The role of chorionic gonadotropin and Notch1 in implantation

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Abstract

Purpose Failed implantation is a major limiting factor in infertility and early pregnancy loss. In primates, human chorionic gonadotropin mediated inhibition of stromal cell apoptosis and their subsequent differentiation into decidual cells is critical for successful embryo implantation. A major regulator of cell survival and differentiation is the Notch receptor, which transduces extracellular signals responsible for cell fate determination during development. Proteolytic cleavage of full-length Notch1 releases an active intracellular peptide, which later translocates to the nucleus and activates gene transcription. Induction of Notch1 during the window of uterine receptivity in stromal fibroblasts in response to chorionic gonadotropin upregulates anti-apoptotic genes and induces α-smooth muscle actin, enabling stromal cells to proliferate and differentiate into a decidualized phenotype. As such, prior to implantation the embryonic signal, chorionic gonadotropin, rescues stromal fibroblasts from normal regression at the end of each ovarian cycle.

Conclusion We are suggesting that chorionic gonadotropin and Notch1 coordinately regulate decidualization by preventing apoptosis of endometrial stromal fibroblasts, averting uterine sloughing, and promoting cell survival and differentiation into the decidualized phenotype, which is critical for the maintenance of pregnancy.

Keywords Chorionic gonadotropin · Decidualization · Implantation · Notch1 · Uterine receptivity

Introduction

Failed implantation is a major limiting factor in assisted reproduction; these events are not clinically recognized as pregnancies [1]. As so, of the pregnancies that are lost, 50–75% represent a failure of implantation [2, 3]. Determining the prevalence of miscarriages is difficult, as many occur even before a woman is aware that she is pregnant [4]. However, prospective studies have found that 25% of pregnancies are miscarried by the sixth week of the last menstrual period [5] and only one-half of all conceptions advance beyond 20 weeks of gestation [6, 7]. Furthermore, by the age of 45, 75% of pregnancies end in miscarriages [8]. A better understanding of the molecular mechanisms responsible for implantation may improve clinician's ability to treat infertility and early pregnancy loss.

One of the most important requirements for the mammalian embryo to establish a successful pregnancy is its ability to implant in the mother's uterus. This process implies an appropriate spatio-temporal synchronic series of events where the embryonic trophoblast establishes contact with the uterus, attaching and intruding to the luminal epithelium. In order to carry out this dialogue, the endometrium must become receptive to signals derived from the embryo. This review explores the correlation between chorionic gonadotropin (CG) and Notch1 during

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Chorionic gonadotropin and Notch1 inhibit stromal cell apoptosis and induce decidualization in the primate endometrium.

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the window of uterine receptivity. We suggest that CG and Notch1 coordinately regulate decidualization by preventing apoptosis of endometrial stromal fibroblasts, averting uterine sloughing, and promoting cell survival and differentiation into the decidualized phenotype, which is critical for the maintenance of pregnancy.

Chorionic gonadotropin

In order to maintain the corpus luteum of pregnancy, different species utilize diverse mechanisms. In the primate, CG is one of the early embryonic signals secreted from the trophoblast cells of the pre-implantation embryo. CG has a direct luteotrophic effect on the corpus luteum, increasing its life span. Concurrently, CG also leads to alterations in morphology and endometrial gene expression by modulating the endometrium in preparation for implantation [9].

Our work has shown that infusion of CG into the uterine cavity of cycling baboons, in a manner which mimics normal blastocyst transit, induces the induction of α-smooth muscle actin (α-SMA) in the stromal fibroblasts. Disruption of α-SMA leads to apoptosis and a decrease in Notch1. Work from our laboratory has shown that treatment with CG rescues these cells from apoptosis and induces Notch1 expression pointing towards the critical role of both CG and Notch1 processes in implantation.

