Evidence for a conserved function of heart and neural crest derivatives expressed transcript 2 in mouse and human decidualization

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Abstract

Previously, we showed that heart and neural crest derivatives expressed transcript 2 (Hand2) mRNA levels dramatically increase in mouse uterine endometrial stromal cells (ESCs) as they undergo decidualization in vivo. However, to date, little is known about the expression and function of this transcription factor in mouse or human uterus decidualization. Therefore, this study was conducted to provide a more detailed assessment of Hand2 gene expression and function in the mouse uterus during the peri-implantation period and also in mouse plus human ESCs during decidualization in vitro. The results show that Hand2 mRNA and protein levels increase in the mouse uterus during decidualization and this does not depend on the presence of a conceptus. Interestingly, Hand2 mRNA and protein are present in ESCs adjacent to the luminal epithelium in the uterus prior to the onset of implantation. We find that progesterone is likely a regulator of Hand2 expression during uterine sensitization of the mouse uterus. Finally, Hand2 expression increases in mouse and human fibroblast cells as they undergo decidualization in vitro. This expression is significantly increased in response to prostaglandin E₂. In particular, reduction of Hand2 expression in these cells using small hairpin RNA or small interfering RNA approaches results in the reduced extent of decidualization as shown by the reduced expression of a subset of decidualization markers. The results of this study support the hypothesis that Hand2 expression not only plays an important role in decidualization but may also play a role in obtaining proper progesterone-dependent uterine sensitization required for implantation to begin.

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Introduction

Implantation begins with the attachment of the embryo to the uterine wall and ends in the formation of the definitive placenta. In mice and humans, this process involves the differentiation of the endometrial tissue into decidual tissue, a process commonly referred to as decidualization (Abrahamsohn & Zorn 1993, Dunn et al. 2003, Salamonsen et al. 2003). In humans, decidualization begins in the late proliferative phase of the menstrual cycle and it continues only if menstruation is prevented due to pregnancy. In mice and other rodents, decidualization does not begin each estrous cycle but rather begins in response to an implantation stimulus. This stimulus can be the presence of a blastocyst or can be artificial such as the injection of sesame oil or blastocyst-sized beads (artificially induced tissue is called the deciduoma to discern it from pregnancy-induced deciduala). Common features of decidualization in mouse and human endometria include progesterone dependence and the requirement for proper hormonal preparation (Ramathal et al. 2010). Hormonal preparation occurs in a transient window in time and is called uterine sensitization or receptivity.

Besides the progesterone receptor (PGR), several other transcription factors play a key role in uterine receptivity and decidualization. These include, but are not limited to, forkhead box O1 (FOXO1A; Buzzio et al. 2006, Grinius et al. 2006), homeobox A10 (HOXA10; Lu et al. 2008, Vitiello et al. 2008), and E26 avian leukemia oncogene 1, 5’-domain (ETS1; Kessler et al. 2006). Some of these, such as FOXO1A, have been shown to play a critical role in uterine sensitization and decidualization in humans but not in mice. On the other hand, some are believed to play a role in both human and mouse endometrial sensitization and decidualization (HOXA10, PGR). Finally, in a recent publication, another transcription factor called heart and neural crest derivatives expressed transcript 2 (Hand2) was shown to play a key role in uterine receptivity in mice (Li et al. 2011). Previously, we showed that this gene is expressed in mouse uterine endometrial stromal cells (ESCs) as they undergo decidualization (Bany & Cross 2006).
However, currently, little detail is known about the expression and function of this transcription factor in the mouse uterus during decidualization. In addition, to the best of our knowledge, nothing has been published regarding Hand2 expression and its role in human ESCs (HESC) during decidualization. Therefore, this study was conducted to provide a detailed assessment of Hand2 expression and function in the mouse uterus during the peri-implantation period and in mouse plus HESC during decidualization in vitro. The results suggest that Hand2 expression plays a role in decidualization of both mouse and HESC. Furthermore, the results also support the hypothesis that Hand2 expression might play a role in the hormonal sensitization of the pre-implantation uterus so that it is receptive to an implantation stimulus.

**Results**

**Hand2 expression dramatically increases in the mouse uterus during implantation**

Steady-state Hand2 mRNA levels were measured in the mouse uterus prior to the onset of implantation (day 3.5) and in implantation site (IS) and non-implantation site (NIS) tissues of uteri after the onset of implantation (days 4.5–8.5) (Fig. 1A). On day 3.5, prior to the onset of implantation and decidualization, Hand2 mRNA was detected in the uterine tissue. Although mRNA levels did slightly change in NIS tissues after the onset of implantation, significant increases were seen in the IS tissue compared with the NIS tissue on days 4.5 ($P<0.01$), 5.5 ($P<0.005$), 6.5 ($P<0.05$), 7.5 ($P<0.001$), and 8.5 ($P<0.001$). This increased expression of Hand2 in IS tissue was accompanied by increases in other markers of decidualization including FK506 binding protein 3 (FKBP3; Fig. 1B), gap junction protein alpha 1 (GJA1; Fig. 1C), and Runx-related transcription factor 1 (RUNX1; Fig. 1D). We used bead-induced decidualoma (BID) in pseudopregnant mice as a model (Herington et al. 2009) to determine whether the increased Hand2 mRNA levels in the IS tissues on days 4.5–8.5 require the presence of a conceptus. No significant difference ($P>0.05$) was detected in Hand2 (Fig. 2E) or Gja1 (Fig. 2F) mRNA levels between the BID and the IS tissues on each day examined.

