REPRODUCTION
REVIEW

Cell cycle regulatory control for uterine stromal cell decidualization in implantation

Sanjoy K Das

Reproductive Sciences, S11.236, Department of Pediatrics, Cincinnati Children's Hospital Medical Center, MLC 7045, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3039, USA

Correspondence should be addressed to S K Das; Email: sanjoy.das@cchmc.org

Abstract

Uterine stromal cell decidualization is integral to successful embryo implantation, which is a gateway to pregnancy establishment. This process is characterized by stromal cell proliferation and differentiation into decidual cells with polyploidy. The molecular mechanisms that are involved in these events remain poorly understood. The current concept is that locally induced factors with the onset of implantation influence uterine stromal cell proliferation and/or differentiation through modulation of core cell cycle regulators. This review will aim to address the currently available knowledge on interaction between growth factor/homeobox and cell cycle regulatory signaling in the progression of various aspects of decidualization.

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Embryo-uterine interactions during early implantation

Implantation, a complex developmental process, involves a reciprocal dialogue between the blastocyst and uterus, and is essential to continued embryonic development within the uterus for achieving successful pregnancy. Synchronized development of the embryo to the active stage of the blastocyst, differentiation of the uterus to the receptive state, and a 'crosstalk' between the blastocyst and uterine luminal epithelium are essential to the implantation process (Psychoyos 1973, Dey *et al*. 2004). Uterine differentiation to support blastocyst implantation is primarily directed by progesterone (P_4) and estrogen. These steroid hormones normally mediate their actions via binding to their cognate nuclear receptors (Beato 1989, O'Malley 1990). Heterogeneous cell types of the uterus respond uniquely to P_4 and/or estrogen. In adult mice, estrogen stimulates proliferation of the luminal and glandular epithelia, while in the stroma this process requires both P_4 and estrogen (Huet-Hudson *et al*. 1989). A similar steroid hormonal regulation also occurs in the uterus during the preimplantation period (days 1–4). On days 1 and 2 (day 1 = vaginal plug), preovulatory ovarian estrogen directs uterine epithelial cell proliferation. On day 3, P_4 from newly formed corpora lutea initiates stromal cell proliferation, which is further stimulated by preimplantation estrogen secretion on the morning of day 4. By contrast, epithelial cells cease to proliferate and become differentiated on this day. In mice, a small amount of estrogen is required to elicit implantation in the P_4-primed uterus, and it has been widely viewed that local mediators amplify estrogenic effects in the uterus at the site of implantation.

For the onset of implantation, uterine preparation is sequentially programmed into three stages, viz., prereceptive, receptive, and nonreceptive (Psychoyos 1973, Yoshinaga 1980). In mice, uteri are prereceptive on day 3 of pregnancy or pseudopregnancy under the increasing ovarian P_4 levels. On day 4, the P_4-primed uterus becomes receptive when complemented with preimplantation ovarian estrogen secretion. Blastocysts implant only in the receptive uterus (day 4), which then automatically enters into the nonreceptive state on day 5 (Dey *et al*. 2004). Similar uterine phases can be produced by ovariectomy on the morning of day 4 of pregnancy or pseudopregnancy before the preimplantation estrogen secretion. Under this condition, blastocysts in the pregnant uterus or transferred blastocysts into the pseudopregnant uterus undergo dormancy and fail to initiate the attachment reaction. This condition can be maintained by continued P_4 treatment when the uterus remains in the neutral (similar to prereceptive phase) state, but is terminated by a single estrogen injection with blastocyst activation and attainment of uterine receptivity. Mechanisms by which estrogen renders the uterus receptive, activates dormant blastocysts, and initiates implantation are not clearly understood, although an extensive literature suggests that a variety of growth regulatory factors, cytokines, and transcription

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factors can function as autocrine/paracrine modulators under the direction of ovarian hormones in the uterus (Lim *et al*. 2002, Paria *et al*. 2002, Dey *et al*. 2004, Wang & Dey 2006).

Aspects of cellular proliferation and differentiation in association with uterine stromal cell decidualization

The initiation of implantation in mice is characterized by a localized uterine vascular permeability at the site of the blastocyst attachment (blue reaction), occurring between 2200 and 2400 h on day 4 of pregnancy. The attachment reaction coincides with the extensive proliferation and differentiation of uterine stromal cells into decidual cells (decidualization) at the site of the implanting blastocyst. By contrast, luminal epithelial cells only at the site of blastocyst apposition progressively undergo apoptosis with the succession of implantation. Uterine decidualization is normally initiated at the antimesometrial pole, which then spreads to the mesometrial pole, the presumptive site of placentation. This orients the implantation chamber in an antimesometrial–mesometrial direction in alignment with the anterior–posterior axis of the embryo. Between the day 5 afternoon and the day 6 morning, stromal cells immediately surrounding the implanting blastocyst cease proliferating and undergo differentiation into decidual cells, forming a zone termed the primary decidual zone (PDZ). Studies have provided evidence that this zone is avascular, epithelioid in nature, and densely packed with decidual cells (Paria *et al*. 1999*a*). By day 6, stromal cells next to the PDZ continue to proliferate and differentiate into polyploid decidual cells forming a zone around the PDZ, termed the secondary decidual zone (SDZ). The SDZ is fully developed by day 7, while the PDZ degenerates progressively up to day 8. After day 8, the placental and embryonic growth slowly replaces the SDZ. Normally, the stimulus for decidualization is the implanting blastocyst. A similar, although not identical, pattern of decidualization can be initiated by the application of artificial stimuli to a receptive pseudo-pregnant uterus, or one that has been appropriately primed by ovarian steroids (Lim *et al*. 1997, Dey *et al*. 2004). Using these model systems, one can examine the cellular and molecular changes that occur during decidualization under the control of P_4 and estrogen.

