Loss of high-mobility group N5 contributes to the promotion of human endometrial stromal cell decidualization

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Abstract

Purpose: High-mobility group N (HMGN) proteins are the only non-histone proteins that specifically bind within the nucleosome between core histones and DNA. Among them, HMGN5 is one of the candidates that could participate in mouse endometrial decidualization; however, the specific role of HMGN5 remains to be clarified in human endometrial stromal cells (HESCs).

Methods: Primary HESCs were isolated from hysterectomy specimens and incubated with or without 8-bromo-cyclic adenosine monophosphate (8-br-cAMP) and medroxyprogesterone acetate (MPA).

Results: We demonstrated that HMGN5 expression in decidualized HESCs stimulated by 8-br-cAMP and MPA decreased significantly. The inhibition of HMGN5 expression by small interfering RNA (siRNA) induced the major decidual marker genes expression, including IGFBP1 (insulin-like growth factor binding protein 1) and PRL (prolactin). In addition, microRNA-542-3p (miR-542-3p), which was identified as a regulatory miRNA of IGFBP1 during decidualization, was significantly suppressed by HMGN5 siRNA. However, the expression of HMGN5 was not alternated by miR-542-3p overexpression.

Conclusions: These findings suggest that the down-regulation of HMGN5 plays a role in the promotion of human endometrial stromal decidualization and acts upstream of miR-542-3p.

KEY WORDS
decidualization, endometrium, HMGN5, IGFBP-1, miR-542-3p

1 INTRODUCTION

Decidualization is the differentiation process of endometrial stromal cells into specialized secretory decidual cells that regulates blastocyst implantation and subsequent placental formation.1,2 This process denotes both a morphological and biochemical transformation that is driven by the post-ovulatory rise in circulating progesterone levels and increasing local cAMP production.2,3 Decidualization is also essential to establish a functional fetomaternal interface because it regulates endovascular trophoblast invasion, tissue homeostasis, and could grant resistance to environmental oxidative stress.4–6 Abnormalities in decidualization can be followed by various pregnancy complications, such as recurrent miscarriages, fetal growth restriction, preterm labor, and preeclampsia.3 While there is
some understanding of this process, it has not been fully elucidated what the key regulatory factors is contributing to differentiation of uterine stromal cells into decidual cells.

High-mobility group N (HMGN) proteins are ubiquitous nuclear proteins among the HMG superfamily that target chromatin and are the only nuclear proteins shown to specially bind to the nucleosome core particle, the building block of the chromatin fiber. HMGN proteins influence epigenetic modifications and modulate the structure and function of chromatin; thus, they play an important role in transcription. HMGN5 (also known as NSBP1) is a new member of the HMGN protein family and has a unique molecular structure compared to the other members of the HMGN protein family. HMGN5 contains a typical HMGN nucleosome-binding domain and comprises a long C-terminal region. When HMGN5 binds to core nucleosome particles, there is a reduction in the compacting of chromatin fiber, leading to altered transcription. HMGN5 is broadly distributed in various tissues and is mainly located in the nucleus. While transcription profiles indicate that HMGN5 affected the expression of more than 2000 genes, the biological role of HMGN5 is not yet fully characterized.

Sakai et al. reported that structural changes of histones play an important role in human endometrium stromal cells (HESCs) during the decidualization process. Histone acetyltransferases and histone deacetylases (HDACs) can induce differentiation and have during the decidualization process. Histone acetyltransferases and modulate the structure and function of chromatin; thus, they have an important role in transcription. HMGN5 (also known as NSBP1) is a new member of the HMGN protein family and has a unique molecular structure compared to the other members of the HMGN protein family. HMGN5 contains a typical HMGN nucleosome-binding domain and comprises a long C-terminal region. When HMGN5 binds to core nucleosome particles, there is a reduction in the compacting of chromatin fiber, leading to altered transcription. HMGN5 is broadly distributed in various tissues and is mainly located in the nucleus. While transcription profiles indicate that HMGN5 affected the expression of more than 2000 genes, the biological role of HMGN5 is not yet fully characterized.

Sakai et al. reported that structural changes of histones play an important role in human endometrium stromal cells (HESCs) during the decidualization process. Histone acetyltransferases and histone deacetylases (HDACs) can induce differentiation and have potential as enhancers of decidualization of HESCs. In addition, it has been shown that HMGN5 regulates the endometrial decidualization process in mice. However, the specific role of HMGN5 in HESCs has not yet been clarified. In the last decade, several studies demonstrated microRNAs (miRNAs) had important roles for endometrial decidualization. In addition, we recently identified miR-542-3p as a negative regulatory miRNA of IGFBP1 which was well known as the decidual marker genes. Therefore, this study was undertaken to investigate the expression, regulation, and function of HMGN5 through miR-542-3p during the decidualization of HESCs.

