

# Hmgn1 acts downstream of C/EBP $\beta$ to regulate the decidualization of uterine stromal cells in mice

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Although Hmgn1 is involved in the regulation of gene expression and cellular differentiation, its physiological roles on the differentiation of uterine stromal cells during decidualization still remain unknown. Here we showed that Hmgn1 mRNA was highly expressed in the decidua on days 6-8 of pregnancy. Simultaneously, increased expression of Hmgn1 was also observed in the artificial and in vitro induced decidualization models. Hmgn1 induced the proliferation of uterine stromal cells and expression of *Ccna1*, *Ccnb1*, *Ccnb2* and *Cdk1* in the absence of estrogen and progesterone. Overexpression of Hmgn1 could enhance the expression of *Pr18a2* and *Pr13c1* which were 2 well-known differentiation markers for decidualization, whereas inhibition of Hmgn1 with specific siRNA could reduce their expression. Further studies found that Hmgn1 could mediate the effects of C/EBP $\beta$  on the expression of *Pr18a2* and *Pr13c1* during in vitro decidualization. In the uterine stromal cells, cAMP analog 8-Br-cAMP could stimulate the expression of Hmgn1 via C/EBP $\beta$ . Moreover, siRNA-mediated down-regulation of Hmgn1 could attenuate the effects of cAMP on the differentiation of uterine stromal cells. During in vitro decidualization, Hmgn1 might act downstream of C/EBP $\beta$  to regulate the expression of *Cox-2*, *mPGES-1* and *Vegf*. Progesterone could up-regulate the expression of Hmgn1 in the ovariectomized mouse uterus, uterine epithelial cells and stromal cells. Knockdown of C/EBP $\beta$  with siRNA alleviated the up-regulation of progesterone on Hmgn1 expression. Collectively, Hmgn1 may play an important role during mouse decidualization.

## Introduction

Embryo implantation involves the intimate interaction between an implantation-competent blastocyst and a receptive uterus.<sup>1,2</sup> Following the onset of embryo implantation, uterine stromal cells surrounding the implanting embryo undergo extensive proliferation and subsequent differentiation into Polyploid decidual cells (decidualization).<sup>2,3</sup> Proper decidualization is essential for continued embryonic development within the uterus and achieving successful pregnancy, because impaired decidualization can lead to miscarriage or even pathological pregnancy such as preeclampsia or intrauterine growth restriction.<sup>2,4</sup> Genome-wide microarray analyses have identified a number of genes that are up-regulated or down-regulated during decidualization, suggesting the presence of complex mechanisms of gene expression.<sup>4</sup> To date, accumulating data have shown that gene expression involves a change of chromatin structure which can be regulated by structural proteins such as the high mobility group nucleosomal binding (Hmgn) family.<sup>5,6</sup>

Hmgn proteins are architectural nonhistone chromosomal proteins that bind specifically to nucleosome core particle, reduce the compaction of the chromatin fiber, alter the structure of chromatin and thereby affect a variety of DNA-dependent activities such as transcription, replication, recombination and DNA repair.<sup>5-7</sup> Hmgn1, previously known as Hmg14, is an abundant member of Hmgn family.<sup>6,7</sup> It can affect the expression of numerous genes and regulate cellular differentiation.<sup>6,8-10</sup> Analysis of Hmgn1-deficient cells indicated that loss of Hmgn1 led to both upregulation and downregulation of gene expression.<sup>9,11,12</sup> During brain development, Hmgn1 could promote astrocyte differentiation of neural precursor cells through modulating of the responsiveness to ciliary neurotrophic factor.<sup>10</sup> Simultaneously, Hmgn1 might also direct chondrocyte differentiation by influencing the expression of *Sox9* gene.<sup>8</sup> Although our (unpublished) microarray data has revealed that Hmgn1 was strongly expressed in day 8 decidua and deciduoma under artificial decidualization compared with the uninjected uterine horn, the effects of Hmgn1 on the expression of decidualization-related genes and

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differentiation of uterine stromal cells during decidualization are still unknown so far.

CCAAT/enhancer-binding protein  $\beta$  (C/EBP $\beta$ ) is a member of basic leucine zipper DNA-binding proteins and has been identified as a regulator of uterine stromal cell proliferation and differentiation.<sup>13-15</sup> C/EBP $\beta$ -null uterine stromal cells were unable to undergo proper proliferation and differentiation in response to a decidual stimulation.<sup>14-16</sup> Ablation of C/EBP $\beta$  gene in female mice resulted in infertility with a complete lack of decidual formation.<sup>2,14</sup> Although it has been proved that C/EBP $\beta$  might regulate the expression of numerous decidualization-

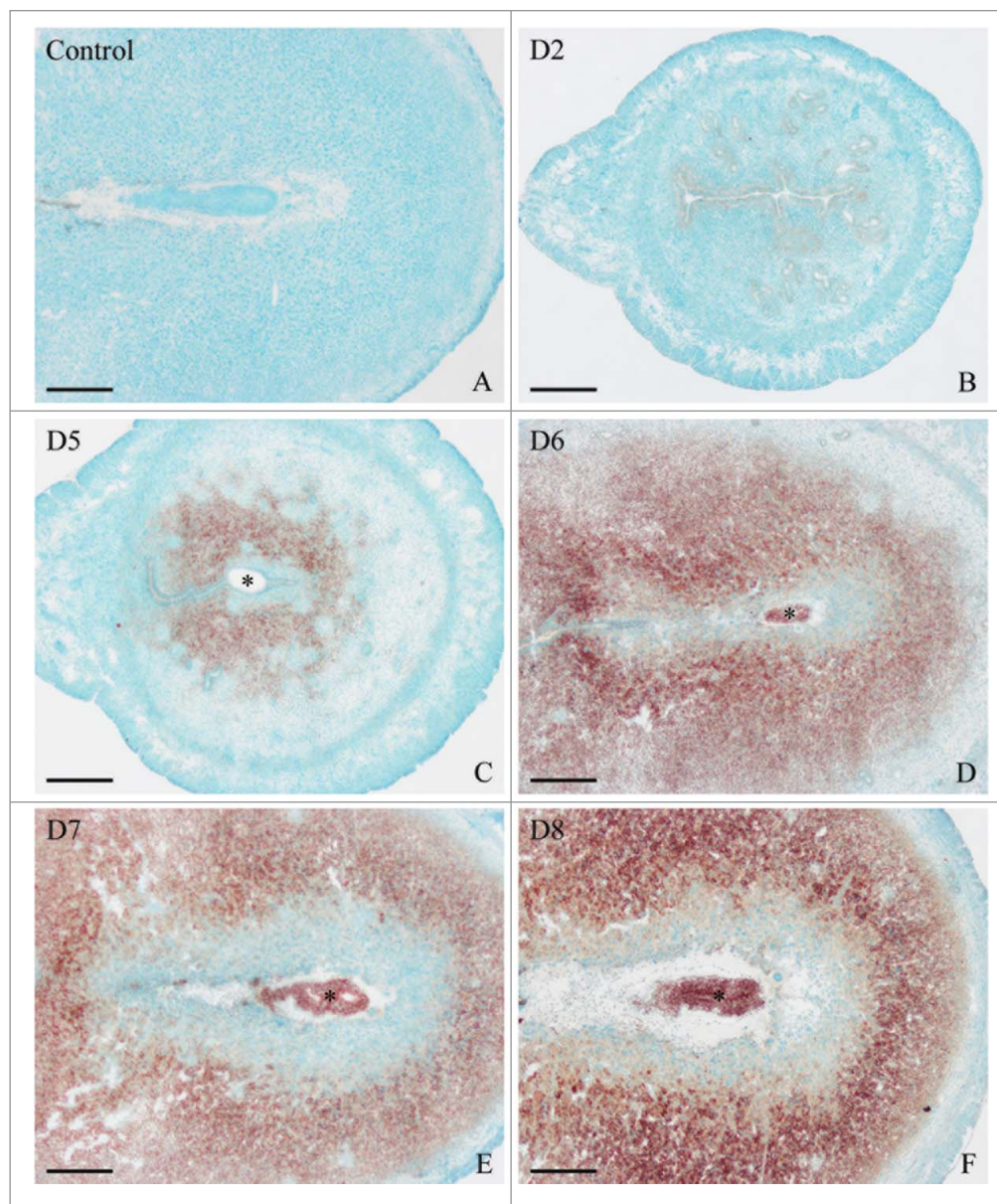
related genes,<sup>13</sup> the relationship between C/EBP $\beta$  and Hmgn1 during decidualization remain poorly understood.

In this study, we showed that Hmgn1 was highly expressed in the decidua and decidualizing stromal cells and important for the proliferation and differentiation of uterine stromal cells during decidualization. Furthermore, our results indicated that Hmgn1 might act downstream of C/EBP $\beta$  to regulate the decidualization of uterine stromal cells.