The development of decidua or deciduoma in rodents is associated with the formation of multinucleate and giant cells (Sachs & Shelesnyak 1955, Ansell *et al*. 1974, Moulton 1979, Tan *et al*. 2002). The polyploid decidual cells are terminally differentiated cells and are shown to be developed through a unique process requiring transition from the mitotic cell cycle to an endoreduplication cycle (endocycle), in which cells undergo a repeated round of DNA replication without successive cell division (cytokinesis). An altered cell cycle regulation has been considered to be associated with the development of cellular polyploidy. In mice, the development of decidual cell polyploidy leads to the generation of large mono- or binucleated cells, consisting of DNA with four, eight, and even higher multiples of the haploid complement (Sachs & Shelesnyak 1955, Ansell *et al*. 1974, Moulton 1979, Tan *et al*. 2002). Previous studies have shown that the antimesometrial decidualizing stroma is comprised of two populations of cells: first one that divides fast before undergoing differentiation and the second one that undergoes differentiation without further division, thus leading to the development of cellular heterogeneity in the decidual bed (Moulton 1979, Tan *et al*. 2002).

It is expected that for normal cell division, cells must receive a complete copy of their genome at each division. To ensure genomic stability, the S phase is tightly regulated so that replication of the chromosomes is initiated only once in each cell cycle. It has been suggested that the loss of this regulation results in polyploidy that allows cells to undergo continuous endocycle without cell division (Edgar & Orr-Weaver 2001). In this regard, it has been speculated that yet another cell cycle variant could be potentially active in decidualization, which may trigger nuclear division without cytokinesis, giving rise to binucleated cells. Indeed, such cell cycle regulation has been shown to be active in mammalian hepatocytes and osteoclasts (Edgar & Orr-Weaver 2001). Although several biological processes, including cell differentiation, expansion, and metabolic activity are shown to be associated with endoreduplication, the definitive physiological significance of decidual polyploidy remains poorly understood. It is believed that stromal cell polyploidy could limit the lifespan of decidual cells in order to accommodate the growing embryo. While it is believed that one of the functions of uterine decidualization is to support embryonic growth and that requires an increased protein synthesis, genomic endoreduplication may ensure the high degree of synthesizing potential for various proteins by increasing the number of gene copies for transcription. Additionally, decidual growth has been considered as a pseudo-malignant state during pregnancy and resembles various aspects of cancer, including its rapid development and the presence of polyploidy.

Core cell cycle regulators during the progression of cellular proliferation and differentiation

The cell cycle is tightly regulated at two particular checkpoints, G1–S and G2–M phases. Normal operation of these phases involves a complex interplay of cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CKIs; Roberts 1999, Sherr & Roberts 1999). Cellular growth is critically regulated at two particular

transitions: G1–S and G2–M (Fig. 1). Various cyclins mediate their actions as positive growth regulators during these transitions by associating with specific CDKs. The well-known regulators of mammalian cell proliferation are the three D-type cyclins (D1, D2, and D3) that are also known as G1 cyclins. The D-type cyclins accumulate during the G1 phase and their association with CDK4 or CDK6 is particularly important to form holoenzymes that facilitate cell entry into the S phase. Retinoblastoma protein (Rb) and its family members RBL1 and RBL2 are negative regulators of the D-type cyclins. Inactivation of these regulators by phosphorylation, dependent on the cyclin/CDK complex activity, allows cell cycle progression through the G1 phase (Riley *et al.* 1994). Overexpression of D-type cyclins shortens the G1 phase and allows rapid entry into S phase (Resnitzky *et al.* 1994). By contrast, cyclins A and B are involved in progression from S to G2–M phase. Binding of cyclin A or cyclin B to CDC2A induces phosphorylation and activation of the complex that is essential for the G2–M phase transition, while cyclin A/CDK2 complex is required during progression in the S phase. In general, the action of CDKs is constrained at least by two CKIs, CDKN2A and CDKN1A. The CDKN2A family includes CDKN2B, CDKN2A, CDKN2C and CDKN2D, and they specifically inhibit the catalytic partners of D-type cyclins (CDK4 and CDK6). The CDKN1A family consists of CDKN1A, CDKN1B and CDKN1C, and they inhibit CDKs with a broader specificity. It is well known that CKIs accumulate in quiescent cells, and are down-regulated with the onset of proliferation. Thus, a critical balance between the positive and negative cell cycle regulators is the key decision-maker for cell division. Changes in the levels of D-type cyclins and CKIs normally occur when quiescent cells are stimulated by mitogenic signals. The general consensus is that the mitogenic signals should converge prior to the entry of a cell to the S phase, but the precise point of convergence and integration in the G1 phase remains unknown.

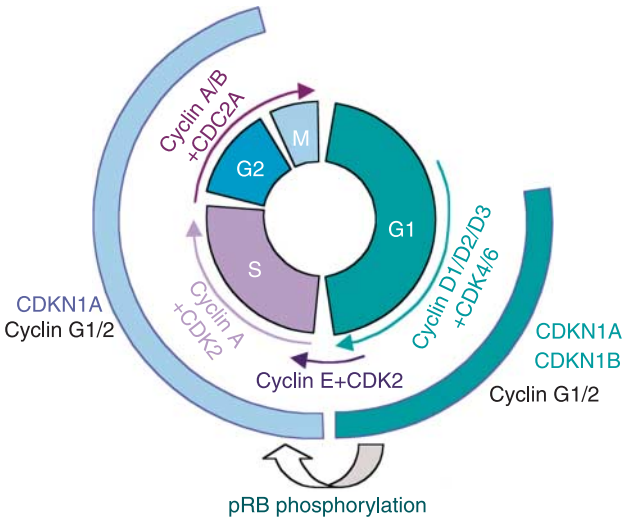

Figure 1 Cell cycle control for G1/S and G2/M phase-specific transitions by different growth regulators.