Hand2 mRNA was localized in the mouse uterus on days 3.5–8.5 of pregnancy using *in situ* hybridization and a color development time of 18 h. Although mRNA could be detected using qRT-PCR in the NIS tissues of days 3.5–8.5 pregnant uteri, levels were too low to be detected by *in situ* hybridization. Representative photomicrographs are shown for NIS tissue from days 3.5, 4.5, and 5.5 pregnant uteri (Fig. 2A–C). In IS tissue of day 4.5 uteri, strong hybridization signals for Hand2 mRNA are seen in the subepithelial ESCs making up the wall of the antimesometrial implantation ‘crypt’ that surrounds the implanting blastocyst (Fig. 2D). This layer of Hand2 mRNA localization was found to be approximately six to seven cell layers deep (Fig. 2E). Lighter hybridization signals are seen in some stromal cells deep into the antimesometrial stroma (Fig. 2E) and also within a small population of ESCs near the luminal epithelium in the mesometrial region (Fig. 2F). A similar area of strong hybridization signals for Hand2 mRNA that surrounds the conceptus (c) in the antimesometrial region is also seen in the endometrial stroma of day 5.5 IS tissue (Fig. 2G). However, this area of intense signal is significantly larger compared with that of day 4.5. A well-formed primary decidual zone (PDZ) is generated at day 5.5. A strong signal in the antimesometrial decidua is up to 20 cell layers into the endometrium from the conceptus (Fig. 2H), which is larger than the fully formed PDZ. It is also notable that the conceptus is now in direct contact with the endometrial stroma in the antimesometrial region, as the luminal epithelium in this region has disappeared. On the other hand, the luminal epithelium is still intact in the mesometrial region of the endometrium and strong hybridization signals for Hand2 mRNA are seen in the subepithelial stroma cell (Fig. 2I). The cells that appear to have strong hybridization signals on day 5.5 appear to be decidual cells, especially those in the PDZ that is well formed at this time point. Cells showing moderate-to-low

**Figure 1** Changes in uterine mRNA levels in the mouse uterus during decidualization. (A) Hand2, (B) Fkbp3, (C) Gja1, and (D) Runx1 mRNA levels in days 3.5–8.5 of pregnant uteri non-implantation (NIS) and implantation segment (IS) tissue. (E) Hand2 and (F) Gja1 mRNA levels in bead-induced decidualoma segment (BID) and IS tissue of pseudopregnant and pregnant uteri. Bars represent the mean ± S.E.M. (N=3–4). *P<0.05, **P<0.01, and ***P<0.005.
hybridization signals seen deeper into the endometrium have smaller nuclei, and are presumably cells beginning the process of decidualization as it slowly spreads throughout most of the endometrium. On days 6.5–8.5, the conceptus noticeably enlarges and the ectoplacental cone seen on days 6.5 (Fig. 3A) and 7.5 (Fig. 3E) then flattens by day 8.5 (Fig. 3I). Hybridization signals for Hand2 mRNA appear well throughout the endometrium on these days, including signals in the polyploid decidual cells of the antimesometrial decidua (Fig. 3B, F, and J) and the mesometrial decidua (Fig. 3C, G, and K). In particular, hybridization signals are not seen in ESCs located adjacent to the circular muscle layer of the myometrium that do not undergo decidualization (Fig. 3D, H, and L). At no time, hybridization signals were seen in trophoblast cells of the conceptus (Fig. 3F) or any other endometrial cells except those that were undergoing or had completed the process of decidualization.

HAND2 protein was localized in the mouse uterus on days 4.5–9.5 of pregnancy by immunohistochemistry. Sections from day 3.5 pregnant uteri, just prior to the onset of implantation, reveal strong HAND2 staining in a subpopulation of ESCs near the luminal epithelium (Fig. 4A). The depth of this staining varies but is up to ~12 cells deep adjacent to the luminal epithelium in the antimesometrial (Fig. 4B) and lateral mesometrial (Fig. 4C) regions. At this stage, no staining is seen in the glandular epithelia (Fig. 4D). NIS tissue from day 4.5 pregnant uteri shows similar staining for HAND2 in the stroma beneath the luminal epithelium, except that more cells stain positive (Fig. 4E). IS tissue from day 4.5 pregnant uteri shows strong HAND2 staining in the developing PDZ cells of the endometrial stroma adjacent to the implanting blastocyst (Fig. 4G and H). Unlike day 3.5, glandular epithelial cells now stain positive in both the NIS (Fig. 4F) and the IS (Fig. 4I) tissues. Unlike the staining in the stromal cells that appears to be localized