Molecular biology of CG

The CG receptor is ubiquitously expressed in reproductive organs [10, 11]. Targeted deletion of the CG receptor results in an altered endometrial phenotype and disrupted gene expression [12]. More so, CG receptor null animals do not demonstrate a complete decidualization response and have lower implantation rates when wild type donor embryos were transferred into the uterus [13]. Luteinizing hormone β (LH-β) knockout mice have been created and have thin uteri. These transgenic females are hypogonadal and infertile. Serum progesterone and estradiol are decreased by 75% and the uteri are hypoplastic consisting of thin endometrial layer [14]. Noteworthy, is the fact that pharmacological rescue of this phenotype was achieved with treatment of CG. Moreover, another LH-receptor knockout (LuRKO), consistent with the LH-β knockouts harbor thin uteri, with limited glands in the endometrium [15].

During implantation and pregnancy CG is primarily secreted by the syncytiotrophoblast and is involved in creating a receptive endometrium by directly modulating the function of both endometrial stromal and epithelial cells [16]. CG is a glycoprotein hormone belonging to the same family as follicle stimulating hormone (FSH), luteinizing hormone (LH) and thyroid stimulating hormone (TSH). The LH/CG receptor is a large cell-surface glycoprotein with the characteristic structure that makes it a member of the superfamily of G protein-coupled receptors (GPCR) [17]. These receptors activate multiple pathways in different cells, acting through various subunits and subsequent second messengers. The LH/CG receptor is composed of 11 exons, and has been mapped to 2p21 [18, 19]. The terminal exon encodes the entire carboxyl-terminal half of the receptor, including all seven transmembrane helices, the three interconnecting extracellular loops, the three interconnecting intracellular loops and the cytoplasmic tail. This carboxyl half of the receptor shares homology with other members of the superfamily of rhodopsin-like G protein-coupled receptors. The first 10 exons of the LH/CG receptor gene encode a large amino-terminal extracellular domain that contains a number of leucine-rich repeat motifs likely to be involved in protein–protein interactions. The extracellular domain of the LH/CG receptor, when expressed in isolation in transfected cells, binds CG with the same high affinity, as does the full-length receptor. Thus, although the extracellular N-terminal domain of the receptor mediates the high-affinity binding of the hormone it does not preclude low affinity interactions with the carboxyl half of the receptor [20].

The LH/CG receptor, like other GPCR undergoes ligand-induced desensitization, becoming less responsive to stimuli within minutes after binding the ligand [21]. Similarly, as pregnancy progresses, subsequent decidualization results in the downregulation of the LH/CG receptor, thought to be a prerequisite for the morphological transformation of stromal cells in the endometrium. This reduction in receptor expression corresponds to a decrease in CG in peripheral circulation [22].

We have investigated the cross talk between CG and progesterone in the endometrium during the period of receptivity. Our results indicate that blocking the action of progesterone on the endometrium, even briefly, has overwhelming effects on the CG-induced response in stromal fibroblasts. In contrast, the diminution of epithelial function in the presence of an ovary requires prolonged inhibition of progesterone action, suggesting a potential paracrine effect on the endometrium from the corpus luteum in response to CG.

Notch1

Uterine and embryonic interactions during implantation are correlative to epithelial–mesenchymal interactions in embryogenesis, and thus involve evolutionary conserved genes across species [23]. The Notch family of receptors mediate a highly conserved pathway that regulates differentiation and pro-survival signals across species from invertebrates

to humans [24]. Notch proteins are ligand-dependant transmembrane receptors that transduce extracellular signals responsible for cell-fate and differentiation throughout development [25, 26]. Notch signaling often restricts the differentiation fates of a cell, directing it to a specific cell fate in cooperation with other signals, while at the same time inhibiting differentiation toward an alternate fate and promoting survival [27].

Experimental evidence indicates that Notch signaling regulates all three branches of the cell fate decision tree: differentiation, cell cycle progression and apoptotic cell death [25, 27, 28]. The effects of Notch signaling on cell fate decisions in vertebrates have been extensively studied in tissue culture, ex vivo systems and transgenic animals [29–32].

