



Published in final edited form as:

Semin Cell Dev Biol. 2008 April ; 19(2): 194–203.

The Hamster as a Model for Embryo Implantation:

Insights into a Multifaceted Process

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Abstract

Defects in preimplantation embryonic development, uterine receptivity, and implantation are the leading cause of infertility, pregnancy problems and birth defects. Significant progress has been made in our basic understanding of these processes using the mouse model, where implantation is ovarian estrogen-dependent in the presence of progesterone. However, an animal model where implantation is progesterone-dependent must also be studied to gain a full understanding of the embryo and uterine events that are required for implantation. In this regard, the hamster is a useful model and this review summarizes the information currently available regarding mechanisms involved in synchronous preimplantation embryo and uterine development for implantation in this species.

Keywords

Hamster; Mouse; Embryo; Uterus; Implantation

Introduction: pathways to implantation

The implantation process is dependent on precisely orchestrated steps that cannot be hurried. Although the details of implantation types and events vary among species, it is generally accepted that both the uterus and the embryo undergo synchronized restructuring and maturation processes to attain implantation competency. As a result of embryo-uterine interaction, the mother recognizes her pregnancy and triggers host immune-surveillance to accommodate the fetal allograft. Thus, implantation is possibly the most eventful stage of pregnancy and the most vulnerable point for pregnancy failure. This review provides a brief overview of the mechanisms of embryo implantation in various animal models as well as recent information suggesting that the hamster is a valuable model for dissection of implantation-specific mechanisms.

Early embryo development initiates the journey to implantation

Following the union of male and female gametes (fertilization) at the ampullary segment of the oviduct, a diploid zygote is formed. The one-cell zygote then divides mitotically to 2-cell, 4-cell, 8-cell, morula and blastocyst stages as they pass through the rest of the ampulla and isthmus to reach the uterus. While the 8-cell stage embryo enters the uterus in hamsters by 62

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h post coitus [1], the late morula or early blastocyst stage enters the uterus at about 72 post coitus in mice [2] Although blastocyst formation (cavitation) occurs within the uterus in most species, blastocyst formation in hamsters occurs after the 4th cleavage (16-cell stage) compared to 5th cleavage (32-cell stage) in mice.

During blastocyst formation, the outer layer of polar trophectoderm (TE) surrounds a small cluster of nonpolar inner cell mass (ICM) cells alongside the blastocoel cavity. Cells in the mouse embryo can differ in their developmental fate and potential as early as the four-cell stage. As the 4-cell embryos divide further, a gradual increase in intercellular adhesion occurs and the embryos become compacted to form the morula possibly due to junction formation between some of the blastomeres. E-cadherin and zonula occludens-1 (ZO-1) first start to assemble at cell contact sites in the 8-cell stage embryos [3;4]. However, embryos lacking either its own or maternal E-cadherin undergo compaction. Thus, the definite role of E-cadherin in polarization of blastomeres is still not clear. The role of ZO-1 and ZO-2 in blastomere polarization is also unclear since these genes have not been mutated in embryos. The role of junctional proteins in morula-to-blastocyst transformation and the mechanism of fluid accumulation inside the hamster embryo are yet to be elucidated. However, our preliminary results suggest the presence of E-cadherin and F-actin proteins in the cell surface of hamster embryos at the morula and blastocyst stages (Fig. 1).

Blastocyst competency: Embryonic preparation for implantation

After blastocyst formation, escape (hatching) from the zona pellucida is the next pivotal step towards achieving implantation competency. Studies have suggested that *in vitro* grown blastocysts hatch zona pellucida either by exerting pressure due to their expansion or by secretion of a trypsin-like protease from the trophectoderm. Several investigators have also proposed that zona hatching *in vivo* involves either uterine zona lysin or lysin from both the embryo and uterus [rev. in: 5]. Methods for zona escape in hamster, however, are different from other species. Gonzales and Bavister [5] demonstrated that blastocysts *in vitro* escape from the zona pellucida through a hole at the site of trophectodermal projections. Hamster blastocysts *in vivo* do not undergo expansion and they hatch by gradual zona thinning and complete dissolution [5;6]. Mishra and Seshagiri [7] demonstrated that possibly embryonic, but not uterine, lysin is involved in lysis of zona pellucida in hamsters since 1-8 cell stage embryos from hamsters, but not mice, rats, sheep, or humans, shed their zona pellucida when cultured with zona-escaping blastocysts.

