CA, USA) and 500 ng Oligo (dT) primers to generate cDNAs. cDNAs were then subjected to qPCR assays using iTaq Fast SYBR Green Supermix with ROX (Bio-Rad Laboratories; Hercules, CA, USA) with the 7500 Fast Real-Time PCR System (Applied Biosystems, Inc.; Foster City, CA, USA).H19-forward, 5'- TCATTGGTGGAACTGCTA-3'; H19-reverse, 5'- TCTCCACAACTCCAACCA-3'; GAPDH-forward, 5'-TGCACCACCAACTGCTTAGC-3'; GAPDH-reverse, 5'- GGCATGGACTGTGGTCATGAG-3'. GAPDH gene was amplified as an internal control.Cycling conditions for PCR were: 95°C for 30 s, followed by 38 cycles of 95°C for 5 s and 60°C for 34 s. Amplification specificity was evaluated by melting curve analysis. Relative mRNA levels were formulated as described previously(18).

Western blot

Forty-eight hours after treatment, MC3T3 cells were harvested and lysed with the SDS lysis buffer (1% SDS; 10 mM EDTA; 50 mM Tris-Cl, pH 8.1; 10 mM sodium butyrate; 1 mM phenylmethyl sulfonylfluoride; 1μg/ml pepstatin A). The concentration of total proteins was determined using the BCA kit (Pierce, Rockford, IL, USA). Equal amounts of protein were loaded to each lane in a 12% SDS-PAGE gel. Thereafter, proteins were transferred to nitrocellulose (NC) membranes (Bio-Rad, Hercules, CA, USA). Then, the membranes were blocked within TBS/0.1% Tween-20 (TBST) containing 5% fat-free milk for 1h, and later incubated with primary antibody overnight at 4°C. After that, membranes were washed with TBST and incubated with the corresponding secondary antibody at 37°C for 1h. Membranes were then processed for chemiluminescent detection by using an ECL detection kit (Pierce, Rockford, IL, USA). β-actin was developed for the loading control.

Detection of histone acetylation levels of OPN promoter

To detect the histone H3 and H4 acetylation levels of OPN promoter, the ChIP assay was performed. MC3T3-E1 cells were harvested and lysed in SDS lysis buffer for 10 min on ice. Samples were sonicated to generate the shorter-length DNA fragments (0.1–0.6kbp, average size 0.3 kbp). Cellular debris was removed by centrifugation and chromatin solutions were distributed into multiple 1ml aliquots that were used subsequently. Chromatin aliquots were precleared with 100μl of DNA-coated protein A/G agarose in the absence of antibody. Samples were then precipitated with 20μl of antiacetyl histone H3, or antiacetyl histone H4 overnight at 4°C on a rotator. A nonspecific antibody (IgG) was used as a negative control. The DNA-histones complexes were mixed with 100μl of a 25% precoated protein A/G agarose suspension for 1h at 4°C while rotating. The immune complexes were then eluted by adding two consecutive 250μl aliquots of a freshly prepared solution of 1% SDS/0.1 M NaHCO3. Samples were consecutively digested with ribonuclease A (10 mg/ml) and proteinase K (20 mg/ml), respectively, to remove RNA and protein. The cross-linking reaction was reversed by overnight incubation of the solutions at 68°C, and the DNA was recovered by phenol-chloroform extractions and ethanol precipitations. DNA pellets were dissolved in 20μl deionized water as template for quantitative PCR analysis.

Statistical analysis

Data shown represent at least three independent replicate experiments performed in triplicate and are expressed as mean ± standard deviation (SD). Statistical significance was determined by the Student *t*-test with the significance set at $p < 0.05$.

Results

LncRNA H19 is significantly elevated in Rh2-treated MC3T3-E1 cells

The murine derived MC3T3-E1 cells, a well known osteogenic cell line and a good model to study in vitro osteoblast function, was selected for our study based on its physiological relevance. To assess the expression levels of lncRNAs in Rh2-treated cells, MC3T3-E1 cells were treated with Rh2 (Rh2 group) or the paired DMSO (control DMSO group).