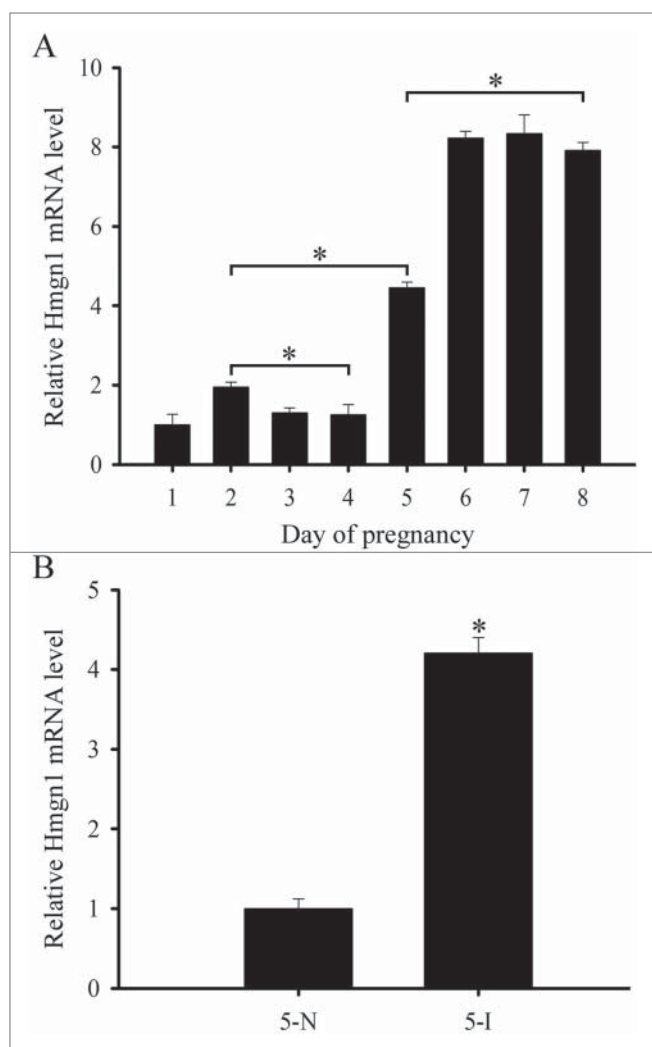
## Results

### Hmgn1 mRNA expression during early pregnancy

Although it has been reported that Hmgn1 was expressed in the uterus,<sup>17</sup> the detailed expression patterns of Hmgn1 in mouse uterus during early pregnancy have not been described. We thus performed in situ hybridization to localize the distribution of Hmgn1 mRNA in mouse uterus. The results showed that there was no visible Hmgn1 mRNA signal from days 1 to 4 of pregnancy (Fig. 1B). On day 5 when embryo implanted, Hmgn1 mRNA was highly expressed in the subluminal stromal cells around implanting blasocyst at implantation sites, but not seen at inter-implantation sites (Fig. 1C). On days 6–8 of pregnancy, Hmgn1 mRNA signal was constantly detected in the decidual cells and embryo (Fig. 1D–F). However, once the DIG-labeled Hmgn1 anti-sense probe was replaced with DIG-labeled Hmgn1 sense probe, there was no corresponding signal in the uterus on day 7 of pregnancy (Fig. 1A). To quantify Hmgn1 mRNA expression, real-time PCR was performed. As expected, a high level of Hmgn1 expression was also detected on days 6–8 of pregnancy (Fig. 2A). Compared with the inter-implantation sites, Hmgn1 mRNA expression was higher at implantation sites on day 5 of pregnancy (Fig. 2B).



**Figure 1.** In situ hybridization of Hmgn1 expression in mouse uteri during early pregnancy on days 2 (B), 5 (C), 6 (D), 7 (E), and 8 (F). No hybridization signals were seen in mouse uterus on day 7 of pregnancy when DIG-labeled Hmgn1 sense probe was used to replace the antisense probe as a negative control (A). Asterisks indicate embryo. Bar = 60  $\mu$ m.



**Figure 2.** Hmgn1 expression in mouse uteri during early pregnancy. **(A)** Real-time PCR analysis of Hmgn1 expression on days 1–8 of pregnancy. **(B)** Real-time PCR analysis of Hmgn1 expression at the implantation sites (5-I) and inter-implantation sites (5-N) on day 5 of pregnancy. Data are shown mean  $\pm$  SEM. Asterisks denote significance ( $P < 0.05$ ).

### Hmgn1 mRNA expression during delayed implantation and activation

To determine whether Hmgn1 expression was dependent on the presence of an active blastocyst, we examined its expression in the delayed-implantation uterus or activated implantation sites and found that there was no obvious Hmgn1 mRNA signal in the delayed uterus (Fig. 3A). After estrogen activated the dormant blastocyst to implant, a high level of Hmgn1 mRNA signal was detected in the subluminal stromal cells surrounding the implanting embryo (Fig. 3B). Simultaneously, a significantly high level of Hmgn1 expression was observed in the activated implantation sites rather than in the delayed uterus by real-time PCR (Fig. 4A).

### Hmgn1 mRNA expression during decidualization

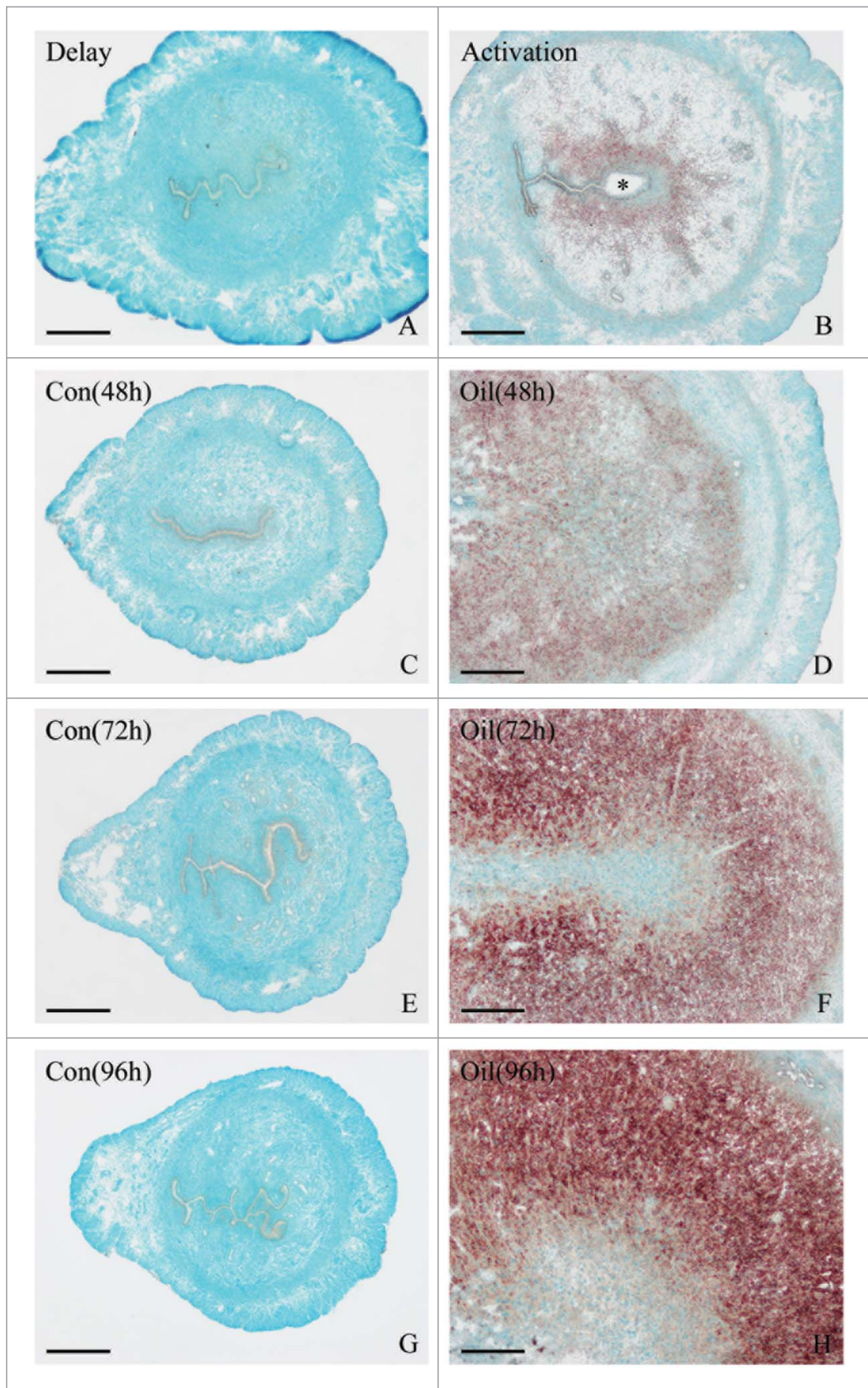
Because Hmgn1 was highly expressed in the decidual cells, we also tested its expression under artificially induced

decidualization in vivo and found that no visible signal was detected in the uninjected control uterus (Fig. 3C, E and G). After the pseudopregnant uterine horn was induced to be artificially decidualized by injecting sesame oil into the uterine lumen, Hmgn1 mRNA signal was obviously seen in the decidualizing stromal cells (Fig. 3D, F and H). By real-time analysis, elevated expression levels of Hmgn1 were observed in the uterine horns undergoing artificially stimulated decidualization with a time-dependent increase and reached a maximum at 96 h after sesame oil injection (Fig. 4B).

Our previous study has confirmed that primary stromal cells isolated from mouse uteri on day 4 of pregnancy were treated with the combination of estrogen and progesterone to induce in vitro decidualization.<sup>18</sup> As the progression of decidualization was increasing, Hmgn1 expression was gradually enhanced and reached a peak at 96 h (Fig. 4C). It has previously established that protein kinase A (PKA) signal transduction pathway is involved in the process of decidualization.<sup>19</sup> Thus we treated the uterine stromal cells with PKA inhibitor H89, then added estrogen and progesterone and monitored the expression of Hmgn1. The results found that H89 could evidently inhibit the expression of Hmgn1 in the uterine stromal cells undergoing decidualization (Fig. 4D).

### Effects of Hmgn1 on decidualization

Decidualization involves extensive proliferation and differentiation of uterine stromal cells.<sup>2,3</sup> Since stromal cell proliferation is the first step of decidualization, we first investigated the effects of Hmgn1 on stromal cell proliferation. After three different Hmgn1 siRNA duplexes were transfected in the uterine stromal cells, expression of Hmgn1 mRNA was determined by real-time PCR. The results showed that Hmgn1 siRNA 1, 2, or 3 could suppress the expression of Hmgn1 mRNA in the stromal cells compared with scrambled siRNA, and Hmgn1 siRNA 2 was the most effective among them (Fig. 5A). Therefore, Hmgn1 siRNA 2 was chosen for further experiments. After transfection with Hmgn1 siRNA, proliferation of uterine stromal cells displayed a significant decline compared with control (Fig. 5B). In contrast, overexpression of Hmgn1 could raise the expression of Hmgn1 in the stromal cells and proliferation activity of stromal cells (Fig. 5C and D). Interestingly, neither overexpression nor inhibition for Hmgn1 had any distinct impact on the proliferation of stromal cells in the presence of estrogen and progesterone (Fig. 5B and D). To understand the molecular basis for the proliferative role of Hmgn1, we subsequently analyzed the influences of Hmgn1 on the expression of cyclin A1 (Ccn1), Ccnb1, Ccnb2, Ccnd1, Ccnd3, Ccne1, cyclin-dependent kinase 1 (Cdk1), Cdk2, Cdk4 and Cdk6. Overexpression of Hmgn1 could strengthen the expression of Ccn1, Ccnb1, Ccnb2 and Cdk1 in the uterine stromal cells, while silencing of Hmgn1 reduced the expression of Ccn1, Ccnb1, Ccnb2 and Cdk1 (Fig. 5E–H). After stromal cells were transfected with Hmgn1 overexpression plasmid or siRNA and then treated with estrogen and progesterone, the expression of Ccn1, Ccnb1, Ccnb2 and Cdk1 did not show any obvious change (data not shown). Similarly, Hmgn1 could not direct the expression of Ccnd1, Ccnd3,



**Figure 3.** In situ hybridization of Hmgn1 expression in mouse uteri. Delay, delayed implantation. Activation, activation of delayed implantation by estrogen. Con, uninjected uterine horn of control. Oil, oil-induced decidualization. Bar = 60  $\mu$ m.