Figure 2 Localization of Hand2 mRNA in the uteri of days 3.5, 4.5, and 5.5 pregnant mice using in situ hybridization. (A) Section from day 3.5 pre-implantation uterus. Sections from (B) day 4.5 and (C) day 5.5 non-implantation segments (NIS). Sections from (D–F) day 4.5 and (G–I) day 5.5 implantation segments (IS). All sections are oriented with mesometrium (m) downward and numbers above scale bars are in microns. am, antimesometrial region; c, conceptus; endo, endometrium; ge, glandular epithelium; le, luminal epithelium; mm, mesometrial region; myo, myometrium. These results are representative of at least three independent samples. Global linear adjustments of the brightness and color level were made on the photomicrographs to more accurately represent what was seen on the slides under the microscope.
almost solely to the nuclei at all stages of implantation, HAND2 appears to be located in the cytoplasm of the glandular epithelial cells. HAND2 protein is detected in the ESCs adjacent to the luminal epithelium in NIS tissue from day 5.5 pregnant uteri (Fig. 4J and K). However, unlike day 4.5 NIS tissues, staining is not seen in the glandular epithelia. HAND2 was localized to PDZ cells as well as some developing secondary decidual zone cells in IS tissue from day 5.5 pregnant uteri (Fig. 4L–N). No staining is seen adjacent to the extreme mesometrial region of this tissue (Fig. 4O). On days 6.5 (Fig. 5A) and 7.5 (Fig. 5E), subepithelial stromal cells continue to stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue. In the IS tissue on these days, almost all areas of the endometrial stroma stain for HAND2 in NIS tissue.
deciduogenic stimulus, such as an intraluminal injection of sesame oil, does not result in a decidualization response (B M Bany, unpublished data). Interestingly, Hand2 mRNA levels show a quite different expression profile (Fig. 6C) compared with that of Lif. Although levels are low on day 5, Hand2 mRNA levels significantly ($P < 0.05$) increase to similar levels in days 6–9 uteri, starting just after the first injection of progesterone, plus estrogen. Hoxa10 expression in the mouse uterus is dependent on progesterone during the peri-implantation period and this expression is required for implantation. As shown in Fig. 6D, the expression profile of Hoxa10 in the sensitization model was similar to that of Hand2. Although levels were low on day 5, after the first injection of progesterone there was a significant ($P < 0.05$) increase in Hoxa10 mRNA to a similar level on days 6–9. Finally, Indian hedgehog (Ihh) expression is also a progesterone-induced gene in the uterus during the peri-implantation period (Matsumoto et al. 2002) and this expression is essential to begin implantation (Lee et al. 2006). As shown in Fig. 6E, Ihh mRNA levels are low on day 5, but after the first injection of progesterone plus estrogen on days 6, there is a significant ($P < 0.05$) increase in these levels. On days 7 and 8, Ihh mRNA levels are still significantly higher compared with that of day 5 but at significantly ($P < 0.05$) lower levels than day 6. Finally, on day 9, the levels of Ihh mRNA reach the same levels as seen on day 5.

Then, we localized HAND2 protein in the uteri of ovariectomized mice during hormone-induced sensitization on days 5–9 of hormone injection. HAND2 is not detected in uterine sections from uteri collected on day 5 of treatment (Fig. 7A). On the other hand, HAND2 protein can be detected in ESCs adjacent to the luminal
epithelium of sections from uteri collected on days 6 (Fig. 7B and C), 7 (Fig. 7D and E), 8 (Fig. 7F and G), and 9 (Fig. 7H and I). This represents the period of time where the animals were injected daily with a combination of both estradiol and progesterone. In particular, the HAND2 protein appears to be mainly localized to the nuclei of endometrial stromal fibroblast cells in these tissue sections. For sections from day 7 uteri, additional staining just above background can be seen in the glandular epithelial cells (Fig. 7D and E). Control day 7 sections stained with goat IgG in place of the goat-anti-HAND2 IgG showed no staining above background.

As it appears that expression of Hand2 significantly increases in the uteri of ovariectomized mice in response to estradiol plus progesterone on days 6–9 of the sensitization protocol, we examined the individual and combined effects of these steroids on Hand2 mRNA levels. Ovariectomized mice received 3 days of estradiol and 2 days of no injections as in the sensitization protocol. However, on days 6, 7, and 8, the mice received estradiol alone, progesterone alone, or estradiol plus progesterone. As shown in Fig. 7K, Hand2 mRNA levels in the uteri of mice treated with estradiol alone remain low. However, in the same tissues of mice injected with progesterone alone or progesterone plus estradiol, there is a significant (P < 0.01) and similar increase in Hand2 mRNA levels. Immunohistochemistry was used to determine whether there is a difference in the localization of HAND2 protein in sections from the uteri of mice treated with progesterone compared with those treated with estradiol plus progesterone. The localization profile of HAND2 protein in these uteri is similar (Fig. 7L and M) and is also similar to the day 8 uteri which is shown in Fig. 7F. In some sections, background staining occurred in the lumens of some...
blood vessels. This is also seen in sections incubated with the same concentration of normal IgG in place of the goat anti-HAND2 IgG (Fig. 7N).