A total of 17 lncRNAs which are considered key regulators of human disease developments were selected in this assay. qRT-PCR analysis of showed that expression levels of all these tested lncRNAs were altered, either largely or slightly. Most importantly, among all these lncRNAs, the lncRNA H19 had the highest expression level, followed by HIF1A-AS1 and HAR1A. H19 in Rh2-treated cells was presented with an up to 4-fold increase of mRNA level relative to the paired control one, while HIF1A-AS1 and HAR1A showed a 3.4-fold increase and a 2.3-fold increase of mRNA level, respectively (Figure 1). These results suggest that lncRNA H19 could be one of the most possible mediators of Rh2 effect. in MC3T3-E1 cells.

LncRNA H19 is promoted by Rh2 in a dose- and time-dependent manner.

Based on its robust upregulation among all the tested lncRNAs in the above experiment, lncRNA H19 aroused our interest and was selected for subsequent analysis. We modulated the concentration and time interval of Rh2 treatments in MC3T3-E1 cells. With the treated concentration of Rh2 increased, the expression level of lncRNA H19 was also increased correspondingly. At the concentration of 100μM Rh2, mRNA level of lncRNA H19 was over four-fold increased as compared with the DMSO-treated cells for 24h (Figure 2A). Furthermore, at the given concentration (10μM) of Rh2 treatment, mRNA level of lncRNA H19 was increasingly elevated as the time interval of Rh2 treatment extended (Figure 2B). These results suggest that lncRNA H19 is upregulated by Rh2 in a dose- and time-dependent manner in MC3T3-E1 cells.

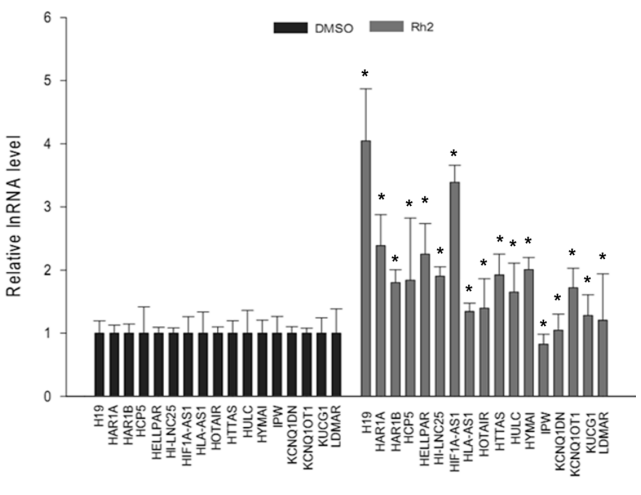

Figure 1. LncRNA H19 is significantly elevated in Rh2-treated MC3T3-E1 cells. A total of 17 lncRNAs were selected for qRT-PCR analysis in the Rh2-treated or DMSO-treated MC3T3-E1 cells. Among them, H19 had the highest expression level in response to Rh2 treatment with an increase of up to four-fold. *, $p< 0.05$ when comparing to DMSO control.

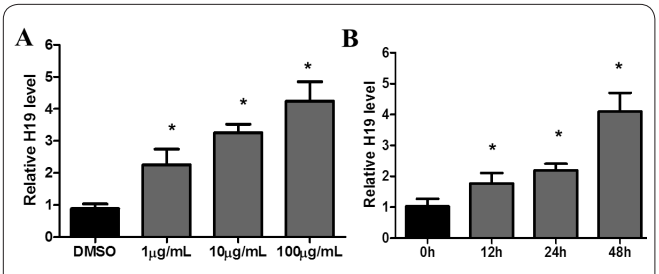

Figure 2. LncRNA H19 is promoted by Rh2 in a dose- and time-dependent manner. (A) Expression level of lncRNA H19 was accordingly elevated as the concentration of Rh2 treatment increased at 24h. (B) At a given concentration (10μM), it was observed that with the extended time interval of Rh2 treatments(0, 12h, 24, and 48h), the expression level of lncRNA H19 was elevated accordingly. At 48h after Rh2 treatment, lncRNA H19 was increased approximately 4-fold. *, $p< 0.05$ when comparing to DMSO or 0hr treatment control.