Ccne1, Cdk2, Cdk4 and Cdk6 in the uterine stromal cells which were untreated or treated with a combination of estrogen and progesterone (Fig. 5E–H).

To explore the effects of Hmgn1 on the differentiation of uterine stromal cells, we monitored the effects of Hmgn1 on the expression of prolactin family 8, subfamily a, member 2 (Prl8a2) and prolactin family 3, subfamily c, member 1 (Prl3c1) which were 2 well-known differentiation markers for decidualization.<sup>20</sup> After stromal cells were transfected with Hmgn1 siRNA, the expression levels of Prl8a2 and Prl3c1 were markedly reduced (Fig. 6A and B). During the induction of in vitro decidualization with estrogen and progesterone, knockdown of Hmgn1 could also decrease the expression of Prl8a2 and Prl3c1 (Fig. 6A and B). On the contrary, overexpression of Hmgn1 could apparently enhance the expression of Prl8a2 and Prl3c1 in the uterine stromal cells in the absence or presence of estrogen and progesterone (Fig. 6C and D).

#### Hmgn1 mediates the effects of C/EBP $\beta$ on the differentiation of uterine stromal cells

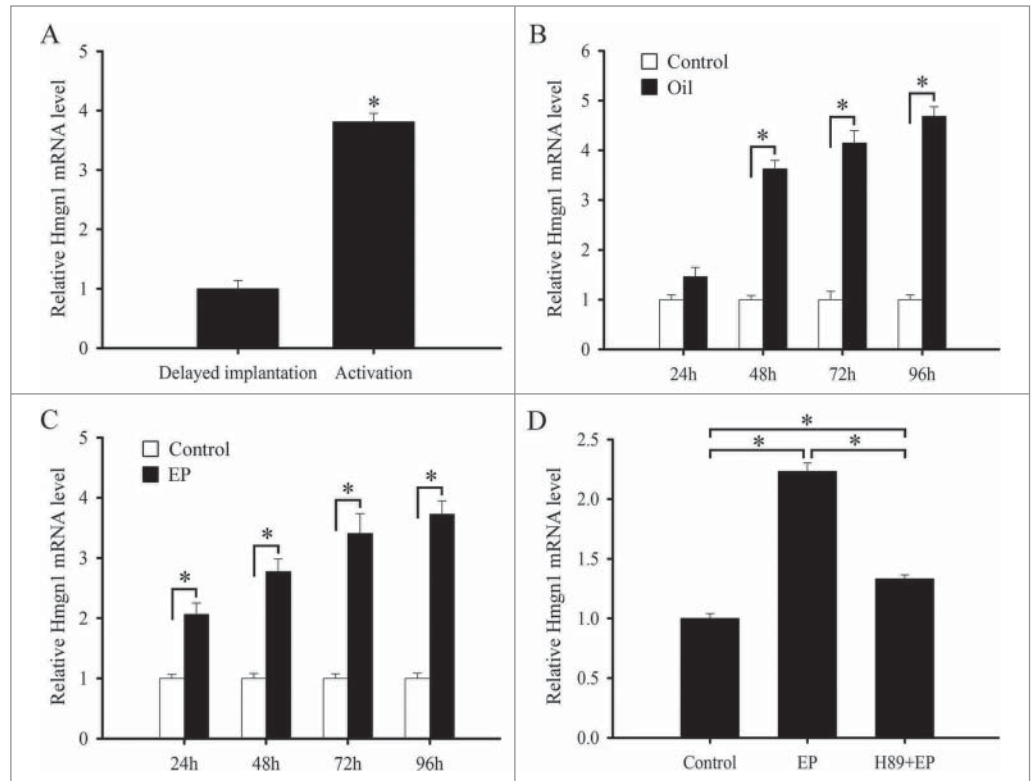
It is well known that C/EBP $\beta$  is critical for the differentiation of uterine stromal cells and regulates the expression of a number of decidualization-related genes.<sup>13,14</sup> Because C/EBP $\beta$  was also expressed in the decidualized cells and overlapped with Hmgn1, we assumed that there was a relationship between C/EBP $\beta$  and Hmgn1 during decidualization. To elucidate the

relationship, we first studied the regulation of Hmgn1 on C/EBP $\beta$ . The results evidenced that neither overexpression nor inhibition of Hmgn1 could impact the expression of C/EBP $\beta$  in the stromal cells undergoing decidualization (Fig. 7A). We next investigated whether C/EBP $\beta$  could modulate the expression of Hmgn1 during in vitro decidualization. Three different C/EBP $\beta$  siRNA duplexes were transfected in the uterine stromal cells to choose the most effective siRNA. Real-time PCR result showed that each siRNA could significantly down-regulate the expression of C/EBP $\beta$  mRNA in the stromal cells, and C/EBP $\beta$  siRNA 3 exhibited obvious inhibition effect (Fig. 7B). Thus, C/EBP $\beta$  siRNA 3 was selected to knock down C/EBP $\beta$  gene in the following study. After transfection with C/EBP $\beta$  siRNA along with the addition of estrogen and progesterone, the expression of Hmgn1 was noticeably declined compared with control (Fig. 7C). Conversely, overexpression of C/EBP $\beta$  could augment the expression of Hmgn1 and C/EBP $\beta$  during in vitro decidualization (Fig. 7D and E).

To test whether Hmgn1 was necessary for the stromal cells differentiation induced by C/EBP $\beta$  overexpression, the uterine stromal cells were co-transfected with C/EBP $\beta$  overexpression plasmid and Hmgn1 siRNA and then subjected to in vitro decidualization in presence of estrogen and progesterone. The results found that knockdown of Hmgn1 could prevent the C/EBP $\beta$ -induced up-regulation of Prl8a2 and Prl3c1 in the stromal cells undergoing decidualization (Fig. 7F and G). We next assessed whether the effects of C/EBP $\beta$  siRNA on the stromal cells differentiation could be rescued by Hmgn1 overexpression. After co-transfection with C/EBP $\beta$  siRNA and Hmgn1 overexpression plasmid, the expression of Prl8a2 and Prl3c1 was evidently enhanced in the uterine stromal cells induced for in vitro decidualization (Fig. 7H and I).

#### Hmgn1 acts downstream of C/EBP $\beta$ to mediate the effects of cAMP on the differentiation of uterine stromal cells

Because cAMP is important for differentiation of uterine stromal cells into decidual cells,<sup>19</sup> we treated the stromal cells with cAMP analog 8-Bromoadenosine-cAMP (8-Br-cAMP, 500  $\mu$ M). The results found that the expression of Hmgn1 was



**Figure 4.** Real-time PCR analysis of Hmgn1 expression in mouse uteri. (A) Hmgn1 expression in delayed and activated uteri. (B) Hmgn1 expression under artificial decidualization. (C) Hmgn1 expression during in vitro decidualization. (D) Hmgn1 expression after uterine stromal cells were induced for decidualization with estrogen plus progesterone in the presence of H89. EP, estrogen plus progesterone.

significantly elevated from 3 to 24 h after 8-Br-cAMP treatment (Fig. 8A). As a ubiquitous second messenger, cAMP prominently activates the PKA signal transduction pathway.<sup>19</sup> After stromal cells were pretreated with PKA inhibitor H89, 8-Br-cAMP-induced Hmgn1 expression was clearly dropped (Fig. 8B). To ascertain whether Hmgn1 could mediate the effects of cAMP on the differentiation of uterine stromal cells, we transfected the stromal cells with Hmgn1 siRNA, then add 8-Br-cAMP and analyzed the expression of Prl8a2 and Prl3c1. The results showed that siRNA-mediated downregulation of Hmgn1 in the stromal cells led to a notable reduction in the expression of differentiation markers Prl8a2 and Prl3c1 (Fig. 8C and D).

It has been well documented that cAMP could stimulate the expression of C/EBP $\beta$  in human endometrial stromal cells.<sup>21</sup> Similarly, administration of 8-Br-cAMP to the mouse stromal cells resulted in a marked increase in the expression of C/EBP $\beta$  (Fig. 8E). H89 pretreatment dramatically abrogated the 8-Br-cAMP-induced expression of C/EBP $\beta$  (Fig. 8F). Knockdown of C/EBP $\beta$  with specific siRNA could obviously attenuate the stimulation of 8-Br-cAMP on the expression of Prl8a2 and Prl3c1 (Fig. 8G and H). We next investigated whether the effects of cAMP on the expression of Hmgn1 was mediated by C/EBP $\beta$ . After the stromal cells were transfected with C/EBP $\beta$  siRNA and then added cAMP analog 8-Br-cAMP, the expression of Hmgn1 was significantly diminished (Fig. 8I).