2 | MATERIALS AND METHODS

2.1 | Isolation of HESCs and cell culture

The Institutional Review Board of the Saitama Medical University Hospital approved this study, and informed consent was obtained before tissue collection from all patients. HESCs were obtained from uterine fibroids patients of normal menstrual cycle, and of not receiving hormonal treatment before operation. HESCs were isolated and cultured as previously described. Decidualization treatments of HESCs were performed by 0.5 mM 8-bromo-cyclic adenosine monophosphate (8-br-cAMP; Sigma-Aldrich, St. Louis, MO, USA) and 10^{-6} M medroxyprogesterone acetate (MPA; Sigma-Aldrich, St. Louis, MO, USA) for each indicated time point.

2.2 | Prolactin measurement

The concentration of PRL in the HESC culture media was measured by the electrogenerated chemiluminescence immunoassay (ECLIA) method using ECLusys Prolactin III reagent and Cobas 6000 (Roche Diagnostics, Basel, Switzerland).

2.3 | Transfection of siRNA and miRNA mimics

The siRNA targeting HMGN5 (HS01077792; Takara bio Inc., Japan) was transfected to HESCs using Xfect RNA Transfection Reagent (Takara Shuzo, Shiga, Japan). The mirVana™ miRNA mimic (#MCM11340; Thermo Fisher Scientific, Waltham, MA, USA) or hsa-miR-542-3p was transfected to HESCs using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Control cultures were transfected with non-targeting siRNA or a control miRNA mimic. After 4 or 6 hours, the medium was changed to control medium or decidual medium.

2.4 | Total RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA from HESCs were extracted by mirNeasy Mini kit (Qiagen, Hilden, Germany). For analysis of mRNAs, the synthesis of cDNA from total RNA by reverse transcription was performed using BioScript reverse transcriptase (Bioline, London, UK) with oligo (dT) 18 primer. qRT-PCR was measured using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences for each gene were shown in Table 1. For analysis of miRNAs, cDNA was synthesized using the Taqman microRNA RT kit (Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was measured by Taqman Universal Master Mix II with UNG (Thermo Fisher Scientific, Waltham, MA, USA). For miR-542-3p assay, TaqMan MicroRNA assays and the endogenous control U6 (Thermo Fisher Scientific, Waltham, MA, USA) were used for reverse transcription and amplification as previously reported. Each qRT-PCR determined using the PikoReal 96 real-time PCR system. The expression level of HMGN5, IGFBP1, PRL, and WNT4 mRNA expression relative to GAPDH or miR-542-3p expression relative to U6 were calculated by the 2^{ΔΔct} method.

| Primer name | Primer sequence (5’ to 3’) |
|-------------|---------------------------|
| HMGN5-forward | CTGCTTGGCTTTTTTCTG |
| HMGN5-reverse | AGACAACTGGCATCTTCTC |
| GAPDH-forward | CGACACTTGTGACTGCTA |
| GAPDH-reverse | AGGGCTTACATGGCAACTG |
| IGFBP1-forward | CTTGCGTGCAAGCTCAG |
| IGFBP1-reverse | CCAAAAGGATGGAAATGCC |
| PRL-forward | CTACATCATGAAACCTCTC |
| PRL-reverse | CGGCTTGCTCTTCTCCT |
| WNT4-forward | CATGCAACAAAGACGCCCAAG |
| WNT4-reverse | AAGCGAACCAGTGAATT |

TABLE 1 | Primer sequence for qRT-PCR
2.5 | Immunofluorescent staining

HESCs were cultured on cover glass before confluence and treatment with or without decidualization medium 3 days. After 3 days, the cells were fixed by 4% paraformaldehyde and stained using primary HMGN5 antibody (Abcam, Cambridge, UK) and the secondary Alexa Fluor 488 antibody (Thermo Fisher Scientific, Waltham, MA, USA). The mounting media was used containing DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized by a fluorescent microscope (Axiocam; Carl Zeiss, Oberkochen, Germany).

2.6 | Western blotting

Cells were lysed by RIPA (Radio-Immunoprecipitation Assay) buffer (WAKO Pure Chemical, Osaka, Japan) with Protein inhibitor, cComplete Tablets EDTA-free (Roche, Mannheim, Germany). The protein extracts were separated by SDS-polyacrylamide gel electrophoresis and transferred to Amersham Protran nitrocellulose blotting membrane (GE Healthcare, Buckinghamshire, UK). anti-HMGN5 rabbit polyclonal antibody (Abcam, Cambridge, UK) or anti-α-Tubulin rabbit polyclonal antibody (Protein Tech, Rosemont, USA) was used for primary antibody. The Western blots were probed with anti-Rabbit IgG, HRP-Linked Whole Ab Donkey (GE Healthcare, Buckinghamshire, UK). The recognized proteins were detected using ECL prime Western blotting detection kits (GE Healthcare, Buckinghamshire, UK) by c-Digit Blot Scanner (LI-COR, NE, USA).