As mentioned above, most cyclins primarily act as positive growth regulators in the cell cycle circuitry; however, there are some exceptions, such as the G-type cyclins that instead participate as negative regulators to inhibit cell proliferation. So far, two G-type cyclins have been identified, cyclin G1 and cyclin G2. Cyclin G1 expression is considered to be constitutive throughout the cell cycle, while that of cyclin G2 fluctuates throughout the cell cycle with a peak level of expression in the G1 phase (Horne *et al.* 1996). The growth inhibitory activity of cyclin G-types is apparent from its abundant expression in highly differentiated tissues and cells undergoing apoptosis due to cell cycle arrest or in response to growth inhibitory stimuli (Bates *et al.* 1996, Horne *et al.* 1997). As mentioned above, several cell cycle regulators have been shown to be expressed in various aspects of uterine decidualization at the site of implantation, which is discussed below.

Cell cycle regulators differentially modulate during the progression of uterine decidualization

Regulation by ovarian steroid hormones

Because the uterus is a dynamic physiological system in which cellular proliferation, differentiation, including the terminal differentiation, and apoptosis occur in a temporal and cell-specific manner during pregnancy and in the cycle, cell cycle molecules have been suggested to be intimately involved. There is evidence that cell cycle regulators play important roles in the uterus during the hormonal stimulation (Geum *et al.* 1997, Prall *et al.* 1997, Tong & Pollard 1999) and reproductive cycle (Shiozawa *et al.* 1996, 1998). In humans, the expression of phase-specific cyclins (cyclins D1, E, A, and B1) and CDKs (CDK4, CDK2, and CDC2A) is known to be induced in uterine epithelial and stromal cells during the proliferative phase. By contrast, during the secretory phase or after P_4 administration, the expression of CDKN1B is strongly induced in the endometrial glands and stromal basalis, which is coincident with their P_4-dependent growth suppression (Shiozawa *et al.* 1996, 1998). In the case of rodents, uterine expression of cyclins D1 and E and CDK4 is primarily regulated by estrogen in association with uterine epithelial cell proliferation (Geum *et al.* 1997, Prall *et al.* 1997, Tong & Pollard 1999). By contrast, P_4-dependent inhibition of uterine epithelial cell proliferation appears to be mediated by the decrease in CDK4 activity, presumably through a depression of its expression, as well as by inhibition of the kinase activity through its concomitant association with CDKN1B (Musgrove *et al.* 1998, Tong & Pollard 1999). There is also evidence that P_4 inhibition of

uterine epithelial cell proliferation is mediated by the inhibition of nuclear translocation of cyclin D1 and CDK4 in association with the activation of cyclins A- and E-dependent CDK2 activity (Tong & Pollard 1999).

Regulation at the site of implantation during decidualization

In order to identify the implantation-related genes, we have originally reported that cyclin D3 is specifically upregulated at the site of implantation in mice (Das *et al.* 1999). A schematic model as shown in Fig. 2 illustrates a possible role for cyclin D3 together with other cell cycle regulatory molecules in the developmental regulation of stromal cell proliferation and differentiation, including the terminal differentiation in association with decidualization. To determine the importance of other G1-phase-specific cell cycle regulators, it has been shown that the expression of other D-type cyclins (cyclins D1 and D2) was low in the uterus prior to and during implantation and decidualization. In the receptive uterus on day 4 of pregnancy, cyclin D3 is very weakly detected only in the stromal cells; however, this gene expression is remarkably induced in the decidualizing stroma either at the site of implantation or in artificially induced decidualization (Das *et al.* 1999). Based on multiple approaches, viz., expression studies for various cell cycle regulators, functional assays for CDKs activity and analysis of cell cycle phase-specific distribution of decidual cells have strongly positioned us to suggest that cyclin D3 is associated with stromal cell proliferation, differentiation, and polyploidy events during decidualization (Tan *et al.* 2002). Coordinate expression of CDK4 and cyclin D3 at the site of implantation in stromal cells on day 5 has suggested that these regulators play roles in cellular proliferation with the onset of implantation. However, the expression of the cell cycle inhibitor CDKN1A, in conjunction with the down-regulation of cyclin D3 and CDK4 in the PDZ on day 5 afternoon supports the view that cell proliferation activity of CDK4/cyclin D3 ceases in the developing PDZ. However, on days 6 and 7, the proliferating stromal cells at the mesometrial pole predominantly express cyclin D3 and CDK4. By contrast, the expression of cyclin D3, but not the CDK4, at the antimesometrial decidual bed (within the SDZ) is further stimulated. Furthermore, in the SDZ, the persistent expression of CDKN1A and a new expression for CDK6 in conjunction with cyclin D3 forming a ternary complex appears to direct the development of decidual cell polyploidy (Tan *et al.* 2002). The presence of cyclin E, cyclin A, and CDK2 with concomitant downregulation of cyclin B and CDC2A in polyploid decidual cells supports the view that these cells are undergoing an incomplete cycle (or endocycle) pathway by inducing an arrest prior to the G_2–M phase transition (Tan *et al.* 2002).