Implantation

Ovarian progesterone (P₄) is required for uterine receptivity, implantation, and maintenance of early pregnancy in all mammals. However, while in mice [8;9] and rats [10] both maternal P₄ and estrogen are critical to implantation, in the majority of species including hamsters [11-13], guinea pigs [14], rabbits [15], and pigs [16] implantation can occur in the presence of P₄ alone; ovarian estrogen is not necessary. Furthermore, observations suggest that uterine receptivity as defined by surface morphology occurs primarily with P₄ in the human and rhesus monkey uterus. There are also reports that luteal phase estrogen is not a requirement for establishment of pregnancy in rhesus monkeys and women [17;18]. In some of these P₄-dependent species, estrogen may be required for implantation, but the source of this nidatory estrogen may be the blastocyst. In this regard, it has been demonstrated that the blastocyst of rabbits [15], pigs [16], hamsters [19] and humans [20] may be capable of producing estrogen. We also show here the presence of aromatase protein in the hamster blastocyst (Fig. 2).

Embryo implantation in hamsters takes place late in the morning of day 4 of pregnancy and blastocysts can no longer be flushed from the uterus. However, apparent increase in vascular permeability at the implantation site after an intravenous injection of blue dye is not clearly

observed until the evening (1600-1800 h) of day 4 (Fig. 3), suggesting that permeability change at the site of implantation takes longer in hamsters than mice. Implantation is induced in ovariectomized-adrenalectomized hamsters with P₄ alone; exogenous estradiol-17 β is not a requirement [12]. However, there are also profound differences in the response of rats and hamsters to exogenous estrogens or progestins. Estradiol-17 β and diethylstilbesterol are only 4% to 5% as active in the hamster as uterotrophic or contraceptive compounds compared with the rat. This correlates with significantly higher levels of circulating estrogen in pregnant hamsters than pregnant rats [13]. In contrast, the hamster is exquisitely sensitive to small amounts of P₄ compared to rats and mice. While hamsters ovariectomized on day 2 of pregnancy need only 62 μ g of daily injection of P₄ to initiate implantation [11], mice ovariectomized on day 3 and 4 of pregnancy need at least 1-3 mg of daily P₄ when superimposed with 10-25 ng of estradiol-17 β [8;21]. Thus, P₄ is the most sensitive steroid in hamsters, and its circulating levels steadily increase from day 1 to 14 of pregnancy. During early pregnancy in the hamster, ovarian venous plasma P₄ levels were fivefold lower than in the rat [13]. Similarly, venous estrogen concentration also increases slowly during early pregnancy in hamsters and its concentration in ovarian venous effluent on day 4 of pregnancy is approximately 20-fold greater than in the rat [22].

Uterine morphological changes in preparation for implantation

Specialized protrusions on the apical surface of uterine epithelial cells, termed pinopodes or uterodomes, appear in mice, humans and other species at the time of implantation [23]. The appearance of pinopodes is also associated with expression of implantation-specific factors including *Lif*, HB-EGF, the homeobox gene *Hoxa-10*, and α v β 3 integrin, and the mid-luteal increase in P₄ levels in women. The function of pinopodes remains unclear, but when they appear, uterine epithelial apical microvilli begin to retract providing a smoothed endometrial surface for embryo-uterine interaction [24]. In contrast to mice and rats, where the epithelial surface becomes flattened and microvilli are no longer visible, epithelial microvilli never completely disappear during implantation in hamsters, but remain until phagocytosed by the trophoblast [6;25]. Beyond pinopode formation and microvilli retraction, the uterine lumen of most of species is lined with glycoproteins such as integrins, selectins, galectins, heparan sulfate proteoglycans (HSPGs), the extracellular matrix molecules osteopontin and laminin, and the trophinin-tastin-bystin complex [26] that can act as cell surface adhesion molecules to position the blastocyst over the implantation site. Mucins are another component of the uterine glycocalyx that function primarily in an anti-adhesive role. MUC-1 is the best-studied member of the reproductive tract mucin family, and is present in the apical border during the pre-receptive state but reduced or absent during receptivity in most species [27]. However, it has been shown that uterine MUC1 persists in women and both MUC-1 mRNA and protein showed several fold increase from the proliferative to the mid-secretory phase in humans [28]. This expression may inhibit the blastocyst attachment process until shedding via interaction with local factors or removal at specific sites such as pinopodes. Similar to women, we observed very little or no reduction of MUC-1 from the uterine epithelium of hamsters (Fig. 4). Furthermore, we observed strong accumulation of MUC-1 at the antimesometrial embryo-uterine contact site on day 5 of pregnancy (Fig. 4). Thus further research is necessary to better understand the precise role of MUC-1 in hamster and human implantation.