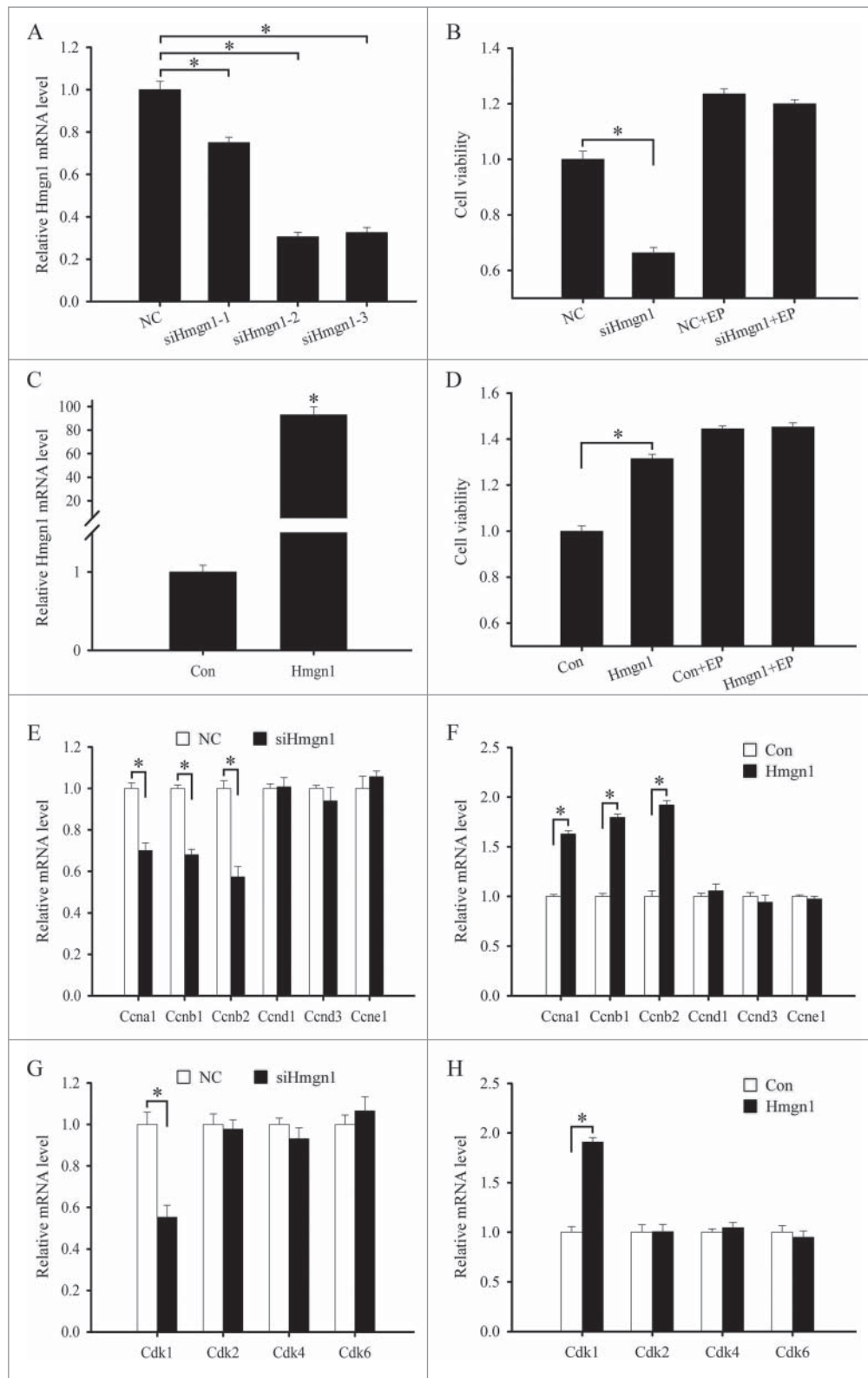
**Hmgn1 mediates the effects of C/EBP $\beta$  on the expression of Cox-2, mPGES-1 and Vegf**

Because Hmgn1 was involved in transcriptional regulation of numerous genes,<sup>6</sup> we examined the effects of Hmgn1 on the

expression of cyclooxygenase-2 (Cox-2), microsomal prostaglandin E synthase 1 (mPGES-1), vascular endothelial growth factor (Vegf), whose expression in the decidual bed overlapped with that of Hmgn1. During in vitro decidualization, overexpression

of Hmgn1 could up-regulate the expression of Cox-2, mPGES-1 and Vegf, while inhibition of Hmgn1 with specific siRNA could down-regulate the expression of Cox-2, mPGES-1 and Vegf (Fig. 9A and B).

As demonstrated above, we have verified that C/EBP $\beta$  regulated the expression of Hmgn1 which directed the expression of



**Figure 5.** Effects of Hmgn1 on the proliferation of uterine stromal cells. **(A)** Effects of Hmgn1 siRNAs on Hmgn1 mRNA expression in the uterine stromal cells. After transfection with control siRNA, Hmgn1 siRNA 1, siRNA 2 or siRNA 3, Hmgn1 mRNA expression was determined by real-time PCR. **(B)** Effects of Hmgn1 siRNA on the proliferation of uterine stromal cells. After transfection with Hmgn1 siRNA, stromal cells were analyzed by MTS assay in the absence or presence of estrogen and progesterone. **(C)** Hmgn1 expression after uterine stromal cells were transfected with Hmgn1 overexpression plasmid. **(D)** Effects of Hmgn1 overexpression on the proliferation of uterine stromal cells. After transfection with Hmgn1 overexpression plasmid, stromal cells were analyzed by MTS assay in the absence or presence of estrogen and progesterone. **(E)** Effects of Hmgn1 siRNA on the expression of Ccna1, Ccnb1, Ccnb2, Ccnd1, Ccnd3 and Ccne1 in the stromal cells in the absence of estrogen and progesterone. **(F)** Effects of Hmgn1 overexpression on the expression of Ccna1, Ccnb1, Ccnb2, Ccnd1, Ccnd3 and Ccne1 in the absence of estrogen and progesterone. **(G)** Effects of Hmgn1 siRNA on the expression of Cdk1, Cdk2, Cdk4 and Cdk6 in the stromal cells in the absence of estrogen and progesterone. **(H)** Effects of Hmgn1 overexpression on the expression of Cdk1, Cdk2, Cdk4 and Cdk6 in the absence of estrogen and progesterone. NC, control siRNA duplex; siHmgn1, Hmgn1 siRNA; Con, empty pcDNA3.1 vector; Hmgn1, Hmgn1 overexpression plasmid.

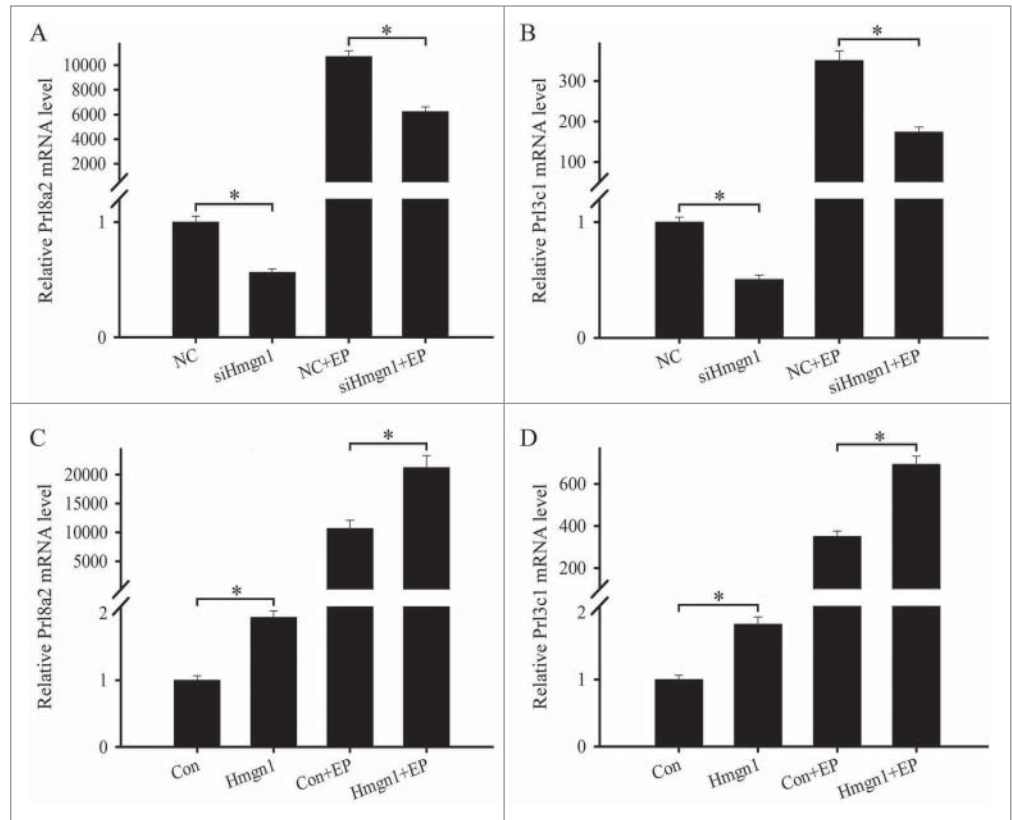
Cox-2, mPGES-1 and Vegf during *in vitro* decidualization. We next asked whether Hmgn1 might mediate the regulation of C/EBP $\beta$  on the expression of Cox-2, mPGES-1 and Vegf. To confirm the possibility, we first studied the effects of C/EBP $\beta$  on the expression of Cox-2, mPGES-1 and Vegf in the uterine stromal cells induced for *in vitro* decidualization. The results found that treatment with siRNA targeted to C/EBP $\beta$  mRNA led to a marked reduction in the expression of Cox-2, mPGES-1 and Vegf, whereas overexpression of C/EBP $\beta$  could augment the expression of these genes (Fig. 9C and D). We next explored whether Hmgn1 could mediate the effects of C/EBP $\beta$  on the expression of Cox-2, mPGES-1 and Vegf in the uterine stromal cells which were subjected to *in vitro* decidualization in presence of estrogen and progesterone. As expected, overexpression of Hmgn1 improved the expression of Cox-2, mPGES-1 and Vegf in the C/EBP $\beta$  siRNA-transfected stromal cells (Fig. 9F). Conversely, siRNA-mediated down-regulation of Hmgn1 inhibited the expression of Cox-2, mPGES-1 and Vegf in the uterine stromal cells transfected with C/EBP $\beta$  overexpression plasmid (Fig. 9E).