**Alterations in Hand2 expression in an in vitro mouse model provide evidence for Hand2 function in decidualization**

Mouse ESCs isolated from optimally sensitized uteri of pregnant or ovariectomized mice have the ability to undergo decidualization in vitro (Bell & Searle 1981). This in vitro decidualization is accompanied by an increased expression of markers of decidualization such as pro lactin family 8, subfamily α, member 2 (Prl8a2; Kimura et al. 2001) and alkaline phosphatase (Chen et al. 2009). ESCs were isolated from sensitized uteri of mice to study Hand2 expression in mouse uterine cells during decidualization in vitro. Incubation of the cells with only steroids for 0–3 days results in the progressive increase in expression of Prl8a2 on days 1–3 of culture (Fig. 8A). Unlike Prl8a2, Hand2 mRNA is detected in the cells on day 0 of culture (Fig. 8B). Thereafter, the levels of Hand2 mRNA significantly (P<0.01) increase in the cells on days 1–3 of culture. Immunocytochemistry was used to verify that HAND2 protein levels were also up-regulated in these cells during decidualization in vitro. HAND2 protein was mainly localized in a few nuclei on days 0

of culture (Fig. 8C). By days 1–3 of culture, there is an increase in the number of cells staining positive for HAND2, and most of this staining appears to be localized to the nuclei (Fig. 8D–F).

Prostaglandins play a key role in implantation and decidualization in rodents (Wang & Dey 2005, Kennedy et al. 2007). Furthermore, prostaglandin E2 (PGE2) can enhance the decidualization of rodent and HESC in vitro (Yee & Kennedy 1993, Frank et al. 1994, Pakrasi & Jain 2008). Therefore, we determined the effect of PGE2 on Hand2, Prl8a2, and alkaline phosphatase 2 (Alpl) mRNA levels in mouse ESCs. Cells were incubated for 2 days in the presence of steroids without or with the addition of PGE2. As shown in Fig. 9A, the mRNA levels for both Hand2 and the decidualization marker Alpl significantly increase (P<0.01) in response to PGE2. On the other hand, Prl8a2 mRNA levels decrease slightly but significantly (P<0.05) in response to PGE2.

In the final experiment using mouse endometrial cell cultures, the effect of reducing Hand2 expression on decidualization was determined. As shown in Fig. 9B, Hand2 mRNA levels significantly (P<0.01) decrease in cells transiently transfected with Hand2 small interfering RNA (siRNA), compared with those treated with a control siRNA. Although decrease in Prl8a2 mRNA levels is not seen, the levels for Alpl mRNA also significantly (P<0.01) decrease in the cells transfected with Hand2 siRNA. Immunohistochemical staining for HAND2 protein seen in control cells (Fig. 9C) is reduced in cells transfected with Hand2 siRNA (Fig. 9D).

**HAND2 expression in human endometrial cells undergoing decidualization in vitro**

To determine whether increased HAND2 gene expression also occurs in HESC during decidualization, we used the HESC cell line. HESC cells were incubated with vehicle, steroid hormones, or steroid hormones plus PGE2 for 0–3 days. To verify an increase in decidualization of the cells, we measured the mRNA levels of several established decidualization markers, including FOXO1A, insulin-like growth factor binding protein 1 (IGFBP1), prolactin (PRL), and tissue inhibitor of metalloproteinase 3 (TIMP3; Buzzio et al. 2006). As shown in Fig. 10A–D, FOXO1A, IGFBP1, PRL, and TIMP3 mRNA levels significantly (P<0.05) increased on day 1 in response to PGE2 plus hormones compared with control and steroid-treated cells. On days 2 and 3 of culture, mRNA levels significantly (P<0.05) increased in response to hormones alone compared with control. On the other hand, mRNA levels significantly (P<0.05) increased even more in response to PGE2 plus hormones compared with those treated with vehicle or hormones. As HESC undergo decidualization, proliferation of the cells dramatically decreases and cyclin E2 (CCNE2) mRNA levels in the cells decrease (Tierney et al. 2003). Therefore, a decrease in CCNE2 mRNA levels was used.
as a marker of decidualization. For days 1–3 of culture, treatment with hormones caused a significant \((P<0.05)\) decrease in \(CCNE2\) mRNA levels compared with those incubated with vehicle (Fig. 10E). \(CCNE2\) mRNA levels significantly \((P<0.05)\) decreased even more in response to \(PGE_2\) plus hormones compared with those treated with vehicle or hormones alone.

Finally, \(HAND2\) mRNA levels were significantly \((P<0.05)\) higher in cells treated with hormones compared with those incubated with vehicle on days 1–3 of culture (Fig. 10F). On these days, \(HAND2\) mRNA levels were significantly \((P<0.05)\) higher in cells treated with \(PGE_2\) plus hormones compared with those incubated with vehicle or hormones alone.

As \(HAND2\) expression in HESC cells rapidly increases in HESC cultures, a shorter time course for induction of expression was examined. Cells were treated with vehicle or steroids plus \(PGE_2\) for 0, 6, 12, 18, or 24 h. As shown in Fig. 11A, \(HAND2\) mRNA levels significantly \((P<0.05)\) increased in the steroid plus \(PGE_2\)-treated cells compared with those treated with vehicle at each time point between 6 and 24 h. This was paralleled with increase in the levels of \(FOXO1A\) mRNA in the steroid plus \(PGE_2\)-treated cells at these time points (Fig. 9H).