Morphological Types of Implantation

Based on the type of blastocyst-uterine interaction, implantation is classified as eccentric in rats, mice and hamsters where blastocysts are small and become lodged in a uterine fold [24]. Initiation of implantation is generally considered a three-phase process that includes blastocyst apposition, adhesion and trophoblast penetration. While the majority of uterine epithelial cells remain normal and intact until invasion by the trophoblast in hamsters, autolysis of uterine epithelial cells precede the invasion of the trophoblast in mice [24]. Ultrastructural studies

examining implantation in mice and rats indicated that some epithelial cells become dissociated from their underlying basal lamina before trophoblast penetration. The hamster differs from mice and rats in that trophoblast processes penetrate directly into epithelial cells, phagocytosing portions of these cells. Thus, it appears that trophoblast of hamsters is more invasive than that of mouse and rat [25]. The formation of microfilaments along the apical surface of the trophoblast helps to form the trophoblastic processes which extend, invade and engulf the epithelium.

Factors involved in implantation

Molecular mediators that govern the implantation process operate within a complex cellular and hormonal milieu. Factors that are expressed within a narrow temporal window are spatially restricted to the implantation site, and are either hormone- or embryo-responsive suggesting their involvement in implantation. In addition, the ability to pharmacologically or genetically perturb their function has led to the identification of genes and pathways that are critical for various implantation events. Emerging information on factors that regulate implantation in the hamster has begun to shed light on the value of this model for implantation studies.

Cytokines—The diverse functions of cytokine family members in proliferation, differentiation, inflammation and immunomodulation imply a potential contribution to uterine preparation, implantation or decidualization. Numerous human and animal studies support roles for interleukin-1 (IL-1), IL-6, IL-15, SOCS3, TNF α , Lif, GM-CSF and CSF-1 in uterine preparation, implantation or decidualization [29;30]. Although gene deletion studies in mice fail to illicit an implantation-specific phenotype for many of these factors, the possibility of redundancy or compensatory function by other family members may mask their contribution. However, signaling of two members of the IL-6 family, Lif and IL-11, appear to be critical for implantation and decidualization [31;32]. Lif is implicated in uterine cyclicity, endometrial function, and the human implantation process and has similar roles in other species. However, the mechanism and definitive site of Lif actions in the uterus remains elusive. *Lif* mRNA and protein expression in hamsters is variable during days 1-6 of pregnancy [33]. Our unpublished results suggest that *Lif* expression is significantly higher on days 1 and 4 of pregnancy. On day 4, Lif is mainly localized in glandular epithelial cells, although low levels were also observed in luminal epithelial cells. While *Lif-r* expression is only observed in luminal epithelium, *gp130* expression was observed in both the luminal and glandular epithelium on day 4 of pregnancy. As the implantation process proceeds from days 5 to 8 of pregnancy, *Lif* expression ceased, but *Lif-r* and *gp130* extended to decidual cells. We also observed that while *Lif* was induced by estrogen, *Lif-r* and *gp130* were induced by P₄ in the ovariectomized hamster uterus [34]. Lif enhances human embryo development [35] and hamster blastocyst hatching *in vitro* [33]. In addition, we have shown that an intraluminal injection of Lif antibody significantly reduces blastocyst implantation sites in hamsters. Taken together, these studies suggest that Lif signaling is also important for blastocyst hatching and implantation in hamsters.

Growth factors—Cell-specific alterations in differentiation, proliferation, and cell turnover are mediated in part by growth factors acting through endocrine, paracrine, or autocrine mechanisms. The knockout phenotype of members of the TGF- β superfamily and their associated intracellular signaling molecules emphasize the importance of this family in decidualization, placentation, and host immune response. In this regard, a recent study showed that mice with conditional ablation of BMP2 from the uterus are infertile due to failure of stromal cell decidualization [36]. However, inhibin-induced inhibition of implantation is attributed to inhibition of estrogen or P₄ production in mice and hamsters, respectively [37; 38]. The insulin-like family of growth factors (IGFs) and their binding proteins are also involved in implantation but may be more important for decidualization and placentation

[39], and have significant impact on the establishment of pregnancy and fetoplacental growth in women, non-human primates, domestic animals, and most laboratory species [40].