#### Steroid hormonal regulation of Hmgn1 expression

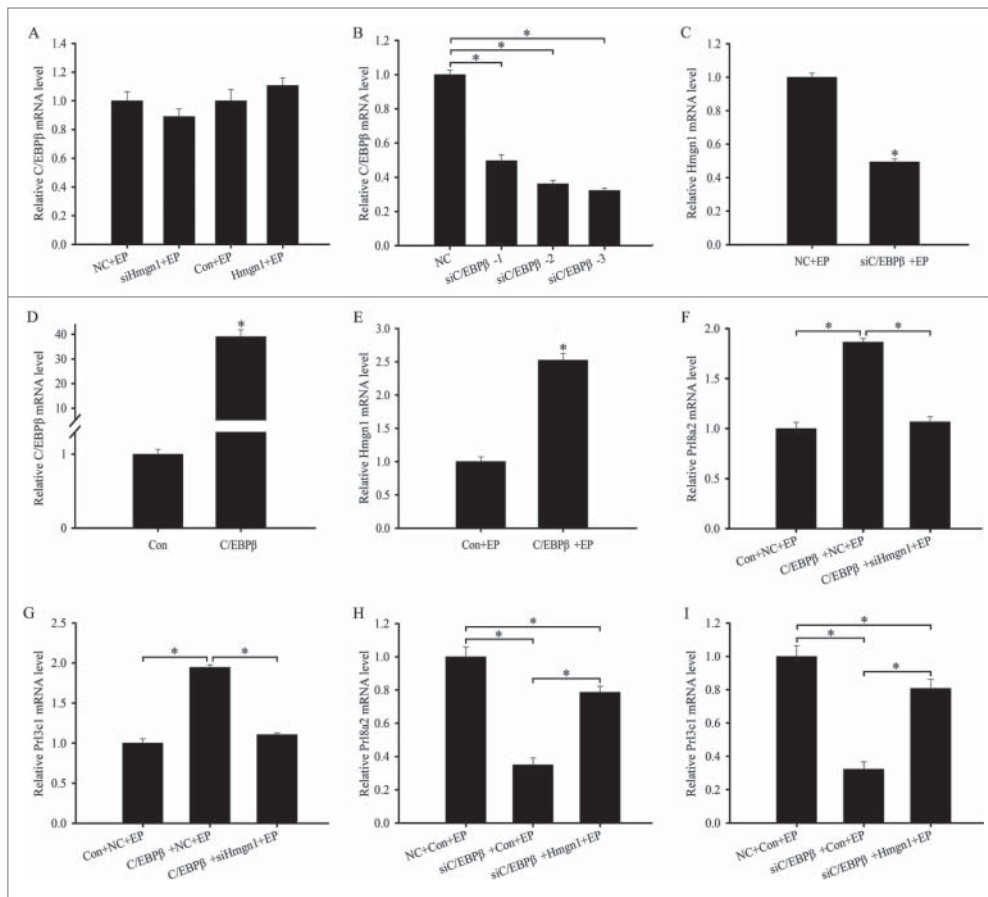
As estrogen and progesterone were essential for embryo implantation and decidualization,<sup>1,2</sup> ovariectomized mice were used to examine whether ovarian steroid hormones could regulate the expression of Hmgn1. In the uteri of ovariectomized mice, a weak hybridization signal was observed in the luminal and glandular epithelium (Fig. 10A). After ovariectomized mice were treated with estrogen for 24 h, Hmgn1 mRNA was highly expressed in the uterine luminal epithelium (Fig. 10B). Progesterone could stimulate the expression of Hmgn1 in the luminal epithelium, glandular epithelium and stromal cells (Fig. 10C). After a combined injection of estrogen and progesterone, the pattern of Hmgn1 expression in uterus was similar to that of progesterone treatment alone but at much higher levels (Fig. 10D). By real-time PCR analysis, both estrogen and progesterone could induce the expression of Hmgn1 in the uteri of ovariectomized mice (Fig. 11A). Based on above observations, the time course of Hmgn1 expression was further examined at 1, 3, 6, 12 and 24 h

after estrogen or progesterone treatment. By *in situ* hybridization, Hmgn1 expression was gradually heightened in the luminal and glandular epithelium after injection of estrogen and reached the highest level at 6 and 12 h (Fig. 10E and F). Similarly, Hmgn1 mRNA was also detected in the luminal and glandular epithelium, and its expression was high at 6 h after injection of progesterone (Fig. 10G–I). Meanwhile, progesterone could induce the expression of Hmgn1 mRNA in the uterine stromal cells at 6, 12 and 24 h (Fig. 10C, H and I). Real-time PCR results showed that Hmgn1 expression was strongly detected at 6 h after estrogen treatment even if it was higher in the uteri of ovariectomized mice treated with estrogen for 3, 6, 12 and 24 h compared with that in the control uteri (Fig. 11B). Progesterone injection could result in an increase in uterine Hmgn1 mRNA levels, which reached a peak at 6 h (Fig. 11C).

To further evaluate the effects of these hormones on the expression of Hmgn1, uterine epithelial and stromal cells were treated with estrogen or progesterone. In the *in vitro* cultured uterine epithelial cells, Hmgn1 mRNA expression was remarkably upregulated at 6 h after treatment with estrogen or progesterone (Fig. 11D and E). However, the upregulation was



**Figure 6.** Effects of Hmgn1 on the differentiation of uterine stromal cells. (A and B) Effects of Hmgn1 siRNA on the expression of Prl8a2 and Prl3c1. After transfection with Hmgn1 siRNA, the expression of Prl8a2 and Prl3c1 was determined by real-time PCR in the absence or presence of estrogen and progesterone. (C and D) Effects of Hmgn1 overexpression on the expression of Prl8a2 and Prl3c1. After transfection with Hmgn1 overexpression plasmid, the expression of Prl8a2 and Prl3c1 was determined by real-time PCR in the absence or presence of estrogen and progesterone.



**Figure 7.** Hmgn1 mediates the effects of C/EBPβ on the differentiation of uterine stromal cells. **(A)** Effects of Hmgn1 siRNA or overexpression on the expression of C/EBPβ. **(B)** C/EBPβ expression after uterine stromal cells were transfected with C/EBPβ siRNAs. **(C)** Effects of C/EBPβ siRNA on the expression of Hmgn1. **(D)** C/EBPβ expression after uterine stromal cells were transfected with C/EBPβ overexpression plasmid. **(E)** Effects of C/EBPβ overexpression on the expression of Hmgn1. **(F and G)** Hmgn1 siRNA abrogated the effects of C/EBPβ overexpression on the expression of Prl8a2 and Prl3c1. After co-transfection with C/EBPβ overexpression plasmid and Hmgn1 siRNA, the expression of Prl8a2 and Prl3c1 was determined by real-time PCR in the presence of estrogen and progesterone. **(H and I)** Overexpression of Hmgn1 improved the effects of C/EBPβ siRNA on the expression of Prl8a2 and Prl3c1.

abrogated by a pretreatment with estrogen receptor (ER) antagonist ICI 182,780 or progesterone receptor (PR) antagonist RU486, respectively (Fig. 11D and E). In the uterine stromal cells, progesterone could stimulate the expression of Hmgn1 at 1, 3, 6, 12 and 24 h, respectively. The highest level was reached at 24 h after progesterone treatment (Fig. 11F). RU486 pretreatment could dramatically attenuate the progesterone-stimulated Hmgn1 expression (Fig. 11G).

#### C/EBPβ mediates the effects of progesterone on the expression of Hmgn1

Previous studies have tested that progesterone could induce the expression of C/EBPβ in the uterine stromal cells.<sup>14</sup> As demonstrated above, we have found that C/EBPβ and progesterone also regulated the expression of Hmgn1 in the stromal cells. We next asked whether C/EBPβ could mediate the effects of progesterone on the expression of Hmgn1. To verify the hypothesis, we

treated the stromal cells with C/EBPβ siRNA, then added progesterone and analyzed the expression of Hmgn1. The results showed that siRNA-mediated attenuation of C/EBPβ could efficiently block the induction of progesterone on the expression of Hmgn1 in the uterine stromal cells (Fig. 11H).

## Discussion

In this study, we investigated the expression and regulation of Hmgn1 in mouse uterus during pre-implantation and then focused our attention on analyzing the physiological role of Hmgn1 in regulating the proliferation and differentiation of uterine stromal cells during decidualization. The results showed that Hmgn1 mRNA was highly expressed in the subluminal stromal cells around implanting blastocyst at implantation sites, but not seen at inter-implantation sites or in the delayed uterus, implying that Hmgn1 might be important for embryo implantation and its expression was dependent upon the presence of active blastocyst. After blastocyst attachment to the uterine luminal epithelium, uterine stromal cells surrounding the implanting blastocyst undergo

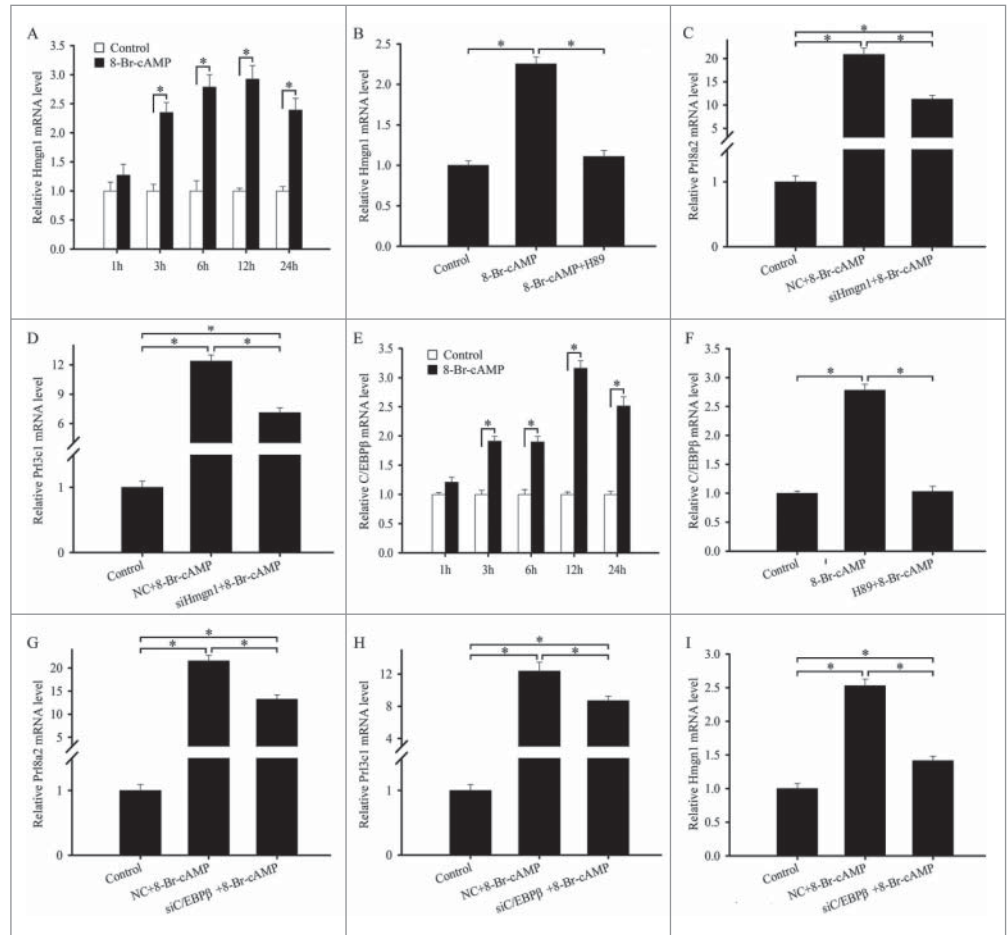
extensive proliferation and subsequent differentiation into decidual cells.<sup>2,3</sup> With the progression of decidualization, the expression of Hmgn1 was gradually enhanced. Overexpression of Hmgn1 was effective to raise the proliferation of uterine stromal cells while inhibition of Hmgn1 with specific siRNA could reduce the proliferation of stromal cells. It is well established that the proliferation of animal cells proceeds through various stages of the cell cycle, G1, S, G2 and M, and is controlled by cyclins and Cdks.<sup>15</sup> In the uterine stromal cells, Hmgn1 could modulate the expression of Ccn1, Ccnb1, Ccnb2 and Cdk1, implying that Hmgn1 was involved in regulating cell cycle progression at the G2-M phase transition. Indeed, Hmgn1-deficient mouse embryonic fibroblasts failed to arrest properly in G2-M phase of the cell cycle after ionizing radiation treatment which was known to activate the G2-M cell cycle checkpoint.<sup>22</sup> However, Hmgn1 did not affect the proliferation of stromal cells and the expression of Ccn1, Ccnb1, Ccnb2 and Cdk1 after uterine stromal cells