2.7 | Statistic analyses

Each experiment was repeated more than three different cell culture preparations (n = 3-6). Statistical analyses were performed by two-tailed Student's t-test, and data were indicated by mean ± standard error. P value of <0.05 was considered significant.

3 | RESULTS

3.1 | HMGN5 expression in HESCs decreased upon decidualization

The efficiency of decidualization of HESCs was confirmed by characteristic morphological changes and evaluating IGFBP1 and PRL mRNA expression, which are widely used as biological decidual

![FIGURE 1](https://example.com/figure1.png)

Morphological transformation and IGFBP1 and PRL expression in decidualizing HESCs. (A) Undifferentiated primary HESCs exhibit a fibroblastic spindle-shaped morphology (CTL). Primary HESCs treated with 8-br-cAMP (0.5 mM) and MPA (10^{-6} M) (8-br-cAMP/MPA) for 3 days transform the spindle-shaped cells into cells with larger nuclei and abundant cytoplasm, which are the typical morphology of decidual cells. Scale bar indicates 200 μm. (B) Primary HESCs were stimulated in the absence (CTL) or presence of 8-br-cAMP and MPA (8-br-cAMP/MPA). Expression of IGFBP1 and PRL mRNA, were measured by RT-qPCR and normalized to GAPDH (n = 4-6). (C) Confluent HESCs were treated with or without 8-br-cAMP and MPA for 3 days. The data represent the mean of PRL protein concentration in the supernatant (n = 6). The data represent mean ± standard error. *P < 0.05, **P < 0.001
As reported previously, HESCs appear as spindle-shaped fibroblast-like cells on light microscopy when cultured without treatment. Treatment with 8-br-cAMP and MPA induced the decidual phenotype, characterized by larger and rounder cells (Figure 1A), as well as significantly induced the expression of IGFBP1 and PRL mRNA in a time-dependent manner (n = 4–6) (Figure 1B). Consistent with qRT-PCR analysis, PRL secretion in HESCs was significantly induced by treatment with 8-br-cAMP and MPA (n = 6) (Figure 1C). To explore the potential role of HMGN5 in the decidualization process of HESCs, the expression and localization patterns of HMGN5 in non-decidualized and decidualized HESCs were examined by qRT-PCR and immunofluorescent staining. The expression level of HMGN5 in 8-br-cAMP- and MPA-treated HESCs was significantly inhibited compared with control cells after 12 and 24 hours (Figure 2). However, this inhibitory effect of HMGN5 mRNA expression in decidualized HESCs disappeared after 3 days of culture (n = 3–6). In agreement with the RNA analysis, confocal microscopy demonstrated that staining of HMGN5 protein in non-decidualized HESCs was localized in the nucleus, and the immunoreactivity decreased upon decidualization (Figure 3A). Furthermore, we confirmed that HMGN5 protein expression was inhibited in HESCs decidualized with 8-br-cAMP and MPA (n = 3) (Figure 3B).

3.2 Loss of HMGN5 promotes decidualization

Next, to determine the functional significance of HMGN5 expression during HESC differentiation, we transfected primary HESCs with either non-targeting siRNA or siRNA targeting HMGN5; we then cultured the cells in medium containing 8-br-cAMP and MPA for 3 days. To evaluate the transfection efficiency, qRT-PCR for HMGN5 was performed. Compared with cells transfected with non-targeting siRNA, HMGN5 expression in response to 8-br-cAMP and MPA treatment was markedly down-regulated by approximately 60% in cells transfected with siRNA targeting HMGN5 (n = 3–4) (Figure 4A). The mRNA expression of the decidual marker genes, IGFBP1 and PRL, but not WNT4, was significantly increased by siRNA targeting HMGN5 (n = 3–4) (Figure 4B–4D).

3.3 miR-542-3p is involved in the decreased expression of HMGN5

HMGN5 binds to core nucleosome particles and can affect transcription. This study demonstrated that loss of HMGN5 expression induces the expression of decidual markers IGFBP-1 and PRL and, as a result, promotes the decidualization process; thus, there is a possibility of a factor responsible for the down-regulation seen between HMGN5 and the decidual marker genes. Recently, we demonstrated that the down-regulation of miR-542-3p promotes decidualization.
by targeting IGFBP1. Therefore, we postulated that HMGN5 may indirectly regulate IGFBP1 expression through miR-542-3p. To elucidate this hypothesis, we investigated miR-542-3p expression under knockdown of HMGN5 in decidualized HESCs. HMGN5 knockdown was sufficient to inhibit miR-542-3p expression (n = 6) (Figure 5A). To examine whether HMGN5 is downstream of miR-542-3p, we investigated the alternation of HMGN5 expression under miR-542-3p overexpression using a miR-542-3p mimic in decidualized HESCs. miR-542-3p overexpression was insufficient to alternate HMGN5 expression during all culture periods (n = 5-6) (Figure 5B), suggesting that HMGN5 indirectly regulates IGFBP1 expression through miR-542-3p (Figure 6).