Members of the EGF family of growth factors also play a significant role in embryo-uterine signaling during implantation. In mice, TGF α , HB-EGF, amphiregulin (Ar), betacellulin (BTC), epiregulin (Er), and several neuregulins are expressed in the peri-implantation uterus and regulated by ovarian hormones [41]. The most compelling evidence for implantation-specific roles of the EGF family comes from HB-EGF. HB-EGF is expressed in the mouse uterus prior to the onset of embryo attachment in response to an undefined embryonic stimulus [42]. Soluble HB-EGF, in turn, stimulates proliferation, zona-hatching, trophoblast outgrowth and phosphorylation of target receptors in the blastocyst of mice, human and hamsters [33; 42;43]. HB-EGF is expressed in human endometrium during the peri-implantation period [44;45] and fluctuates in response to cycle stage and ovarian steroids [46;47]. HB-EGF is also expressed on the surface of pinopodes and is able to stimulate other biomarkers of uterine receptivity in women [47;48]. The actions of HB-EGF are mediated by erbB1 and erbB4, which are expressed in both the uterus and embryo [42;49-53]. A recent study also showed that HB-EGF may improve implantation by stimulating expression of integrin $\alpha\beta 3$ in preimplantation mouse embryos [54]. In hamsters, HB-EGF is produced by both the blastocyst and uterus and its expression is not observed in uterine decidual cells after initiation of implantation [33;53; 55]. Expression of amphiregulin, on the other hand, is quite different in hamsters and mice. It is not expressed in the uterine epithelium around the time of implantation and is also not regulated by P₄. Instead, it is primarily expressed in decidual cells adjacent to the implanted embryo from day 5 to day 8 of pregnancy [56]. While HB-EGF induces stromal cell proliferation and differentiation in mice [57], amphiregulin induces stromal cell proliferation in hamsters [56]. Taken together, it appears that HB-EGF and amphiregulin have species-specific actions in hamsters, and that a better understanding of these differences may give broader insight into the roles these growth factors play in the implantation process.

Vasoactive mediators—The presence of increased vascular permeability at the site of implantation [58] is a common feature among diverse species [59]. Numerous vasoactive substances are present at the implantation site, including platelet activating factor (PAF), various kinins, histamine, vascular endothelial growth factor (VEGF), angiopoietins, nitric oxide, prostaglandins and other eicosanoids, as well as direct hormone-induced changes in vascular architecture [60]. Histamine and prostaglandins have long been considered as important mediators of embryo-uterine interactions. Normal implantation in mast cell deficient mice [61] and virtual absence of mast cells from the mouse endometrium and deciduum [62] argue against the role of mast cell-derived histamine in implantation. Histidine decarboxylase (HDC) is the rate-limiting enzyme in histamine formation and it is a P₄-responsive epithelial gene in the mouse uterus [63]. In contrast to mice, the expression of HDC in hamster uterus occurs only on day 4 of pregnancy in the glandular epithelium. Its expression in the hamster uterus is not regulated by P₄ or estrogen. Furthermore, its expression in the day 4 uterus requires the presence of embryos, suggesting influence of the embryo in uterine preparation for implantation [56]. It is possible that local uterine histamine may be required for uterine relaxation [64] in hamsters to provide ample time and uterine surface for blastocyst-uterine interaction and attachment for implantation.

Prostaglandins are proinflammatory mediators derived from membrane-associated lipid precursors via phospholipase (cPLA₂) and the Cox-1 and Cox-2 enzymes. Impaired fertility following treatment with inhibitors of prostaglandin synthesis first suggested the importance of prostaglandins during implantation in multiple species including hamsters [65-68]. Deletion of cPLA₂ alters the timing and orderly spacing of implantation, whereas Cox-2 deletion results in impaired ovulation, fertilization, implantation, and decidualization [69;70]. Evidence indicates that Cox-2-derived prostacyclin (PGI₂) is important for implantation in mice [71].

Cox-1 deleted mice have no implantation problem, although Cox-1 assists in uterine preparation for implantation, and Cox-1 and Cox-2 can compensate for the absence of the opposite isoform to sustain the implantation process [72;73]. Other lipid-derived vasoactive mediators, including lipoxigenase products [74;75] and lysophospholipids [76;77] appear to have significant roles in implantation. Both the Cox-1 and Cox-2 enzymes are expressed in the hamster preimplantation uterus in a pattern similar to mice [68]. A reduction of Cox-1 expression from uterine epithelial cells and a gradual induction of Cox-2 expression exclusively in luminal epithelium and adjacent decidual cells occurred at the implantation site of the hamsters. Most interestingly, however, Cox-2 expression is mainly observed in decidual cells immediately surrounding the implantation chamber on day 6. In contrast to mice, PGE₂, but not PGI₂, is the preferential product of cyclooxygenases in hamsters [68]. Because PGE₂ is a potent inflammatory prostaglandin, our finding suggests that this lipid mediator is involved in local implantation-associated changes in the uterus at the site of implantation in hamsters.