**Depletion of \(HAND2\) in a human in vitro cell model suggests a regulatory role for this transcription factor in decidualization**

In order to assess the function of \(HAND2\) expression in HESC decidualization, we established HESC cells that stably express either control non-target or \(HAND2\)-targeting small hairpin RNA (shRNA). These cells were treated with steroids plus \(PGE_2\) for 2 days
and the mRNA levels of several genes were determined. As shown in Fig. 11A, HAND2 shRNA caused a significant ($P<0.01$) decrease in HAND2 mRNA levels. This was accompanied by significant decrease in FOXO1A ($P<0.05$) and IGFBP1 ($P<0.001$) mRNA levels. Unexpectedly, HAND2 shRNA significantly ($P<0.05$) increased PRL mRNA levels. To verify that HAND2 shRNA-expressing cells also have a reduced level of nuclear HAND2 protein in the HESC cells, we used immunocytochemistry. Cells expressing non-targeting control shRNA (Fig. 11B) robustly stained for HAND2 protein whereas those expressing HAND2 shRNA did not (Fig. 11C).

**Discussion**

Hand2 expression dramatically increases in the mouse uterus during decidualization. Previously, using northern blot methods and limited in situ hybridization studies, we have shown that Hand2 mRNA levels in the mouse uterus increase during decidualization (Bany & Cross 2006). In these experiments, we extended this approach to include the more sensitive technique of qRT-PCR and find using this method that there is a dramatic increase in Hand2 mRNA levels in the mouse uterus during decidualization. Furthermore, we verified that the magnitude and timing of the induction of Hand2 expression in a pregnant mouse is similar to expression in a pseudopregnant uterus undergoing bead-induced decidualization. This suggests that blastocyst-specific molecular or physical stimuli are not required for Hand2 expression in the mouse uterus during decidualization. Besides assessing the localization of Hand2 mRNA, this study also assessed the localization of HAND2 protein. Increased expression at the mRNA as well as protein levels occurred in cells undergoing decidualization. Furthermore, HAND2 protein is mainly localized to the nuclei of these cells. Therefore, the protein is located...
Reproduction estrogen and progesterone play key roles in this process and decidualization responses. Certainly, both dependent changes in order to mount normal implant-genes that play a key role in the decidualization process. potentially regulating the expression of downstream in the proper subcellular compartment for it to be potentially regulating the expression of downstream genes that play a key role in the decidualization process.

The uterus must go through a series of hormone-dependent changes in order to mount normal implantation and decidualization responses. Certainly, both estrogen and progesterone play key roles in this process of uterine sensitization or receptivity (Psychoyos 1973, 1976, 1986). A recent study using uterine-specific Hand2 knockout mice showed that Hand2 is expressed in the subepithelial stroma during the receptive phase and that this expression is essential for stromal–epithelial signaling to obtain a receptive uterus (Li et al. 2011). This study confirmed that Hand2 is expressed in the mouse uterus prior to the onset of implantation. Although expression levels are much lower compared with that during decidualization, this expression was localized to a subpopulation of stromal cells adjacent to the luminal epithelium. Ovariectomized mice can be treated with a specific regimen of injections with estrogen and progesterone is a major regulator of in the luminal epithelium during hormone-induced uterine sensitization of ovariectomized mice. This is consistent with previous work on the effects of RU486 on reducing Hand2 expression in the preimplantation mouse uterus on day 3.5 of pregnancy (Li et al. 2011). Although all these results strongly suggest that Hand2 expression is downstream of the PGR, it still remains to be determined whether Hand2 transcription is directly regulated by PGRs or by other downstream targets.

Progesterone is a key regulator of uterine gene expression during the uterine sensitization period. For example, Hoxa10 expression in the mouse and human endometrium is induced by progesterone prior to the onset of implantation (Ma et al. 1998, Taylor et al. 1998) and plays a key role in endometrial receptivity (Gui et al. 1999, Vitiello et al. 2008). Recent work also suggests that progesterone is a major regulator of Hand2 expression in the mouse uterus during the receptive phase (Li et al. 2011). Using ovariectomized mice and an exogenous hormone sensitization model, this study shows that Hand2 expression increases in response to progesterone. The localization of Hand2 expression in the stromal cells adjacent to the luminal epithelium on day 3.5 of pregnancy or in optimally sensitized uteri of ovariectomized steroid-treated mice is consistent with the localization of PGR protein in these cells on day 3.5 of pregnancy (Tan et al. 2011). Using ovariectomized and Alpl mice and an exogenous hormone sensitization model, this study shows that Hand2 expression increases in response to progesterone.

Studies to date suggest that the endometrial expression of Hoxa10 plays a critical role in uterine sensitization.
and decidualization (Das 2010), and several observations raise the possibility that HOXA10 might be the direct regulator of Hand2 expression in the uterus during the early peri-implantation period. This study shows that the expression level profiles of Hand2 and Hoxa10 are similar in the mouse uterus during hormone-induced uterine sensitization in ovariectomized mice. Progesterone is the major regulator of Hoxa10 expression in the mouse and human uterus (Ma et al. 1998, Taylor et al. 1998, Lim et al. 1999, Godbole et al. 2007). On day 3.5 of pregnancy in the mouse, expression of Hoxa10 is restricted to the endometrial stroma and then dramatically increases in areas undergoing decidualization (Das 2010). In a similar fashion, Hoxa10 expression is also seen in mouse and HESC as they undergo decidualization in vitro (Godbole & Modi 2010). Finally, the promoter region of the mouse and human Hand2 genes contain conserved putative Hoxa10- but not PGR-binding sites (Voth et al. 2009). Therefore, future work determining how Hand2 gene expression is potentially controlled by the direct actions of HOXA10 at the Hand2 promoter region is warranted.