An alteration in cell cycle molecules in association with the development of polyploidy has been reported for other cell lineages (Kiess *et al.* 1995, Kikuchi *et al.* 1997, Zimmet *et al.* 1997, MacAuley *et al.* 1998). For example, overexpression of cyclin D3 leads to polyploidization of megakaryocytes with reduced kinase activity of cyclin B1-dependent CDC2A (Zimmet *et al.* 1997). Similarly, CDKN1A is also implicated in polyploidization during megakaryocytic differentiation (Kikuchi *et al.* 1997). Furthermore, cyclin D3 expression in myoblast cell lines correlates with their terminal differentiation into multinucleated quiescent myotubes (Kiess *et al.* 1995). A classic example of endoreduplication during pregnancy in rodents is the differentiation of trophoblast giant cells that is accompanied by the switch of cyclin D3 to cyclin D1 expression with the initiation of S phase during endocycles and appears to involve cyclins E and A (MacAuley *et al.* 1998, Geng *et al.* 2003, Parisi *et al.* 2003).

We have recently observed that i.v. administration of recombinant adenovirus-Cre (rAd-Cre) on day 5 of pregnancy in Rosa $26^{loxp\text{-}lacZ}$ mice (Soriano 1999) shows LacZ expression in antimesometrial decidualizing stromal cells on days 7 and 8 of pregnancy, while control rAd-GFP virus failed to exhibit such a response (Wang *et al.* 2006*a*). Utilizing this technique, we have also shown that suppression of cyclin D3 by an antisense adenovirus approach causes compromised decidualization at the site of implantation on days 7 and 8 of pregnancy in mice (Tan *et al.* 2004). Thus, our overall observation of cyclin D3 upregulation during

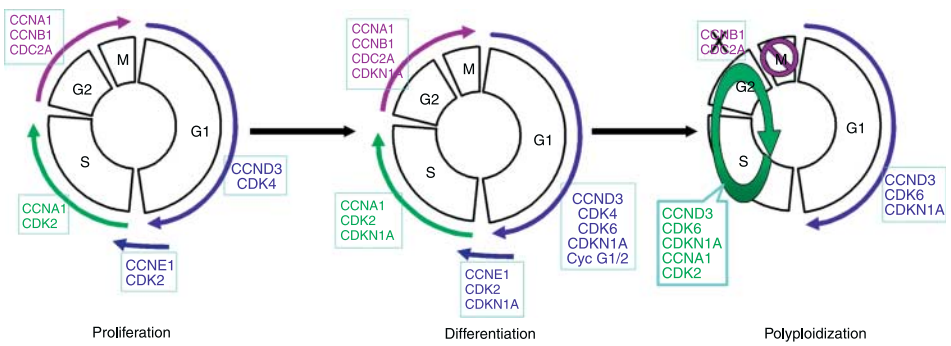

Figure 2 A potential model for stromal cell proliferation, differentiation, and terminal differentiation for polyploidization during the progression of uterine decidualization.

decidualization and attenuation of this process by dampening cyclin D3 signaling leads us to believe that cyclin D3 is an important player in decidualization.

Our findings are at odds with a report of apparently normal female fertility in cyclin D3$^{-/-}$ mice (Sicinska *et al.* 2003). However, this report did not provide any supporting data. Combined deletion of all three mammalian D-type cyclins (D1, D2, and D3; Kozar *et al.* 2004), or D-type CDK partners, CDK4 and CDK6 (Malumbres *et al.* 2004), has been shown to have little effect on fibroblast cell proliferation, but, interestingly, these fibroblasts lack oncogenic potency for transformation. These observations suggest that D-type cyclin-dependent function is not essential for normal cell proliferation, but instead is required for oncogenic cell proliferation and transformation. This raises an interesting question as to the role of cyclin D3 in uterine decidualization, which is considered a pseudo-malignant state during pregnancy. More importantly, after carefully monitoring the experiments, we have recently observed that cyclin D3 deficiency significantly compromises pregnancy outcome resulting from defective decidualization in mice (Fig. 3). To explore whether cyclin D3 knock-out (KO) females show defective reproductive phenotypes, we examined term pregnancy for KO and WT littermate females on the same genetic background (129Sv/C57BL/6J) after crossing with fertile males. A significant number (16%) of plug-positive cyclin D3$^{-/-}$ females failed to produce offspring (Fig. 3A). Furthermore, the litter size was significantly smaller (4.8±2.0) from KO females that underwent term pregnancy compared with that of WT females (8.6±2.3; unpaired *t*-test, $^*P<0.01$), suggesting loss of embryos in KO mothers during pregnancy. Analysis of the number of implantation sites on day 5 did not show any apparent defects in the initiation of implantation for KO females as determined by blue dye injection (Psychoyos 1973; Fig. 3B). By contrast, weight of implantation sites on day 8 (D8) was significantly reduced as compared with WT mice (Fig. 3C), suggesting impaired stromal cell decidualization. Indeed, histological examination clearly revealed retarded decidualization with signs of embryo resorption (arrow) in KO mice (Fig. 3D). In addition, analysis of the weight of