Morphogens and developmental patterning genes—Efforts to better understand the implantation process led to the identification of genes with recognized roles in early embryo development and body plan organization, including the HOX genes, a family of developmental stage-specific transcription factors. Mice lacking *Hoxa10* have altered segmental identity of the utero-tubal region, implantation and decidualization failure, and impaired endometrial responses to P₄ [78-81]. HOXA11 null mice are also infertile, with reduced endometrial development and diminished LIF production [82;83]. Human studies reveal similarly important functions for HOXA10 and HOXA11 in embryo development, uterine differentiation, and fertility [84]. Wnt genes including Wnt2, Wnt4, Wnt5a, Wnt7a and others, also appear to have important functions in the implantation process across species [85-88].

Among several of the Hedgehog (hh) family of morphogens (Sonic hh, Desert hh, and Indian hh), *Ihh* is the sole member expressed in the peri-implantation mouse uterus [89;90], and is regulated by P₄ or the presence of blastocysts or growth factor-saturated beads [90;91]. Global deletion of *Ihh* results in mice with dwarfism and perinatal lethality, whereas mice with conditional *Ihh* deletion are infertile due to implantation and decidualization failure [92]. Similarly, conditional deletion of COUP-TFII, an epithelial *Ihh*-regulated gene, was found to cause implantation failure [93]. These findings suggest that P₄ action via induction of epithelial *Ihh* production plays an essential role in induction of implantation in mice. *Ihh* is also a P₄-regulated gene in hamsters that is critical for uterine preparation for implantation. Its expression during early pregnancy closely follows the pattern observed in mice [56].

Hamsters as preferred models for implantation studies

The Syrian or golden hamster, *Mesocricetus auratus*, was once widely used in animal research because of their tolerance to tissue transplants as well as human tumors, viruses and bacteria. Although the relative use of hamsters in research has declined over the years, they are uniquely suited for reproductive studies. Female hamsters are polyestrous, and their sexual maturity begins around 4 weeks of age [94]. In mice, both uterine horns open in a common vagina [95], but a dual vagina is present in rats, rabbits, guinea pigs and hamsters [96]. While vaginal cytology shows regular 4 day estrous cycles (estrus, metestrus, diestrus and proestrus) in hamsters, the average individual cycle length in mice lasts 4-5 days, but this is highly variable and easily influenced by environment [97]. In hamsters, the day of estrus is easily predictable by external copious vaginal discharge that is not observed in mice. In addition, a female's receptivity for mating in laboratory mice is not limited to estrus but may occasionally take place during proestrus or metestrus. Among all eutherian mammals, the gestation period of golden hamsters lasts only 16 days [98] while in mice it lasts for 19-21 days [97]. Ovulation time is highly predictable in hamsters and the female shows rapid development and regression of corpora lutea during a single cycle, unlike the mouse and rat where there is retention of

several sets of corpora lutea from previous cycles [99]. Like mice, superovulation can be performed in hamsters by exogenous gonadotrophins. Although preimplantation embryos of mice, humans and hamsters can be cultured *in vitro*, hamster embryos are extremely sensitive to culture conditions [33]. Thus, hamster embryos can be used to test media components to improve the quality of the blastocyst. Early cleavage of hamster embryos *in vivo* is very similar to mice but hamster embryos enter into the uterus at the 8-cell stage on day 3 of pregnancy approximately 60 h after fertilization [53], while in mice, embryos enter into the uterus at the late morula and early blastocyst stage early on the morning of day 4 approximately 72 h after fertilization [2]. Although blastocyst formation mostly occurs inside the uterus in all mammals, unlike mice where blastocysts undergo expansion before implantation, blastocysts of hamsters do not undergo expansion *in vivo* [5]. While in hamsters initiation of implantation begins around late morning of day 4 [100], implantation begins in mice around midnight of day 4 or early morning on day 5 [42]. In addition, early implanted embryos cannot be flushed out from the uterus of hamsters suggesting firm trophoblast attachment and invasion compared to those of mice and rats. Pseudopregnant and pregnant hamsters also undergo stromal cell decidualization in response to artificial stimuli or implanting blastocysts like mice and rats [101]. Finally, hamsters might be considered as the species of choice for early pregnancy research considering the fact that implantation in this species is absolutely dependent on ovarian P₄, but not estrogen, similar to rabbits, guinea pigs, agricultural animals, primates, and possibly humans. Thus, important new information might be generated studying the mechanisms of embryo development and implantation using the hamster model.