Currently, little is known about the function of Hand2 expression in the mouse or human uterus during uterine sensitization and decidualization. Hand2 knockout mice are embryonic lethals (Srivastava et al. 1997), so approaches other than the use of generic mouse knockout models are needed to study HAND2 function in mouse ESC decidualization. A recent study using uterine tissue-specific Hand2 knockout mouse clearly shows that HAND2 expression is critical for proper uterine receptivity (Li et al. 2011). These mice showed no decidual swellings and this is likely due to failure of the embryo to begin implantation and thus pregnancy fails prior to the onset of decidualization. This study has used ESC culture models to assess the function of Hand2 expression during the decidualization of mouse and human endometrial stromal fibroblast cells. We find that HAND2 expression significantly increases during the decidualization of these cells. Suppressing Hand2 expression in mouse or human endometrial cells may cause a decrease in the decidualization of these cells as indicated by the reduction of the expression of markers of decidualization. However, this may be an oversimplification. For example, our results support the hypothesis that HAND2 plays a key role in the expression of IGFBP1 and FOXO1A, established markers of decidualization in humans. On the other hand, PRL expression increased when HAND2 expression was suppressed in human cells. As PRL is also a marker of decidualization, this suggests HAND2 might somehow suppress PRL expression and decidualization. In a similar fashion, the expression of Alpl but not Prl8a2 decreased in the mouse ESCs when Hand2 expression was suppressed. Although this study suggests that it plays a key role in decidualization of mouse and HESC, the identity and function of downstream target genes of Hand2 expression need to be elucidated further.

PGE2 is a regulator of Hand2 expression in endometrial cells undergoing decidualization. PGE2 plays a key role in decidualization and ESC decidualization in rodents (Kennedy & Ross 1997, Cong et al. 2006, Pakrasi & Jain 2008). The results of this study suggest that Hand2 expression in mouse and HESC during decidualization is enhanced by PGE2. PGE2 has four potential receptors (prostaglandin E receptors 1–4) that activate distinct intracellular signaling pathways (Sugimoto & Narumiya 2007). The identity of which one(s) is important in Hand2 expression in the cells during decidualization is not known. However, recent evidence suggests that

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Figure 10 Changes in gene expression in human HESC cells undergoing decidualization in vitro. Cells were treated with vehicle (Veh), steroid hormones (H; estradiol-17β, 10 nM; medroxyprogesterone, 1 μM), or hormones plus PGE₂ (10 μM; H + PGE₂) for 0, 1, 2, or 3 days and relative (A) FOXO1A, (B) IGFBP1, (C) PRL, (D) TIMP3, (E) CCNE2, and (F) HAND2 mRNA levels were determined by qRT-PCR. Bars with a different letter on a given day denotes a significant (P<0.05) difference in mRNA levels. Cells were treated with vehicle (Veh), or steroid hormones plus PGE₂ (H + PGE₂) for 0, 6, 12, 18 or 24 h and relative (G) HAND2 and (H) FOXO1A mRNA levels were determined by qRT-PCR.
prostaglandin E receptor 2 may be the key receptor involved in rat uterine decidualization (Pakrasi & Jain 2008). On the other hand, potentially, more than one receptor might play a role in the mouse (Yang et al. 1997). Regardless, the effect of PGE2 on enhancing the decidualization of rodent and HESC has been attributed to increased cAMP levels in the cells (Yee & Kennedy 1991, 1993, Brar et al. 1997). It possible that cAMP may be able to modulate transcription through a highly conserved cAMP response element-binding protein (CREB) site described previously in the Hand2 promoter region (Voth et al. 2009). However, further work is needed to determine with certainty whether increased cAMP production and activation of related downstream intracellular signaling pathways, including CREB-mediated transcriptional regulation, are involved in mediating the effects of PGE2 on ESC Hand2 expression during decidualization.

In conclusion, this study was conducted to better characterize the expression and function of Hand2 expression in the uterus during mouse implantation and in decidualization of mouse and HESC. The results of this study support the hypothesis that Hand2 expression in mice plays an important role both in uterine sensitization prior to the onset of implantation and in decidualization after the onset of implantation. Furthermore, our results suggest that HAND2 may play a key role in the decidualization of HESC. Hand2 expression might play a conserved role in the mouse and HESC decidualization. These results should form the basis for further research on Hand2 function in the uterus during implantation.

Materials and Methods

Animals and tissue collection

All procedures involved the use of female (9–12 weeks old) and male (3–10 months old) CD1 mice (Charles Rivers Laboratory, Wilmington, MA, USA) and were approved by the Southern Illinois University Institutional Animal Care and Use Committee. Mice were allowed free access to food (Formulab 5008 LabDiet, Purina Mills, Gray Summit, MO, USA) and water while housed under controlled light conditions (lights on: 0700 to 1900 h). Females were mated with fertile males, and in the morning, a vaginal plug was observed and termed day 0.5 of pregnancy. The model used to obtain bead-induced deciduomas for this study has been described in detail elsewhere (Herington et al. 2009). Briefly, females were mated with vasectomized males, and in the morning, a vaginal plug was observed and considered day 0.5 of pseudopregnancy. At 1300–1500 h on day 2.5, ConA-coated agarose beads were transferred into the uterine lumen. This deciduoma model was chosen rather than other artificial decidualization models because it better recapitulates the decidual changes that occur in the pregnant uterus compared with other