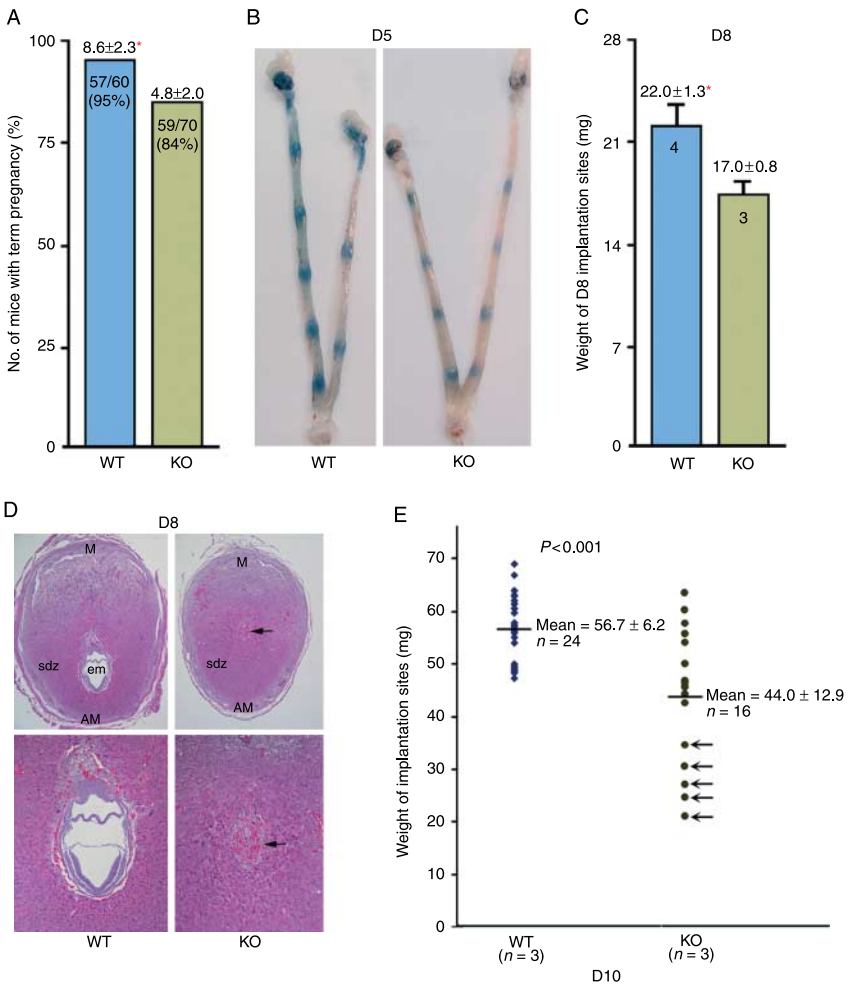

Figure 3 Cyclin D3 deficiency compromises decidualization in mice. (A) Term pregnancy was recorded for cyclin D3 knock-out (KO) and wild-type (WT) littermate females after mating with fertile males. Plug-positive mice were monitored for delivery of pups at term, and litter size was recorded. Although term pregnancy occurred in cyclin D3 KO mice, the litter size was significantly smaller compared with wild-type females (unpaired *t*-test, $^*P<0.001$). (B) The number of implantation sites is comparable between KO and WT mice as examined by the blue dye method on day 5 (D5). (C). Weight of implantation sites was recorded on D8. A significant reduction in weight was noted for KO mice compared with WT mice (unpaired *t*-test, $^*P<0.001$). The number of mice used is shown inside bars. (D) Hematoxylin and eosin stained histology of representative uterine sections on D8. Photomicrographs shown in the upper and lower panels are at 4× and 10× respectively. M, mesometrial pole; AM, anti-mesometrial pole; em, embryo; sdz, secondary decidual zone. Arrow indicates the position of an embryo undergoing resorption in the KO mice. (E) Weight of individual implantation sites was compared between WT and KO mice ($n=3$ in each group) on D10. Arrows indicate the degenerating embryos (5 out of 16) in the KO.

implantation sites on day 10 revealed similar results with signs of degenerating embryos in KO mice (shown by arrows; Fig. 3E). This is clearly a significant reproductive phenotype considering female reproduction is of such a major biological system, albeit cyclin D3$^{-/-}$ females are not totally infertile. For example, there are some implantation sites that appeared to be normally developed in the cyclin D3$^{-/-}$ mice, as examined on day 10 of pregnancy (Fig. 3E). Although, we do not know the exact cause of why certain sites in the mutant uterus can accommodate embryos while others cannot, this is not an uncommon finding. There is ample evidence that many gene-deleted mice show reduced litter sizes, but not complete failure of pregnancy. In this regard, our preliminary studies show that cyclin D2 compensates for the loss of cyclin D3 at the site of implantation during decidualization, which is indeed interesting (L Ping & SK Das, unpublished observation). In this regard, it is worth mentioning that D-type cyclins possess functional redundancy in different biological systems in mice (Lam *et al*. 2000, Chen & Pollard 2003). It is thus reasonable to speculate that the results obtained from double KO mice for cyclin D3 plus cyclin D2 might address this issue, and we have already initiated development of these mice.

Although the above findings suggest that CDKN1A is important for the onset of stromal cell differentiation, the studies utilizing the CDKN1A$^{-/-}$ mice demonstrate that embryo development and implantation are apparently normal (Deng *et al*. 1995). Consistent with this notion, our analyses of decidual progression in CDKN1A null females revealed similar decidual growth and development and stromal cell polyploidy, when compared with that of wild-type littermates, suggesting that a compensatory mechanism might be involved in decidualization under the condition of CDKN1A deficiency. Indeed, our recent findings have demonstrated that CDKN1A deficiency leads to the compensatory upregulation of P_4 receptor-B (PGR) isoform specifically during the progression of decidualization (Ping *et al*. 2008). Studies further revealed that the double KO females for CDKN1A and PGR-B were infertile and the defects primarily associated with the uterine decidualization.