Where do we go from here?

Finding the means to bridge and extrapolate data from animal implantation models to the setting of human implantation is a daunting challenge. Implantation is not only a multiphase but also a species-specific event. Use of morphological, pharmacological, biochemical and molecular techniques has enabled us to gain greater understanding of the signaling pathways involved in implantation. However, multiple redundant mechanisms exist to ensure the success of pregnancy. Moreover, studies in various animal models suggest that common pathways may be involved between divergent species. Thus, the importance of a molecule might only be established by identifying its role in several species. Although animal models have their own limitations, they represent an important and indispensable tool to derive a better understanding of the underlying molecular and genetic mechanisms of implantation. Furthermore, comparative studies can help to answer a variety of questions about species specificity of the signals, the components of signaling systems and the differing functions that the communication system may have in the establishment of pregnancy.

Acknowledgements

B. C. Paria is supported by National Institutes of Health (NIH) grant HD 044741 and UO1 HD042636. We are grateful to Dr. Qian Zhang for Muc1 immunostaining.

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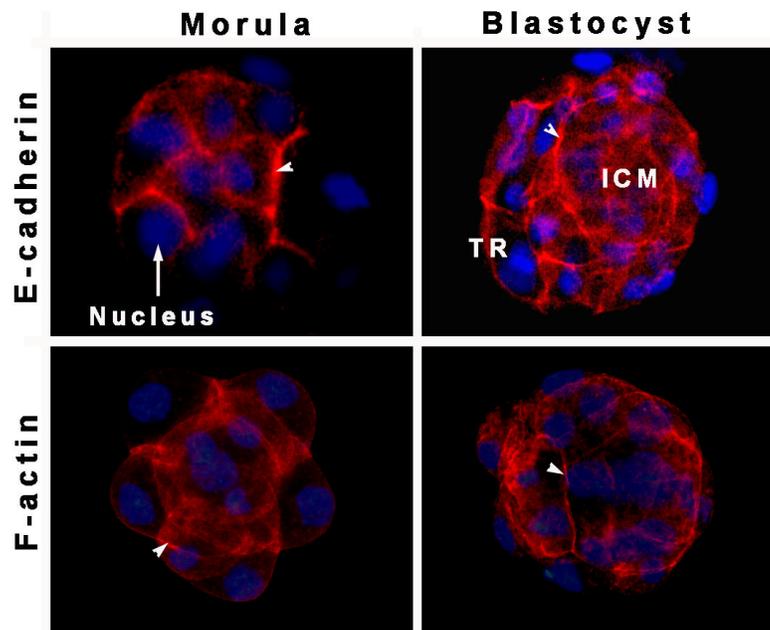


Figure 1. Membranous E-cadherin and F-actin localization in the morula and blastocyst stage embryos of hamsters

Arrowheads indicate E-cadherin (TRITC, red) and F-actin localization at cell-cell contact sites between blastomeres. A rat monoclonal E-cadherin antibody and Texas red-labeled phalloidin were used for E-cadherin and F-actin staining, respectively. Nuclei were stained with DAPI (blue). ICM, inner cell mass; TR, trophoblast