were induced for in vitro decidualization. These results suggest that Hmgn1 is important for the proliferation of uterine stromal cells during the initiation of decidualization. After this marked proliferation, stromal cells begin to terminally differentiate into decidual cells with acquisition of polyploidy.<sup>2,23</sup> The present study showed that Hmgn1 could stimulate the expression of Prl8a2 and Prl3c1 which were 2 well-known markers for decidual cell differentiation. Taken together, these results revealed an important role of Hmgn1 in maintaining the proliferation and differentiation of uterine stromal cells during decidualization.

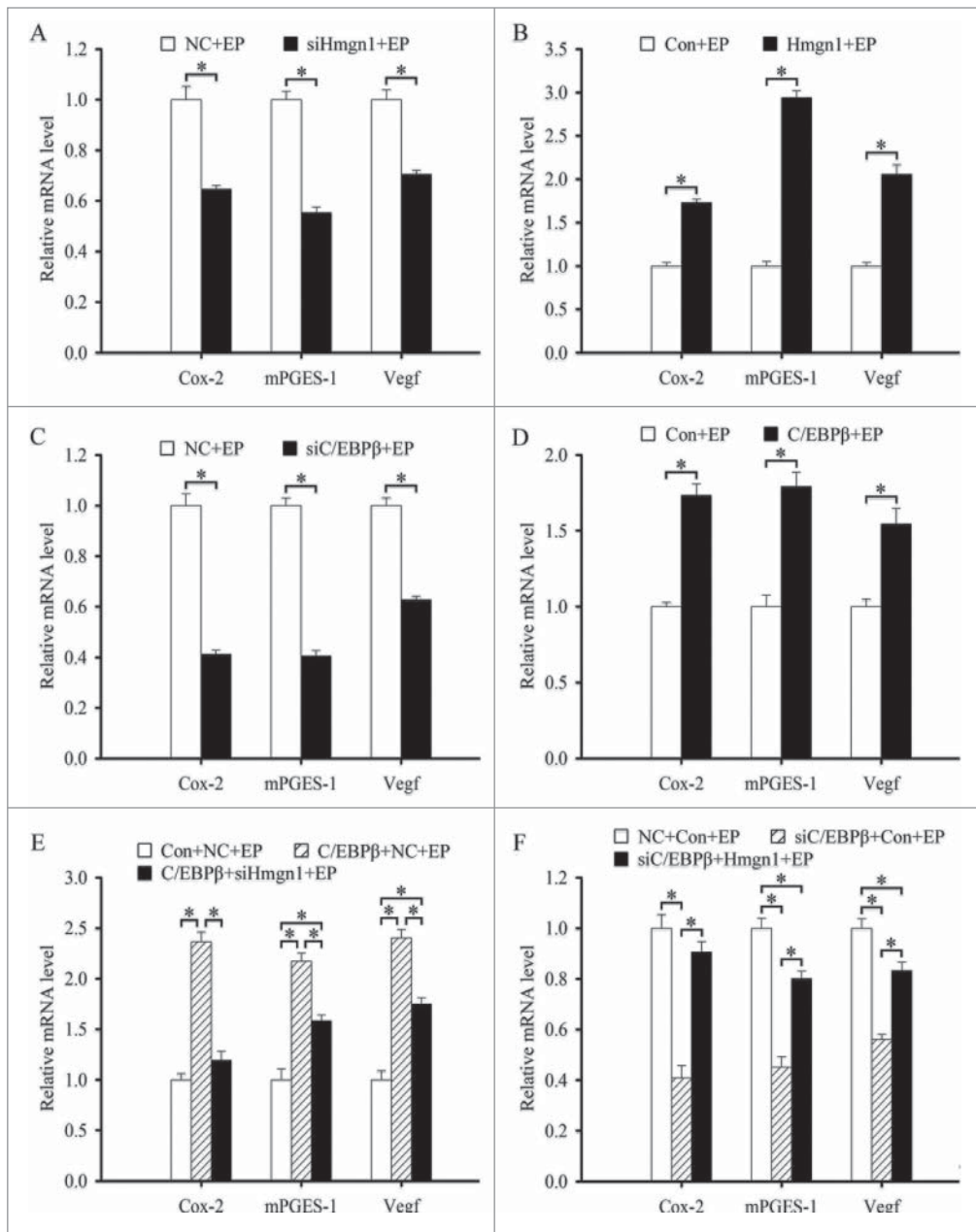
Previous studies have evidenced that the process of decidualization were accompanied by an increase of intracellular cAMP level which could initiate the differentiation of uterine stromal cells prominently via the PKA signal transduction pathway.<sup>19,24</sup> PKA inhibitor H89 could block the expression of Hmgn1 in the uterine stromal cells undergoing decidualization and the stimulation of cAMP on Hmgn1 expression. The siRNA-mediated downregulation of Hmgn1 in the stromal cells could attenuate the effects of cAMP on the expression of differentiation markers Prl8a2 and Prl3c1, indicating that Hmgn1 is a critical regulator of cAMP-induced stromal differentiation during decidualization. Further analysis revealed that the effect of cAMP on Hmgn1 expression was mediated by C/EBP $\beta$  which was essential for decidualization and controlled the differentiation of stromal cells during decidualization.<sup>14,15</sup> In the uterine stromal cells undergoing decidualization, C/EBP $\beta$  might induce the expression of Hmgn1. Moreover, overexpression of Hmgn1 could improve the expression of Prl8a2 and Prl3c1 in the C/EBP $\beta$  siRNA-transfected stromal cells, whereas knockdown of Hmgn1 prevented the C/EBP $\beta$ -induced up-regulation of Prl8a2 and Prl3c1. These results provide convincing evidence that Hmgn1 is the downstream target of regulation by C/EBP $\beta$  during uterine stromal differentiation.

It has been well established that Hmgn1 was involved in transcriptional regulation of numerous genes.<sup>6</sup> In the uterine stromal cells treated with estrogen plus progesterone, Hmgn1 could



**Figure 8.** Hmgn1 acts downstream of C/EBP $\beta$  to mediate the effects of cAMP on the differentiation of uterine stromal cells. (A) Hmgn1 expression in the uterine stromal cells after 8-Br-cAMP treatment. (B) Hmgn1 expression after uterine stromal cells were treated with 8-Br-cAMP, or both 8-Br-cAMP and H89. (C and D) Effects of cAMP on the expression of Prl8a2 and Prl3c1 through Hmgn1. After transfection with Hmgn1 siRNA and addition of 8-Br-cAMP, the expression of Prl8a2 and Prl3c1 was determined by real-time PCR. (E) C/EBP $\beta$  expression in the uterine stromal cells after 8-Br-cAMP treatment. (F) C/EBP $\beta$  expression after uterine stromal cells were treated with 8-Br-cAMP, or both 8-Br-cAMP and H89. (G and H) Effects of cAMP on the expression of Prl8a2 and Prl3c1 through C/EBP $\beta$ . (I) Effects of cAMP on the expression of Hmgn1 via C/EBP $\beta$ .

stimulate the expression of Cox-2 which was a rate-limiting enzyme in the biosynthesis of prostaglandins (PGs) and implicated in mouse decidualization.<sup>25,26</sup> Targeted disruption of Cox-2 led to defective decidualization.<sup>25</sup> Under artificially induced decidualization, Cox-2 inhibitor nimesulide diminished the production of uterine PGE<sub>2</sub> which was important for decidualization.<sup>26,27</sup> mPGES-1 might catalyze the conversion of PGH<sub>2</sub> to PGE<sub>2</sub> and was also modulated by Hmgn1 during in vitro decidualization.<sup>26</sup> Cumulative evidences have declared that deficiency of Cox-2 or mPGES-1 caused a reduction in uterine Vegf mRNA level.<sup>28,29</sup> Vegf was a well-known angiogenesis promoter and could profoundly influence the uterine angiogenesis which was crucial to decidualization.<sup>28,30</sup> In fact, Hmgn1 could also direct the expression of Vegf in the uterine stromal cells undergoing decidualization. Collectively, these results suggest that Hmgn1 may impact the uterine decidualization by affecting the



**Figure 9.** Hmgn1 mediates the effects C/EBPβ on the expression of Cox-2, mPGES-1 and Vegf. **(A)** Effects of Hmgn1 siRNA on the expression of Cox-2, mPGES-1 and Vegf. **(B)** Effects of Hmgn1 overexpression on the expression of Cox-2, mPGES-1 and Vegf. **(C)** Effects of C/EBPβ siRNA on the expression of Cox-2, mPGES-1 and Vegf. **(D)** Effects of C/EBPβ overexpression on the expression of Cox-2, mPGES-1 and Vegf. **(E)** The expression of Cox-2, mPGES-1 and Vegf after stromal cells were co-transfected with C/EBPβ overexpression plasmid and Hmgn1 siRNA. **(F)** The expression of Cox-2, mPGES-1 and Vegf after stromal cells were co-transfected with C/EBPβ siRNA and Hmgn1 overexpression plasmid.

expression of Cox-2, mPGES-1 and Vegf genes. Previous studies have tested that silencing of C/EBPβ with siRNA weakened the expression of Vegf in the process of decidualization of human endometrial stromal cells.<sup>13</sup> The evidence presented in this study demonstrated that C/EBPβ might regulate the expression of Cox-2, mPGES-1 and Vegf genes during decidualization of mouse stromal cells. Furthermore, Hmgn1 might act

downstream of C/EBPβ to regulate the expression of Cox-2, mPGES-1 and Vegf genes during in vitro decidualization.