In addition to the D-type cyclins, cyclin Gs also appear to play roles in uterine decidualization (Yue *et al*. 2005). Our studies show that cyclins G1 and G2 are differentially expressed in the peri-implantation uterus under the direction of P_4. In brief, in the preimplantation uterus, cyclin G1, but not cyclin G2, is upregulated in the uterine epithelial cells on days 3 and 4 of pregnancy. However, with the onset of implantation notably on day 5, cyclin G2, but not cyclin G1, distinctly localized in the luminal epithelium and PDZ at the site of the implanting blastocyst toward the antimesometrial pole. By contrast, on days 6 and 7 of pregnancy, the expression of cyclin G1 is primarily localized at the SDZ and mesometrial decidual bed, while that of cyclin G2 is robustly detected in the PDZ. Overall, the results suggest that cyclin G1 is primarily associated with uterine epithelial cell differentiation prior to implantation. However, following the onset of implantation, the expression of cyclin G1 is primarily associated with stromal cell differentiation during decidualization, while that of cyclin G2 is consistent with the progression of terminal differentiation and apoptosis of the luminal epithelial and PDZ cells at the site of implanting blastocyst. The generation of cyclin G1 null mice has been reported (Kimura *et al*. 2001, Jensen *et al*. 2003); these mice survive to adulthood and display reduced tumor susceptibility due to increased tumor-suppressive function of TP53. In this regard, it is interesting to note that TP53 is also detected in a similar fashion like that of cyclin G1 during decidualization on days 6–8 of pregnancy in mice (Yue *et al*. 2005). However, reproductive phenotypes in these null mice have not yet been reported and we are currently exploring this possibility. To the author's knowledge, no report of cyclin G2 deletion in mice is available.

Upstream regulators for the control of uterine cyclin D3

Molecular signaling by HBEGF

Heparin-binding EGF-like growth factor (HBEGF) is a member of the epidermal growth factor (EGF) family and it binds with EGF receptor (EGFR) and ERBB4 for their activation (Elenius *et al*. 1997, Lim & Dey 2009). HBEGF has been implicated in several physiological and pathological processes in mice, including the blastocyst implantation, lung development, heart valve development, eyelid closure, heart muscle homeostasis, skin wound healing, skin hyperplasia, and tumorigenesis (Das *et al*. 1994, Raab *et al*. 1996, Nishi & Klagsbrun 2004, Xie *et al*. 2007).

HBEGF is synthesized as a type I transmembrane protein (proHBEGF). ProHBEGF can function as a biologically active molecule through juxtacrine signaling to the neighboring cells. ProHBEGF is cleaved at its juxtamembrane domain by metalloproteases, in a process called ectodomain shedding. This process yields soluble ectodomain of HBEGF (sHBEGF) and a carboxy (C)-terminal fragment (HBEGF-C). sHBEGF is a potent mitogen and chemoattractant for cells expressing its cognate ERBB receptor. Mutant mice expressing an uncleavable form indicate that the major functions of HBEGF in the biological systems are mediated by sHBEGF (Yamazaki *et al*. 2003). HBEGF-C is also considered as a signaling molecule; following the shedding, it undergoes phosphorylation and nuclear translocation, where it binds to and regulates several nuclear factors (Nanba *et al*. 2003, Wang *et al*. 2006*b*). HBEGF has a high affinity for heparin and heparan sulfate proteoglycan, and they are considered as important regulators of HBEGF functions (Higashiyama *et al*. 1993).

Recent genetic and molecular biology approaches in mice have identified many growth factors, cytokines, homeotic factors, transcription factors, lipid mediators, and morphogens that are crucial to implantation (Dey *et al*. 2004, Wang & Dey 2006). Among these, HBEGF is particularly interesting because of its expression pattern in the uterus and its paracrine and juxtacrine interactions with the embryonic ERBBs during implantation in mice and humans (Das *et al*. 1994, Raab *et al*. 1996, Martin *et al*. 1998, Paria *et al*. 1999*b*). In mice, HBEGF is first expressed in the uterine luminal epithelium at the site of the blastocyst apposition 6–7 h before the attachment reaction, and persists through day 5 (Das *et al*. 1994). However, on day 6, HBEGF becomes more wide spread in the decidualizing stroma (Fig. 4) and persists through days 7 and 8. Furthermore, studies have shown that gelatin beads pre-soaked in HBEGF, but not by EGF or transforming growth factor-α, elicit implantation-like responses when transferred into the uterine lumen in pseudopregnancy (Paria *et al*. 2001). HBEGF promotes embryonic growth via EGFR and/or ERBB4 expressed on the blastocyst cell surface (Dey *et al*. 2004). The ligand–receptor signaling mediated by HBEGF is also operative in the receptive human uterus and promotes growth of IVF-derived embryos (Yoo *et al*. 1997, Martin *et al*. 1998, Leach *et al*. 1999, Chobotova *et al*. 2002, Dey *et al*. 2004). Moreover, HBEGF has been implicated in two-way signaling between the uterus and blastocyst during implantation (Wang *et al*. 2002). Collectively, results suggest that HBEGF plays an important role in embryo development and implantation in various species including humans.

Because beads pre-soaked in HBEGF induced implantation-like responses, we also analyzed whether this growth factor directly influences cyclin D3 in association with uterine decidualization events by both *in vitro* and *in vivo* studies (Tan *et al*. 2004). Utilizing the primary culture of uterine stromal cells, we indeed showed that HBEGF is specifically capable of inducing expression of cyclin D3 in conjunction with the increase in cellular growth, polyploidy, and binucleation (Tan *et al*. 2004). Furthermore, we also showed that uterine delivery of HBEGF via beads also augments stromal cell decidualization and polyploidy in association with an overexpression of cyclin D3. To further explore the role of HBEGF on uterine stromal cell decidualization events via cyclin D3, we used the adenoviral gene delivery system to suppress the expression of cyclin D3 in both *in vitro* and *in vivo* studies. Indeed, our studies strongly revealed that suppression of cyclin D3 abrogates HBEGF-driven stromal cell decidualization and polyploidy (Tan *et al*. 2004). Collectively, these results suggest that HBEGF directs stromal cell polyploidy and decidualization via cyclin D3 during implantation.