Knockdown of lncRNA H19 significantly abolishes Rh2-mediated MC3T3-E1 cell proliferation.

To assess the role of lncRNA H19 in Rh2-mediated osteoblastic cell proliferation, specific siRNAs against lncRNA H19 (siRNA1# and siRNA2#) were employed. Knockdown efficiency of synthesized siRNAs was first evaluated, and it was shown that the expression level of lncRNA H19 was significantly reduced by the specific siRNAs (Figure 3A), verifying the specificity of our designed siRNAs. On this basis, we investigated the effects of lncRNA H19 knockdown on Rh2-mediated cell viability. As manisfested by the MTT assay, MC3T3-E1 cells began to exhibit higher cell viability on day 3. On day 4, the viability of Rh2-treated cells were approximately twice of that in the DMSO-treated cells or scramble siRNA-transfected cells. This observation supported that Rh2 had beneficial effects on osteoblast. Importantly, the viability of Rh2-treated cells was significantly inhibited when lncRNA H19 was reduced by either specific siRNA1 or siRNA2, supporting the hypothesis that lncRNA H19 is a key mediator of Rh2 promoted osteogenic cell viability. As a crucial control, merely lncRNA H19 knockdown (siRNA1 and siRNA2

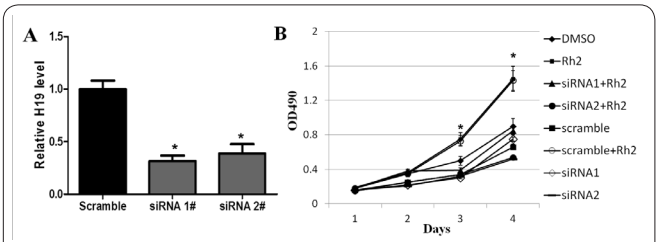

Figure 3. Knockdown of lncRNA H19 significantly abolishes Rh2-mediated MC3T3-E1 cell proliferation. (A) Specific siRNAs against lncRNA H19 (siRNA#1 and siRNA#2) were synthesized. The knockdown efficiency was verified by RT-PCR in MC3T3-E1 cells. (B) Effect of lncRNA H19 knockdown on Rh-2 mediated cell viability was assessed. Treatment of Rh2 promoted MC3T3-E1 cell viability by up to 2-fold on day 4. Knockdown of lncRNA H19 with specific siRNAs significantly abolished Rh2-mediated osteoblastic growth. *, $p< 0.05$ when comparing the scramble+ Rh2 group to DMSO or scramble control.

groups) did not generate distinguishable MC3T3-E1 cell viability compared to the scramble control on day 3 and day4, (Figure 3B) reinforcing the idea that the regulated lncRNA H19 is responsible for Rh2 induced cell viability status in MC3T3-E1 cells.

These data indicated that knockdown of lncRNA H19 blocked Rh2-mediated osteoblastic growth.

LncRNA H19 regulates osteopontin by promoting its histone acetylation under Rh2 treatments

Osteopontin (OPN) is a critical mediator of bone formation(19-21). To investigate whether OPN was involved in Rh2-lncRNA H19 axis in MC3T3-E1 cells, the lncRNA H19 knockdown was done using previously designed specific siRNAs (Figure 4A). It was observed that treatment of Rh2 with and without scramble siRNA significantly increased the mRNA level of OPN. Interestingly, knockdown of lncRNA H19 significantly diminished the above effects. In fact, the mRNA level of OPN was reduced to the level in the DMSO-treated cells when lncRNA H19 was inhibited in Rh2-treated cells (Figure 4B). Comparable results were also observed in the protein level of OPN (Figure 4C). These results indicated that lncRNA H19 probably mediated Rh2effect by targeting downstream OPN. Furthermore, treatment of Rh2 (with or without the scramble siRNA) significantly increased the histone H3 and H4 acetylation levels of OPN promoter in MC3T3-E1 cells. Importantly, knockdown of lncRNA H19 decreased Rh2-induced histone acetylation levels of OPN promoter to the control levels (Figure 4D), indicating that lncRNA H19 regulated OPN through histone acetylation modifications of OPN promoter. These results suggested that lncRNA H19 positively regulates OPN in Rh2-treated cells.