4 | DISCUSSION

Decidualization is characterized by the transformation of endometrial cells into specialized secretory cells, with a distinctive process of vascular remodeling and an influx of immune cells into the stroma, that is, exclusively uterine natural killer cells and macrophages. This process is closely controlled by several factors, including ovarian steroids, growth factors, cytokines, and other signal molecules. Abnormalities in decidualization result in various pregnancy complications, such as recurrent miscarriages, fetal growth restriction, preterm labor, and pre-eclampsia. However, key regulatory factors contributing to the differentiation of uterine stromal cells have not been fully elucidated. Thus, it is worthwhile to clarify the precise mechanism underlying decidualization to facilitate the development of therapeutic strategies for these complications.

In this study, we demonstrated that HMGN5 mRNA expression decreases upon decidualization. The inhibition of HMGN5 expression by HMGN5 siRNA promoted the induction of major decidual marker genes, including IGFBP1 and PRL. Moreover, miR-542-3p expression, which is a known regulator of IGFBP1 expression during decidualization, was significantly suppressed by HMGN5 siRNA. These findings suggest that the down-regulation of HMGN5 expression contributes...
to the promotion of human endometrial stromal decidualization, acting upstream of miR-542-3p (Figure 6A, B).

Shirakawa et al. demonstrated that HMGN5 is highly expressed in mouse trophoblasts, and its expression is related to placental formation. It has recently been reported that HMGN5 expression is induced by decidual stimulation in a mouse in vitro system; however, this finding contradicts the results of this study. This discrepancy may be due to differences in the species studied or the decidualization protocol used, that is, Li et al. adopted a combination of estradiol and progesterone to induce decidualization, which is a much weaker stimulus than treatment with a cAMP analog and MPA. To the best of our knowledge, this study is the first report to demonstrate that HMGN5 regulates differentiation of HESCs through decidual gene targeting via miR-542-3p expression.

As small non-coding RNAs, miRNAs were identified as regulatory RNAs that act by silencing translation or reducing the stability of mRNA and have been reported to regulate various biological processes, including cell growth, proliferation, and differentiation. It is well known that abnormal miRNA expression is related to various endometrial disorders, such as endometriosis, endometrial hyperplasia, and carcinoma. To date, several studies have revealed that miRNA plays an important role during endometrial decidualization. Recently, we reported that the down-regulation of miR-542-3p expression in HESCs enables the morphological and biological differentiation of the endometrium. Initially, we hypothesized that HMGN5 directly regulates decidual gene expression; however, contrary to our expectation, a reciprocal relationship was found between the expression of HMGN5 and IGFBP-1 during decidualization in this study (Figure 2). This finding raised the possibility of a factor responsible for the down-regulation seen between HMGN5 and IGFBP-1. Therefore, we focused on miR-542-3p as a candidate.

In this study, we demonstrated that HMGN5 expression in decidualized HESCs was down-regulated early during the culture period compared with non-decidualized HESCs. Previous studies have suggested that decidual transformation is at least a biphasic process, characterized initially by an acute auto-inflammatory phase, followed by a profound anti-inflammatory response. Therefore, HMGN5 may regulate genes associated with the acute auto-inflammatory phase. In addition, we showed that the mRNA expression of decidual marker genes, IGFBP1 and PRL, but not WNT4, increased significantly by siRNA targeting HMGN5. However, we previously showed that the overexpression of miR-542-3p inhibited IGFBP1, PRL, and WNT4 mRNA expressions in decidualized HESCs. Other unknown regulatory factors of WNT4 may account for this finding, and further studies are required to resolve this discrepancy.

In conclusion, this study demonstrated that the down-regulation of HMGN5 promotes the differentiation of HESCs through miR-542-3p expression. Our results indicate that HMGN5 is involved in human endometrial decidualization by regulating miR-542-3p expression.

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DISCLOSURE

Conflict of interest: The authors declare no conflict of interest. Human Rights Statement and Informed Consent: All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institution and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients included in the study. The
Institutional Review Board of the Saitama Medical University Hospital approved this study. Animal Studies: This article does not contain any studies with animal subjects performed by any of the authors.

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