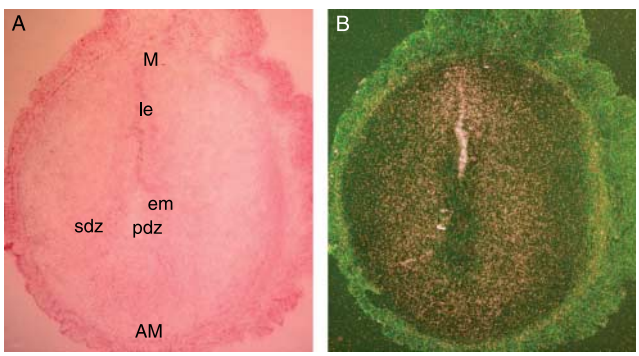

Figure 4 *In situ* hybridization of HBEGF at the implantation sites on day 6 of pregnancy in wild-type mice. (A) Bright- and (B) dark-field photomicrographs of a representative cross section are shown at 40×. M, mesometrial pole; AM, anti-mesometrial pole; e, embryo; pdz, primary decidual zone; sdz, secondary decidual zone; le, luminal epithelium.

Two groups independently generated HBEGF$^{-/-}$ mice (Iwamoto *et al*. 2003, Jackson *et al*. 2003). We have obtained the line generated by the Japanese group (Iwamoto *et al*. 2003) and these mice are being studied and maintained in our facility. Using these mice in our recently reported studies (Xie *et al*. 2007), we observed significantly reduced litter size in HBEGF null females as compared with that in wild-type littermates, suggesting that HBEGF influences normal reproductive outcome. In addition, studies further show that HBEGF$^{-/-}$ females display deferral of on-time implantation after mating with fertile males. More importantly, analysis of the weight of implantation sites in those HBEGF$^{-/-}$ mice showing implantation on day 8 suggests compromised decidualization when compared with wild-type littermates (L Ping & SK Das, unpublished observation). These results are consistent with the observation of signs of embryo resorption at the site of implantation (L Ping & SK Das, unpublished observation). Collectively, the results suggest that defective attachment reaction and the subsequent onset of decidualization are deranged in the absence of HBEGF, suggesting its critical role in these processes.

Molecular signaling by Hoxa10

Hoxa10 is a member of the homeobox or Hox multigene family of transcription factors (Satokata *et al*. 1995). This gene is highly expressed in the uterine stroma in mice and humans during the receptive phase. The expression pattern follows the wave of proliferation and decidualization in the stroma and is controlled by P_4 in a PGR-dependent fashion (Ma *et al*. 1998, Taylor *et al*. 1998, Lim *et al*. 1999, Daikoku *et al*. 2004). Gene KO studies in mice have shown that Hoxa10-deficiency leads to female infertility, and defective decidualization is the primary reason for this cause (Benson *et al*. 1996). A small percentage (∼40%) of Hoxa10 mutant mice can initiate the implantation reaction (Benson *et al*. 1996), suggesting that uterine defects in these mice do not completely antagonize functions of luminal epithelial cells for blastocyst attachment. In this respect, the expressions of several genes (i.e. *Lif*, *Hbegf*,

amphiregulin and *Cox1*) in the receptive uterine epithelium are not affected before implantation, while the expression of P_4 responsive genes *Ptger3* and *Ptger4*, but not *Pgr*, are affected in stromal cells (Lim *et al.* 1999). While stromal cell proliferation is depressed in Hoxa10 mutant mice in response to P_4 and estradiol-17β (E_2), epithelial cell proliferation is normal in response to E_2.

Since Hox genes are implicated in cell proliferation and differentiation (Duboule 1995, Thorsteinsdottir *et al.* 1997), cell cycle regulation is likely to be involved. Indeed, studies have shown that cyclin D3 expression remains downregulated in Hoxa10$^{-/-}$ uteri during the progression of decidualization (Das *et al.* 1999, Tan *et al.* 2004). Furthermore, it has been shown that Hoxa10 deficiency causes loss of pole-specific expression for CDK4 and CDK6 in the mesometrial and antimesometrial regions of the decidual bed respectively (Tan *et al.* 2004). In addition, based on microarray studies for the comparison of decidualization in Hoxa10$^{-/-}$ mice, as compared with that of wild-type littermates, revealed alteration of numerous genes that are involved in cellular proliferation and differentiation (Rahman *et al.* 2006). Interestingly, many of these genes such as growth differentiation factor 10 (*Gdf10*), hepatocyte growth factor (*Hgf*), *Snail2* and several granzymes are shown to be aberrant in respect of their region-specific distribution in the decidual bed for the Hoxa10$^{-/-}$ mice as compared with that of wild-type mice, suggesting Hoxa10 deficiency alters region-specific decidual development during the post-implantation period. In this regard, it is worth mentioning that uterine NK cells are believed to function to appropriately regulate and establish the maternal–fetal barrier at the implantation site (Croy *et al.* 1996, Moffett-King 2002, Dosiou & Giudice 2005). Interestingly, our recent studies have also provided strong novel evidence that *Hoxa10* deficiency causes dysregulation of uterine NK cell differentiation, but the uterine recruitment of precursor NK cells appears to be unaffected (Rahman *et al.* 2006).