Osteopontin expression inhibition blocked Rh2 promoted MC3T3-E1 cell viability in culture

To access whether the potential lncRNA H19

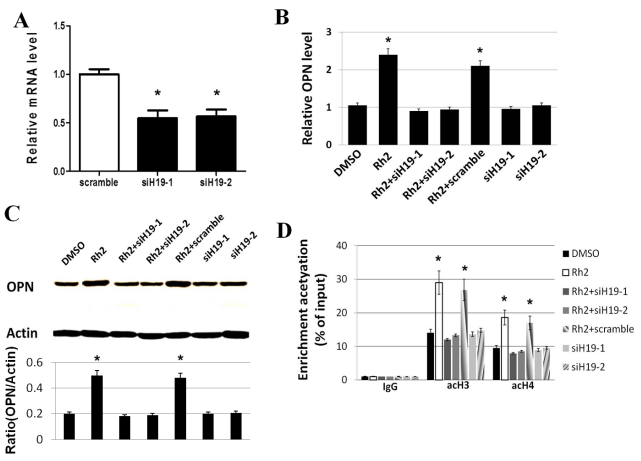

Figure 4. LncRNA H19 positively regulates osteopontin under Rh2 treatments. (A) Knockdown efficiency of the specific siRNAs was verified by RT-PCR. (B, C) treatment of Rh2 (with or without scramble) increased both mRNA (B) and protein levels (C) of osteoponin (OPN). Importantly, knockdown of lncRNA H19 significantly abolished the above effects. (**D**) Rh2 promoted the histone H3 and H4 acetylation levels of OPN promoter, whereas knockdown of lncRNA H19 significantly abolished the above effects. *, $p< 0.05$ when comparing to DMSO or scramble control.

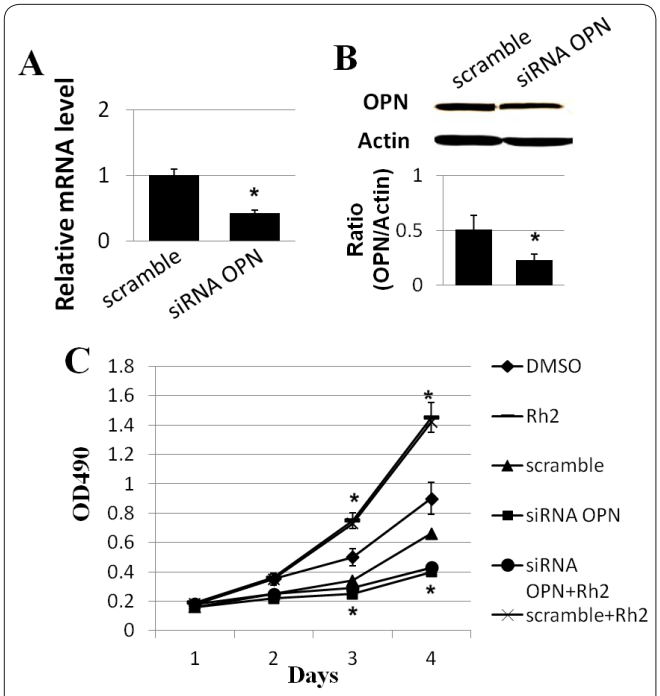

Figure 5. Osteopontin downstream lncRNA H19 mediated Rh2 treatment induced MC3T3-E1 cell proliferation. Knockdown efficiency of the siRNA against OPN was verified by RT-PCR **(A)** and western blotting (**B**). (C): Knockdown of OPN significantly abolished Rh2 treatment (with or without scramble) induced MC3T3-E1 cell proliferation elevation at day 3 and day4. *, $p< 0.05$ when comparing to both DMSO and scramble controls. (* was demonstrated for the scramble+Rh2 group and siRNA OPN+Rh2 group in the Figure).