Figure 11 Gene expression in HESC cells stably expressing control shRNA and HAND2 shRNA after incubation with steroids (estradiol-17β, 10 nM; medroxyprogesterone, 1 μM) plus PGE2 (10 μM) for 2 days. (A) Relative mRNA levels determined by qRT-PCR. Bars represent mean ± S.E.M. (N=4). Difference in mRNA levels significant, *P<0.05, **P<0.01, and ***P<0.001. Immunocytochemical localization of HAND2 protein in HESC cells expressing (B) control and (C) HAND2 shRNA.
Hand2 plays a key role in decidualization

A 314 bp Hand2 cDNA was generated by RT-PCR using TopTaq mastermix (Qiagen) and primers (IDT DNA Technologies, upstream 5'-CGAGGAGACCCCTACTTCC-3' and downstream 5'-CGCAGTGTCCTGATCTTGGA-3') as directed by the manufacturer (Qiagen). The cDNA was purified using a Cycle-Pure kit (Omega BioTek, Norcross, GA, USA), then cloned into pGEM-Teasy vector (Promega), and grown in bacteria as directed by the manufacturer. Plasmid preparation and purification was carried out using the Omega BioTek Maxiprep kit as recommended by the manufacturer (Omega Biotek). The sequence and orientation of the cDNA clone was then verified (W. M. Keck Center for Comparative and Functional Genomics, University of Illinois Urbana-Champaign). To generate sense and antisense probes, transcription templates were generated by PCR using the appropriate combination of M13 forward or reverse plus upstream or downstream Hand2 primers. The PCR amplicons were isolated using an E.Z.N.A. Cycle Pure Kit as directed by the manufacturer (Omega Biotek). Preparation and purification of the DIG-labeled sense and antisense probes using these transcription templates was carried out as previously reported (Bany & Cross 2006).

Paraffin sections mounted on Superfrost plus glass slides (Fisher Scientific) were cleared in xylene and then hydrated in graded ethanol solutions followed by water. The sections were then digested with proteinase K (Amresco, Solon, OH, USA) and treated with triethanolamine (Sigma) as described previously (Bany & Cross 2006). Hybridization, post-hybridization washes, immunological detection of DIG, and colorimetric development using BCIP/NBT were carried out exactly as described previously (Simmons et al. 2008). After counterstaining in nuclear fast red, the sections were covered with glass coverslips and observed under a Leica Microscope equipped with a Retiga Camera and QImaging Pro software (QImaging, Surrey, BC, USA). Nuclei and Hand2 mRNA stain red and purple respectively. Sections incubated with sense riboprobes in place of antisense riboprobe exhibited no positive Hand2 mRNA staining (data not shown).

HAND2 immunohistochemistry

Paraffin sections mounted on Superfrost plus glass slides were cleared in xylene and then hydrated in graded ethanol solutions followed by water. The sections were then exposed to high-temperature antigen unmasking and washed in PBS containing 0.05% Tween-20 (PBST). The sections were then incubated with blocking buffer containing 2% normal horse serum (Vector Labs, Burlingame, CA, USA) in PBST at room temperature for 1 h followed by 0.1 μg/ml goat anti-HAND2 IgG (Santa Cruz, Santa Cruz, CA, USA) in blocking buffer overnight at 4 °C. After washing in PBST, the sections were incubated with biotinylated horse anti-goat IgG (Vector Labs, 2 μg/ml) in blocking buffer for 1 h at room temperature. After washing in PBST, the sections were incubated with streptavidin–alkaline phosphatase conjugate (Vector Labs) in PBST for 30 min at room temperature. After washing with PBST, the sections were incubated with Alkaline Phosphatase Blue or

qRT-PCR

Total RNA isolation and qRT-PCR were carried out exactly as described previously (Herington et al. 2009). Oligonucleotide primers for mouse Alpl mRNA and 18S rRNA have previously been published (Herington & Bany 2007a, Herington et al. 2009). Primers used for human FOXO1A, H36B4, HAND2, IGFBP1, PRL, and TIMP3 mRNAs have previously been published (Buzzio et al. 2006). Mouse Hand2 (upstream 5'-AGATCAAGAGACGGACGTGA-3', downstream 5'-CTGTCCGGGCTTGTGTTTTC-3'); mouse Prlha2 (upstream 5'-GCTGCTAATCCCTGATGTC-3', downstream 5'-CCCTCACAATGTCGG-3'); mouse Fkb3p (upstream 5'-ATGGGCCCTACGGAAAGAA-3', downstream 5'-CCACTTCA-3'); mouse Gja1 (upstream 5'-TTGTGCTTCAGCGTCCTCCAGG-3', downstream 5'-CATGTCCTGCGGACCTCT-3'); mouse Runx1 (upstream 5'-CTCCGTCCTACCACTC-3', downstream 5'-TGACGTGGGACCAGTAGTC-3') and human CCNE2 (upstream 5'-CCCAACCGAGCCGATA-3', downstream 5'-CAGGTTGGCCAAATCTCC-3') primers were obtained from IDT DNA Technologies (Corvalis, IA, USA). Briefly, high-capacity RNA-to-cDNA kits (Applied Biosystems, Foster City, CA, USA) were used to generate the cDNA and 2 × iQ SYBR Green Supermix plus a CFX real-time PCR machine (Bio-Rad) was used to perform the quantitative PCR. PCR primers were used at a final concentration of 200 nM and cycle conditions were 3 min at 94 °C followed by 40 cycles of 94 °C, 62–64 °C for 20 s, and 72 °C for 1 min for melting, annealing, and extension steps respectively. Cycle thresholds (Ct) were determined using the CFX software (Bio-Rad) and relative mRNA levels were determined as described previously after normalization to mouse 18S rRNA (Herington & Bany 2007a) or human H36B4 (Buzzio et al. 2006).
BCIP/NBT chromogenic substrates (Vector Labs) as directed by the manufacturer. After counterstaining in nuclear fast red, the sections were covered with glass coverslips and observed under a Leica Microscope. Nuclei and HAND2 protein stain red and blue/purple respectively. Recently the same antibody used in this study was shown to stain similar cells in the receptive mouse uterus and was lost in a uterine-specific knockout mouse (Li et al. 2011) verifying the specificity of the antibody. In this study, sections incubated with the same concentration of normal goat IgG in place of the anti-HAND2 IgG exhibited no positive staining except where noted (data not shown).