Overall, these results suggest that impaired stromal cell proliferation is a potential cause for defective decidual response in Hoxa10 mutant mice. Furthermore, Hoxa10 as a transcriptional factor may convey P_4-dependent responsiveness in the uterine stroma by regulating local gene expression. Indeed, a recent study using microarray analysis of P_4-responsiveness in uteri of ovariectomized Hoxa10 mutant mice has shown that many genes, including the cell cycle inhibitory genes *Cdkn2b* and *Cdkn1c*, are upregulated (Yao *et al.* 2003). Our recent results also have shown that negative cell cycle regulators, cyclins G1 and G2, are upregulated in Hoxa10$^{-/-}$ uteri (Yue *et al.* 2005), suggesting that cyclin Gs negatively impacts cell proliferation. While it is known that increased stromal cell proliferation, but not the apoptosis, is considered an initiator of decidualization, establishment of a full-grown functional decidualization depends on both proliferation and differentiation. Overall, these results suggest that aberrant activities of the cell cycle regulatory molecules enforce inhibition of stromal cell proliferation in the uteri of Hoxa10 mutant mice.

Other signaling mediators

Stathmin is a cytosolic phosphoprotein involved in the regulation of microtubule dynamics during the cell cycle progression. Studies have shown that the stathmin gene is expressed in the antimesometrial decidual bed of the implantation site in mice (Yoshie *et al.* 2006). Mice deficient in stathmin show reduction of cyclin D3 expression in the decidual bed and reduced fertility, as compared with that of wild-type littermate females. Furthermore, it is well established that uterine decidualization involves interleukin 11 (IL11) and its receptor IL11 receptor-α (IL11RA) mediated signaling in mice (Bilinski *et al.* 1998, Robb *et al.* 1998). In this regard, recent studies have also provided evidence that IL11/IL11RA signaling utilizes cyclin D3 and CDKN1A in conjunction with BIRC5 (Survivin) as downstream targets during the developmental progression of decidualization (Li *et al.* 2008). In addition, basic transcription element-binding protein-1 (KLF9), a member of the Sp/Krüppel-like family of transcription factors, functionally interacts with PGR isoforms (PGR-A and PGR-B) and is

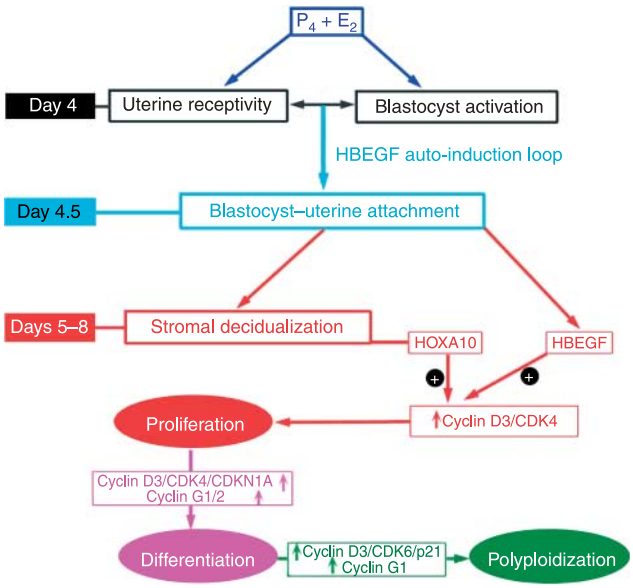

Figure 5 A scheme depicting potential roles of HBEGF, cyclin D3, Hoxa10, and cyclin Gs at various stages of implantation. We speculate that Hoxa10 directs in an opposing manner two contrasting classes of cell cycle regulators to critically balance stromal cell proliferation and differentiation for optimal decidualization; a tipping of this balance will lead to defective decidualization. Hoxa10 and HBEGF signaling pathways converge to a downstream cell cycle regulatory circuitry consisting of cyclin D3 and cyclin Gs to appropriately balance proliferation and differentiation for a full complement of decidualization.

expressed in decidual cells at the site of implantation in a PGR-A-dependent fashion (Simmen *et al*. 2004). In this regard, it is interesting to note that the expression of cyclin D3 and Hoxa10 was downregulated in *Bteb1*-null mutants during decidualization (Simmen *et al*. 2004).

Working model

Based on the above discussions, a working model for implantation and decidualization is presented in Fig. 5. This model proposes potential roles of Hoxa10, HBEGF, cyclin D3, and cyclins G1 and G2 at various stages of implantation, including stromal cell proliferation, differentiation, and polyploidy. These regulators are expressed in the uterus either prior to or during decidualization. We speculate that Hoxa10 and HBEGF mediated signaling pathways working independently, but in parallel, to appropriately balance the growth promoting and growth inhibitory activities to support the full complement of decidualization. However, this model should not preclude in any way the roles of other signaling molecules that may control cell cycle regulatory activities during stromal cell decidualization.

Concluding remarks

Despite significant developments in IVF technology, the pregnancy success rate remains low because of higher incidence of implantation failure and unexplained pregnancy loss. Indeed, failure of events during preimplantation embryo development, implantation, and placentation are major determinants for pregnancy loss. Basic research to better understand these events will alleviate problems of infertility. In this respect, a better understanding of molecular mechanisms that participate during implantation/decidualization is critical to ensure healthy pregnancy outcome. The process of decidualization is common to both mice and humans, although stromal cells in the human uterus undergo decidualization during the receptive phase (secretory phase) of each menstrual cycle in the absence of embryo. Since both HBEGF and Hoxa10 are expressed in the receptive human uterus and since decidualization occurs during this period in the absence of the embryo, these two molecules are very attractive candidates for in-depth investigation. It is not possible to use humans to study embryo–uterine interactions during implantation to explore the underlying mechanism(s). Mouse models serve to provide mechanistic approaches in defining the molecular basis of implantation/decidualization. Physiological functions of specific factors can be examined more mechanistically by overexpression, silencing, or targeted deletion of their genes. No doubt, the research using mice as an animal model will provide information that will be valuable to better understand the intricacies of human pregnancy.

Declaration of interest

The author declares that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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