It has long been recognized that progesterone was essential for the initiation and maintenance of decidualization.<sup>2,3</sup> The actions of progesterone were executed by its receptor PR.<sup>2,3</sup> In this study, progesterone could induce the expression of Hmgn1 in the ovariectomized mouse uterus, uterine epithelial cells and stromal cells. Moreover, the induction was abrogated by PR antagonist RU486, suggesting PR requirement for this induction. It has previously been reported that C/EBPβ was a critical downstream target of progesterone during decidualization.<sup>14</sup> In C/EBPβ-transfected stromal cells, stimulation of progesterone on Hmgn1 expression was obviously attenuated, indicating that C/EBPβ mediates the expression of Hmgn1 in response to progesterone. Additionally, Hmgn1 expression in the ovariectomized mouse uterus and uterine epithelial cells was also induced by estrogen which was required for embryo implantation and decidualization.

In summary, Hmgn1 may act downstream of C/EBPβ and function via Cox-2, mPGES-1 and Vegf to regulate the decidualization of uterine stromal cells. Progesterone and cAMP regulate the expression of Hmgn1 through C/EBPβ.

## Materials and Methods

### Animals

Matured mice (Kunming white strain) were caged in a controlled environment with a cycle of 14L:10D. All animal procedures were approved by the Institutional Animal Care and Use Committee of Jilin University. To confirm reproducibility of results, at least 3 mice per group were used in each stage or treatment in this study.

### Pregnancy and pseudopregnancy

Adult female mice were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy by co-caging, respectively (day 1=day of vaginal plug). On days 1–4, pregnancy was confirmed by recovering embryos from the oviducts or uterus. The implantation sites on day 5 were identified by intravenous injection of 0.1 ml of 1% Chicago blue (Sigma, St. Louis, MO) in 0.85% sodium chloride.

### Delayed implantation and activation

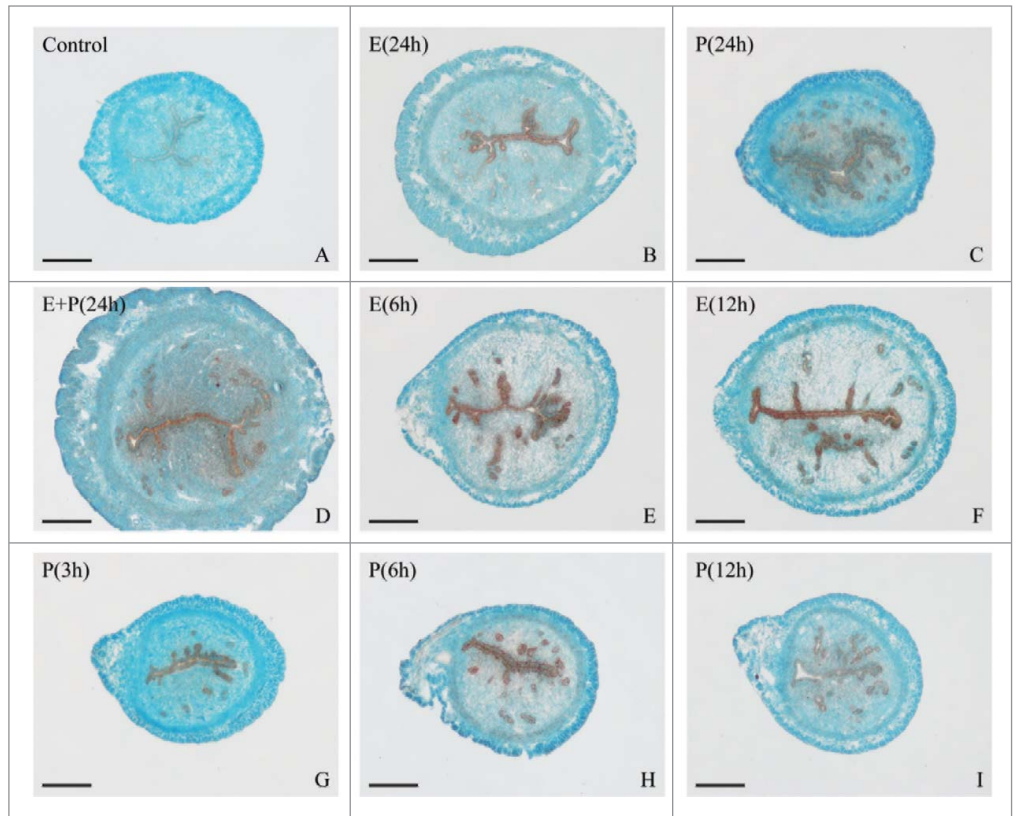
To induce delayed implantation, pregnant mice were ovariectomized under ether anesthesia at 08:30–09:00h on day 4 of pregnancy. Progesterone (1 mg/mouse; Sigma) was injected subcutaneously to maintain delayed implantation from days 5 to 7. Estradiol-17 $\beta$  (25 ng/mouse, Sigma) was given to progesterone-primed delayed-implantation mice to activate blastocyst implantation. The mice were sacrificed to collect uteri at 24 h after estrogen treatment. The implantation sites were identified by intravenous injection of Chicago blue solution. Delayed implantation was confirmed by flushing the blastocysts from the uterus.

### Artificial induced decidualization

Artificial decidualization was induced by intraluminally infusing 25  $\mu$ l of sesame oil into one uterine horn on day 4 of pseudopregnancy, while the contralateral uninjected horn served as a control. The mice were killed to collect uteri at 24, 48, 72 or 96 h after artificial induced decidualization. Decidualization was confirmed by weighing the uterine horn and histological examination of uterine sections.

### Steroid hormonal treatments

Mature female mice were ovariectomized and, after 2 weeks, given a single injection of estradiol-17 $\beta$  (100 ng/mouse), progesterone (2 mg/mouse) or a combination of the same doses of estradiol-17 $\beta$  and progesterone, respectively. All steroids were dissolved in sesame oil and injected subcutaneously. Control mice received the vehicle only (sesame oil, 0.1 ml/mouse). Mice were sacrificed at different time to collect uteri after the hormonal injections.



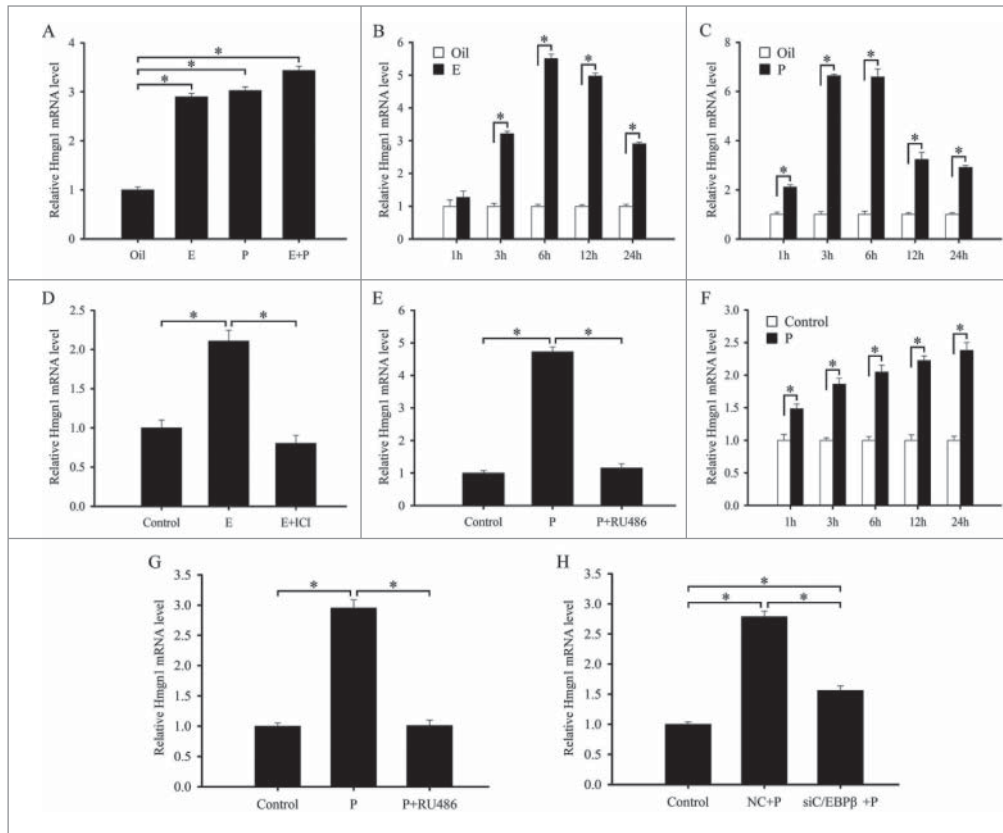
**Figure 10.** In situ hybridization of Hmgn1 expression after ovariectomized mice were treated with sesame oil (Control), estrogen, progesterone or a combination of estrogen and progesterone. E, estrogen; P, progesterone. Bar = 60  $\mu$ m.