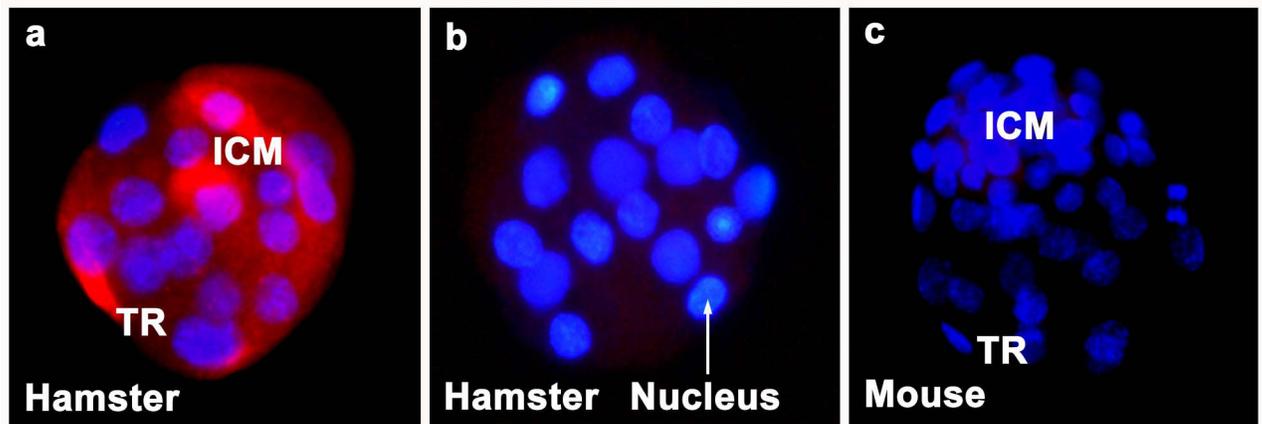


Figure 2. Immunofluorescence of cytochrome P450 aromatase (Cyp19) protein in day 4 hamster blastocysts

Blastocysts of hamsters (a) and mice (c) were incubated with polyclonal anti-human aromatase antibody. As a negative control, hamster blastocysts (b) were stained without primary antibody. In comparison, mouse blastocysts (c) show no expression of Cyp19. Simultaneous staining was performed for Cyp19 antigen (TRITC, red) and nuclei (DAPI, blue). ICM, inner cell mass; TR, trophoblast.

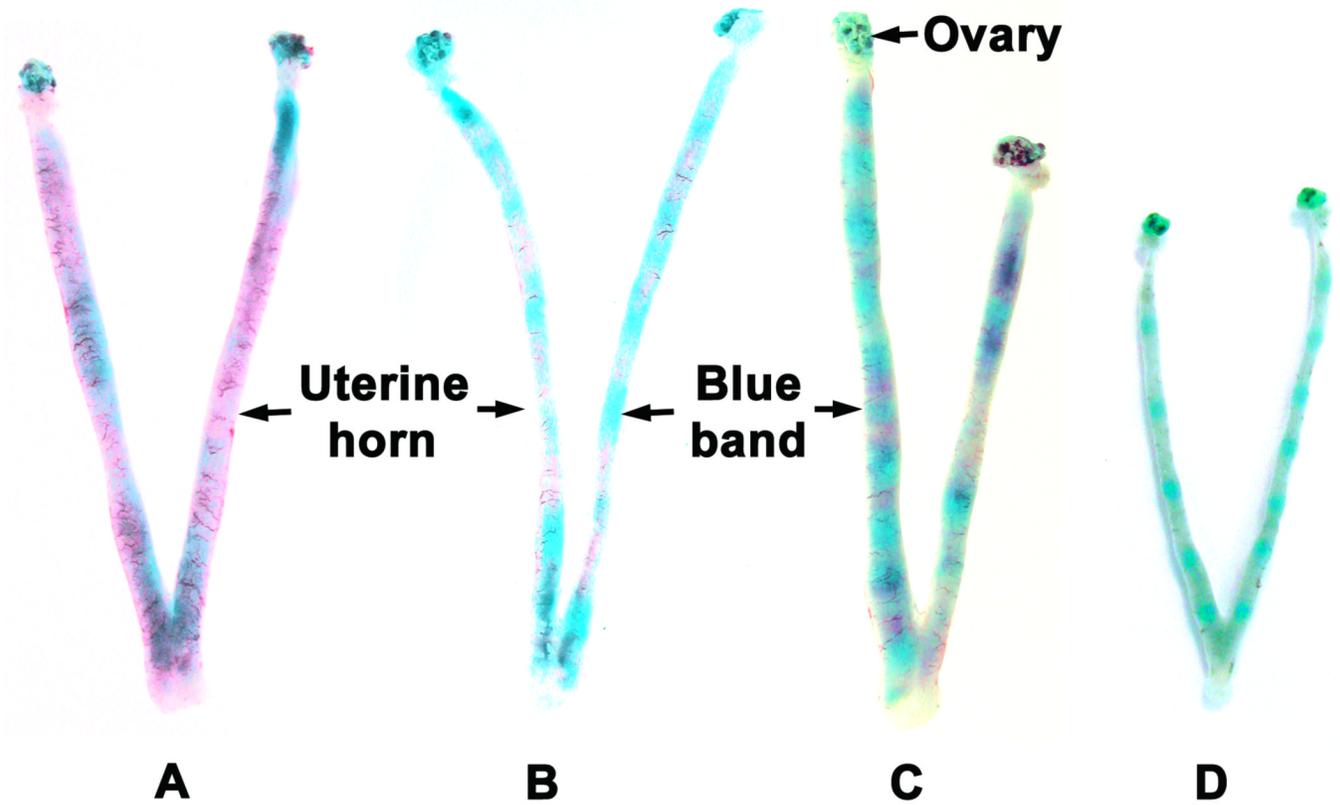


Figure 3. Implantation sites on days 4 and 5 of pregnancy as detected by intravenous injection of Chicago blue B dye injection
 Blue bands along the uterine horn of hamsters (A, day 4 1200 h; B, day 4 1800 h; C, day 5 0900 h) and mice (D, day 5, 0800 h) are indicators of implantation sites.