**Primary cultures of mouse ESCs**

To obtain primary endometrial cell cultures, we ovari-ectomized mice and treated them with steroids to sensitize the uterus for a deciduogenic stimulus exactly as described in Fig. 6A. At 3 h after the steroid injection on day 8 of hormone treatment, the mice were killed and the uteri were dissected. ESCs were isolated and plated exactly as described previously (Bany et al. 2000, Bany & Schultz 2001). Once plated, the cells were incubated with DMEM:F12 (Invitrogen) containing 10% charcoal stripped serum, 100 U/l penicillin, 0.1 g/l streptomycin and 1.25 mg/l fungizone (Invitrogen) with or without treatments. Unless indicated, the medium contained 17β-estradiol (10 nM) plus medroxyprogesterone (1 μM) (Sigma) with or without PGE2 (10 μM, Cayman Chemicals, Ann Arbor, MI, USA). Cells were incubated with treatments for 1, 2, or 3 days with fresh medium supplied every 24 h.

In some experiments, cells were transiently transfected with mouse control non-targeting or Hand2 siGENOME SMARTpool siRNA (Dharmacon, Inc., Lafayette, CO, USA). Transfections were carried out for 6 h on freshly plated primary cell cultures using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions using 100 nM of siRNA pool. The cells were then incubated with steroids plus PGE2 as above for 2 days with fresh medium supplied every 24 h.

**HAND2 immunocytochemistry**

Cells were washed with PBS and fixed with PBS containing 4% paraformaldehyde for 10 min. After washing in PBS, the cells were incubated in blocking buffer (PBS, 0.2% Triton-X100, 10% horse serum) for 1 h. Then, the cells were incubated overnight at 4 °C with blocking buffer containing 0.1 μg/ml goat anti-HAND2 IgG (Santa Cruz). After washing in PBS, the cells were incubated in blocking buffer containing 0.2 μg/ml biotinylated donkey anti-goat IgG (Vector Labs) for 30 min. After washing in PBS the cells were incubated with 1 mg/ml Cy3-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA, USA) for hours. Finally, after washing and staining the nuclei with 4,6’-diamidino-2-phenylindole (Pierce, Rockford, IL, USA), the cells were observed under a Leica inverted fluorescence microscope equipped with the appropriate filters and Retiga camera controlled by QImaging software (QImaging). Positive staining for HAND2 protein and nuclei were red and blue respectively.

**In vitro decidualization model of HESC**

The previously described (Krikun et al. 2004) immortalized HESC line was obtained from ATCC (Washington, DC, USA) and cultured, passaged, and preserved as recommended by the supplier. Once the cells reach 70% confluence, they were incubated with various treatments in phenol-red-free DMEM:F12 containing 3.1 g/l glucose and 1 mM sodium pyruvate (Sigma) supplemented with 1.5 g/l sodium bicarbonate (Invitrogen), 1% ITS+ Premix (Becton-Dickinson, Franklin-Lakes, NJ, USA), and 10% charcoal-stripped serum (Fisher Scientific). Treatments included estradiol-17β (10 nM) plus medroxyprogesterone (1 μM, Sigma) and PGE2 (10 μM, Cayman Chemicals). Medium was replaced every 24 h of culture.

**Electroporation and establishing HESCs that stably express shRNA**

Sure silencing shRNA expression vectors targeting human HAND2 (HAND2 shRNA: GTGCGTTTGTGAAGTGATCTC) or the vectors that were a negative non-targeting control (GGAACTCTACCTGATGCATA) (SABiosciences, Frederick, MD, USA) were electroporated into HESC cells. These vectors carry neomycin-resistance sequences allowing for positive selection of cells that stably express the shRNAs. Electroporation was carried out using a Gene Pulser Xcell Electroporation System (Bio-Rad). Suspensions of HESC cells (3 × 10⁶) in InGenio Electroporation Solution (Mirus, Madison, WI, USA) were mixed with 20 μg shRNA vector, and then exposed to a 15 ms pulse of 2.5 V. After electroporation, the cells were recovered and plated in medium as above but containing 0.4 mg/ml neomycin. After four passages under neomycin selection, the HESC cells were stored in liquid nitrogen until used.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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