downstream target OPN is essential for mediating the above observed MC3T3-E1 cell viability status change by Rh2, we applied siRNA gene knockdown strategy to modulate the expression level of OPN. We successfully downregulated OPN mRNA expression by 2.3 fold in MC3T3-E1 cells. (Figure 5A) The protein expression change of OPN was further verified with western-blotting. (Figure 5B)

In the cultured MC3T3-E1 cells, OPN siRNA knockdown fully blocked Rh2 promoted cell viability elevation at day 3 and day 4, (Figure 5C) even generating a relative lower viability level compared to DMSO and scramble controls, indicating that OPN expression was responsible for mediating the observed cell viability phenotype induced by Rh2.

Discussion

Human bone mass homeostasis is dynamically regulated by coupled actions of osteoblasts (bone formation) and osteoclasts (bone resorption), termed as bone remodeling (22). Recently, it has been documented that the active component of Ginseng, Rh2, possesses potent inhibitory effect on osteoclast formation (8, 9), whereas it stimulates osteoblastic differentiation and mineralization (10), it is considered to possess potent anti-osteporotic effects(11, 12). However, despite the potent effects, the detailed mechanisms underlying Rh2-mediated osteoblastic activity remain to be elucidated.

In this study, we are the first to report that lncRNA H19 is a potential target of Rh2 in mediating osteoblastic MC3T3-E1 cell proliferation. Among the 17

lncRNAs, lncRNA H19 showed the highest upregulated mRNA level in response to Rh2 treatments (Figure 1). The elevated expression level of lncRNA H19 was Rh2 treatment dose- and time-dependent (Figure 2), indicating that lncRNA H19 was under positive regulation of Rh2. Importantly, knockdown of lncRNA H19 by siRNAs significantly abolished Rh2-mediated MC3T3-E1 cell proliferation,(Figure 3B). Taken together, these results strongly suggest that lncRNA H19 contributes to Rh2-mediated osteoblastic proliferation.

Recent discoveries of lncRNAs have greatly revolutionized our understanding of disease initiation and development. LncRNA H19 was discovered in 1991 by Bartolomei and later shown to be highly expressed in the embryo proper, extra embryonic tissues and most fetal tissues. However, its expression is dramatically reduced after birth (23, 24), unless in the reported cancer tissues (16, 25). In fact, the role of lncRNA H19 in cancer development has been widely reported, with its potential role in developmental aspects barely studied. Our in vitro findings provide evidence that lncRNA H19 is also a critical contributor to the physiological bone formation.

LncRNA H19/let-7 and lncRNA H19/miRNA-675 axis are two major pathways underlying lncRNA H19 biological effects (26, 27). In the present study, we found that lncRNA H19 targeted OPN and promoted OPN expression in the osteoblastic MC3T3-E1 cells (Figure 4B and 4C). Further evidence supported that knockdown of lncRNA H19 led to the decreased acetylation levels of histone H3 and H4 of OPN promoter (Figure 4D). The effect of chromatin structure on gene transcription is determined, at least in part, by the post-transcriptional modifications of the histones, such as acetylation and methylation. Acetylation is believed to facilitate transcription whereas deacetylation reverses such effects and thus, reinforces the repressive effect of chromatin(18, 28). The decreased acetylation levels of H3 and H4 by lncRNA H19 inhibition collaborated our results that lncRNA H19 induced OPN expression in MC3T3-E1 cells under Rh2 treatment. OPN is an extracellular glycoprotein, which has important roles in bone remodeling (29). Our results in Figure 5 demonstrated that inhibition of OPN expression fully abolished Rh2 mediated cell proliferation. Thus all of these results would collectively lead us to conclude that lncRNA H19-induced OPN expression was responsible for Rh2-mediated osteogenic cell proliferation.

All in all, we indentified a novel mechanism that contributed to Rh2-mediated osteoblastic MC3T3-E1 cell proliferation. The Rh2-mediated effects were attributed to lncRNA H19 ugregulation, which consequently led to the increase of OPN expression. Our findings may provide novel insights into Rh2-mediated bone formation process.

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