Uterine epithelial and stromal cells from day 4 of pregnancy were isolated and cultured as previously described.<sup>31</sup> Cultured epithelial and stromal cells were also treated with 1  $\mu$ M of progesterone or 0.1 nM of estradiol-17 $\beta$ , respectively. For further studies, cells were pretreated with RU486 (1  $\mu$ M) for 2 h or ICI 182,780 (100 nM) before the addition of progesterone or estrogen, respectively. Progesterone and antagonist were dissolved in ethanol. Controls received the vehicle only.

### In situ hybridization

Total RNA from the mouse uteri was reverse-transcribed and amplified with Hmgn1 primers. Hmgn1 forward primer 5'-ATACTGAATCAGGAGAGGCT and reverse primer 5'-CAG-GACTTCCATCTTTCACA were designed according to Mus musculus high mobility group nucleosomal binding domain 1 gene (Genbank accession number NM\_008251). The amplified fragment (291 bp) of Hmgn1 was cloned into pGEM-T plasmid (pGEM-T Vector System 1, Promega, Madison, WI) and verified by sequencing. Hmgn1-containing plasmid was amplified with the primers for T7 and SP6 to prepare templates for labeling. Digoxigenin (DIG)-labeled antisense and sense cRNA probes were transcribed in vitro using a DIG RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany).

Frozen sections (10  $\mu$ m) were mounted on 3-aminopropyl-triethoxy-silane (Sigma)-coated slides and fixed in 4%



**Figure 11.** Hormonal regulation of Hmgn1 expression. **(A)** Real-time PCR analysis of Hmgn1 expression after ovariectomized mice were treated with sesame oil, estrogen, progesterone or a combination of estrogen and progesterone for 24 h. **(B)** Real-time PCR analysis of Hmgn1 expression in ovariectomized mouse uteri after injection of estrogen for 1, 3, 6, 12 and 24 h. **(C)** Real-time PCR analysis of Hmgn1 expression in ovariectomized mouse uteri after injection of progesterone for 1, 3, 6, 12 and 24 h. **(D)** Real-time PCR analysis of Hmgn1 expression after uterine epithelial cells were treated with estrogen or both estrogen and ICI 182,780. **(E)** Real-time PCR analysis of Hmgn1 expression after uterine epithelial cells were treated with progesterone or both progesterone and RU486. **(F)** Real-time PCR analysis of Hmgn1 expression in uterine stromal cells treated with progesterone for 1, 3, 6, 12 and 24 h. **(G)** Real-time PCR analysis of Hmgn1 expression after uterine stromal cells were treated with progesterone or both progesterone and RU486. **(H)** Effects of progesterone on the expression of Hmgn1 via C/EBPβ.

paraformaldehyde solution in PBS. Hybridization was performed as described previously.<sup>18</sup> Sections were counterstained with 1% methyl green. The positive signal was visualized as a dark brown color. The sense probe was also hybridized and served as a negative control. There was no detectable signal from sense probes.

#### Real-time PCR

Total RNAs from mouse uteri or cultured cells were isolated using TRIPURE reagent according to the manufacturer's instructions (Roche) and reverse-transcribed into cDNA using M-MLV reverse-transcriptase (Promega). Reverse transcriptase was performed at 42°C for 60 min with 2 μg total RNA in 25 μl volume. For real-time PCR, cDNA was amplified using FS Universal SYBR Green Real Master (Roche) on BIO-RAD CFX96™ Real Time Detection System. The conditions used for real-time PCR were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were

run in triplicate. The result was analyzed using CFX Manager Software. After analysis using the  $2^{-\Delta\Delta C_t}$  method, data were normalized to Gapdh expression. Primer sequences for real-time PCR were listed in Table 1.

#### In vitro decidualization

Mouse in vitro decidualization was performed as previously described with modification.<sup>18</sup> Briefly, uterine stromal cells were induced for in vitro decidualization with fresh medium supplemented with progesterone (1 μM) and estradiol-17β (10 nM) in DMEM-F12 with 2% charcoal-treated FBS (Biological Industries Ltd., Kibbutz Beit Hemek, Israel).

#### Plasmid Construction and Transfection

Full-length Hmgn1 and C/EBPβ cDNA fragments were amplified by PCR from mouse uterus using the following primers: Hmgn1 (5'-GAATTC (EcoRI) ATGCCCAAGAG-GAAGGTTAG and 5'-GATATC (EcoRV) GAGACAGTCACAGCCTCTCC) and C/EBPβ (5'-GAATTC (EcoRI) ATGGAAGTGGC-CAACTTCTACT and 5'-CTCGAG (XhoI) CTAG-CAGTGGCCCCGCCGA). The amplified products were purified and cloned into pGEM-T vector. The pGEM-T-Hmgn1, pGEM-T-C/EBPβ and pcDNA3.1 vector were cut by EcoRI/EcoRV or EcoRI/XhoI (TaKaRa, Dalian, China) at 37°C for 1 h, and then the fragments were ligated into pcDNA3.1 with T4 ligase (Promega) at 4°C overnight to construct pcDNA3.1-Hmgn1 and pcDNA3.1-C/EBPβ, respectively. An empty pcDNA3.1 expression vector was served as control.

Transfection of uterine stromal cells was performed according to the manufacturer's protocol for lipofectamine 2000 (Invitrogen). After transfection with control plasmid (empty pcDNA3.1 vector) or overexpression plasmids, stromal cells were collected or induced for in vitro decidualization for 48 h.

#### RNA interference

The small-interfering RNA (siRNA) duplexes for targeting Hmgn1 and C/EBPβ as well as a scrambled sequence (control

**Table 1.** Primers for real-time PCR

Gene	Primer sequence	Accession number	Size (bp)
Hmgn1	CGGAAAGGATAAAGCATCA TGGACTCTGGTTTCCGTCT	NM_008251	138 bp
Ccna1	GCCCCAGCTGGATGAGTTT AGGAGGAATTGGTTGGTGGTT	NM_007628	132bp
Ccnb1	CTGAGCCTGAGCCTGAACCT AGCCCCATCATCTGCGTCT	NM_172301	191bp
Ccnb2	GTAGTCTCCAAGGATCGTC CTGCAGAGCTGAGGGTTCTC	NM_007630	141bp
Ccnd1	GGGATGTGAGGGAAGAGGTGA GCAGCGAAAACAACGTGAAA	NM_007631	154bp
Ccnd3	CCTCTACTTCCAGTGCCTG GGCAGACGGTACCTAGAAGC	NM_007632	195bp
Ccne1	AATGGATGGTTCGGTTCGC TGGGTCTGGATGTTGTGGG	NM_007633	103bp
Cdk1	CTGGGCACTCTAACAACGAAG TCCAAGCCGTTCTCGTCCAG	NM_007659	128bp
Cdk2	ACAGGGCAAGGTGAAAGAC AGGAGGACGGCAATGAGG	NM_016756	183bp
Cdk4	GTGGCTGAAATGGTGTCCG TAACAAGGCCACCTCACGAA	NM_009870	156 bp
Cdk6	TCCTGTCCAGTCCAGCTAT CCACGTCTGAACCTCCACGA	NM_009873	108bp
Prl8a2	AGCCAGAAATCACTGCCACT TGATCCATGCACCCATAAAA	NM_010088	119bp
Prl3c1	GCCACACGATATGACCGGAA GGTTTGGCACATCTTGGTGT	NM_001163218	162bp
C/EBPβ	CCAAGAAGACGGTGGACAAGC TGAACAAGTCCCGAGGGTG	NM_001287738	207bp
Cox-2	CATCCCCTCTCTGCGAAGTT CATGGGAGTTGGGCAGTCAT	NM_011198	178bp
mPGES-1	TCCTCGGCTTCTGTACTCA GAGAAGTGGGCCAGGACATAG	NM_022415	157bp
Vegf	ACGTCAGAGAGCAACATCACC CTGTGCTGTAGGAAGCTCATCTC	NM_001025257	90bp
Gapdh	GCCTCCGTGTTCTACCC TGCCTGCTTACCACCTTC	NM_008084	102bp

siRNA duplex, negative control) were designed and synthesized by GenePharma. The sequences were shown as follows: 5'-GGGAAAGGAUAAAGCAUCATT and 5'-UGAUGC-UUUUAUCCUUUCCTT (Hmgn1 siRNA 1); 5'-GUGCAUUCGUGUCU GAAAUTT and 5'-AUUUCAGACA CGAAUGCATT (Hmgn1 siRNA 2); CCUGUCAGAAC UGUGAUGUTT and 5'-ACAUCACAGUUCUGACAGGTT (Hmgn1 siRNA 3); 5'-GCGACG AGUACAAGAUGC GTT and 5'-CGCAUCUUGUACUCGUCGCTT (C/EBPβ siRNA 1); 5'-ACAAGGCCAAGAUGC GCAATT and 5'-UUGCGCAUCUUGGCCUUGUTT (C/EBPβ siRNA 2); 5'-GGAAUUGUUAAGCAGCUTT and 5'-AGCUGCUUGAA CAAGUUCCTT (C/EBPβ siRNA 3); 5'-UUCUCCGAACGUGUCACGUTT and 5'-ACGUGACACGUUCGGAGAATT (nonspecific scrambled siRNA, negative control). Transfections for siRNA were performed according to Lipofectamine 2000 protocol. After transfection with Hmgn1 siRNAs or C/EBPβ siRNAs, uterine stromal cells were collected or induced for in vitro decidualization for 48 h.

### Cell proliferation

Proliferation assays were performed using MTS reagent (Promega) according to the manufacturer's directions. Uterine stromal cells were seeded at a density of  $1 \times 10^5$ /well in 96-well plates and cultured in the DMEM/F12 medium containing 2%

heat-inactivated FBS. After transfection with Hmgn1 overexpression plasmid or siRNA, stromal cells were cultured or induced for in vitro decidualization for 48 h. Finally, 20  $\mu$ l of MTS reagent was added to each well and incubated for 4h. Absorbance was measured at 490 nm using a 96-well plate reader. Every experiment was performed in triplicate.

### Statistics

All the experiments were independently repeated at least 3 times. The significance of difference was analyzed by one-way ANOVA or Independent-Samples T Test using the SPSS software program (SPSS Inc., Chicago). The differences were considered significant at  $P < 0.05$ .

### Disclosure of Potential Conflict of interest

No potential conflicts of interest were disclosed.

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