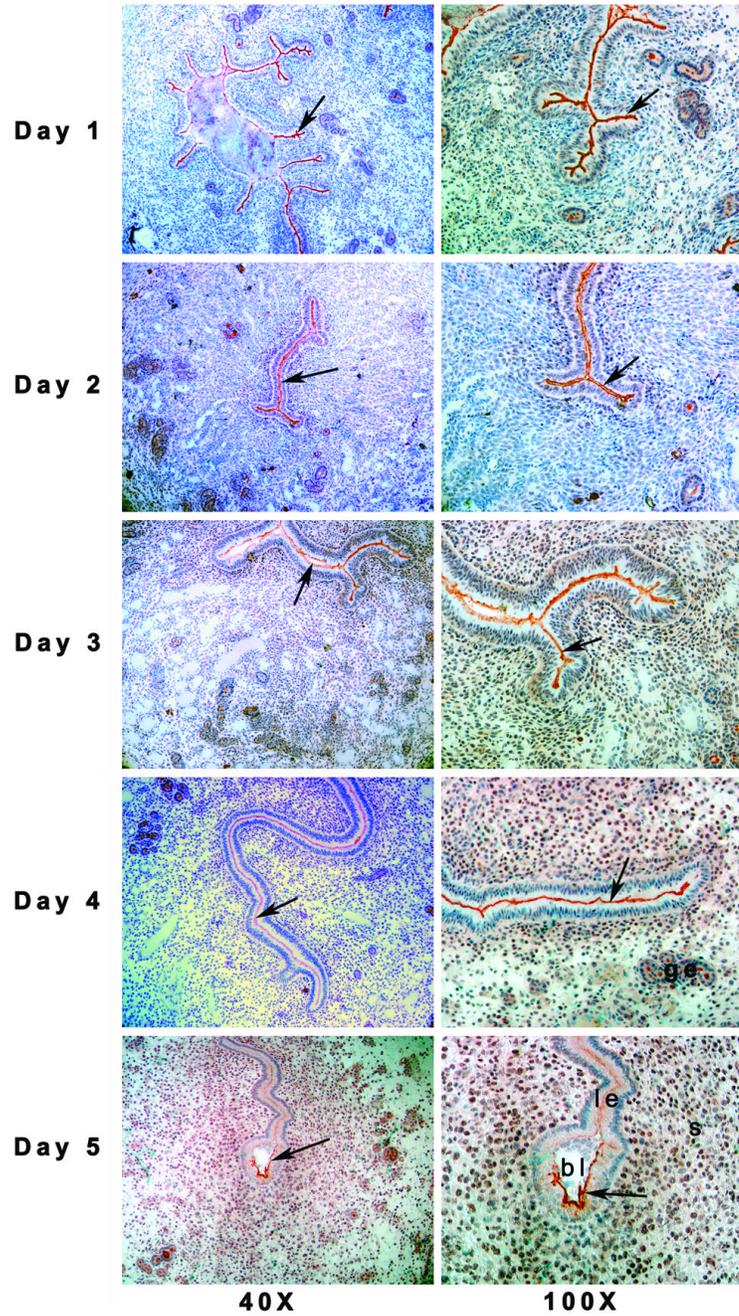


Figure 4. MUC-1 immunostaining of the luminal epithelium of hamsters on days 1-5 of pregnancy CT-1, a rabbit polyclonal antibody, was a generous gift from Dr. Daniel D. Carson, University of Delaware, DE, and used to detect MUC-1. CT-1 was the antibody of choice since it is not species specific nor affected by the glycosylation state of MUC-1 and recognizes all MUC-1 isoforms except the secreted form lacking the cytoplasmic tail. Immunostaining was performed using Zymed kit for rabbit primary antibody. Sections were lightly counterstained with hematoxylin. Arrows indicate apical MUC-1 staining. bl, blastocyst; ge, glandular epithelium; le, luminal epithelium; s, stroma.