Molecular biology of Notch1

The Notch proteins encompass a family of four homologous heterodimeric transmembrane receptors. Ligand-activated proteolytic cleavage of full-length Notch releases an active intracellular peptide, which later translocates to the nucleus and activates gene transcription [33]. Synthesized as a single polypeptide precursor in the endoplasmic reticulum, the Notch receptor is cleaved into a bipartite protein by a furin-like convertase in the *trans*-Golgi [34, 35]. These transmembrane receptors are made up of an extracellular and transmembrane subunits non-covalently held together by calcium-dependant interactions. Ligand binding at the extracellular domain results in dissociation of the extracellular subunit from the transmembrane subunit, followed by two sequential cleavages of Notch. The first is catalyzed by an ADAM protease and the second by γ-secretase [36]. This latter cleavages releases the active intracellular domain (NICD), which migrates to the nucleus and activates transcription by binding to ubiquitous transcription factor CBF1/RBPJκ and recruiting coactivator MAML1 (mastermind-like 1). The extracellular subunit of Notch receptors contains tandem epidermal growth-factor-like repeats that are involved in ligand recognition [37, 38]. The transmembrane domain includes a RAM domain that interacts with CBF1/RBPJκ [39]. This causes dissociation of a large co-repressor complex, which is replaced by a coactivator complex including MAML1, triggering transcriptional activation of Notch target genes. After transcriptional activation the C-terminal PEST sequence in Notch NICD gets ubiquitiinated and triggers receptor turnover [40]. Ligands for Notch are members of the DSL (Delta, Serrate, Lag-2) family of transmembrane proteins. The five mammalian ligands include: Jagged1, Jagged2, Delta1, Delta3, Delta4 [41]. Like the Notch receptors, these ligands have an extracellular domain comprised primarily of EGF-like repeats.

Notch1 knockouts are embryonic lethal because of vascular and somatic defects [42, 43]. It has been shown in mice that Notch1 is essential for post-implantation development. Crossing animals heterozygous for the Notch1 mutation yields mostly non-viable offspring and embryos that did survive beyond 11.5 days of gestation revealed widespread cell death, not attributable to defects in vascularization [44]. Similarly, targeted disruption of Notch ligand genes result in severe developmental defects or embryonic lethality [45, 46].

Window of uterine receptivity

After fertilization, the conceptus begins its migration from the oviduct into the uterus where it attempts to implant. Implantation requires a series of very precisely timed responses from both the embryo and the uterus. This occurs within a short period of time, known as the *window of uterine receptivity*. If implantation is successful, the endometrial stromal compartment forms the decidua, a morphologically and functionally gestation-specific tissue, representing the maternal side of the feto-maternal interface. This transformation requires a modified uterine milieu that promotes differentiation and metabolically active cells called decidual cells.

The concept of endometrial receptivity was first established in rodents and then extended to other species [47]. Although estrogen and progesterone have long been believed to be essential for developing an appropriate endometrial environment for blastocyst implantation, it is now evident that peptide hormones, such as CG, and growth factors further modulate these effects secreted by a variety of cell types within the uterine endometrium. In preparation for the implanting blastocyst, the endometrium becomes increasingly vascular, inducing α-SMA in stromal fibroblasts. More so, endometrial glands respond to CG by displaying an enhanced secretory activity and a plaque response develops in the luminal epithelium. The *window of receptivity* ranges from day 6 to day 10 post-ovulation in primates.

In addition to the quality of the embryo, inhibition of stromal cell apoptosis by (h)CG, and subsequent cell differentiation into fully differentiated decidual cells is critical for successful implantation. Differentiation into decidual cells is important for control of endometrial vascularization and involves vasodilation and angiogenesis in the endometrium [48]. During the process of decidualization, endometrial stromal fibroblasts transform morphologically and biochemically into polygonal, secretory cells and begin to express specific decidual proteins such as prolactin and insulin like growth factor binding protein-1 (IGFBP-1) [49]. Although the morphological changes are

useful predictors of successful implantation, the molecular mechanisms underlying them are largely unknown and remain to be elucidated.

Notch activation modulates the response of a cell to a differentiation stimulus, rather than directly specifying cell fate. In some cell types, as initially discovered in murine erythroleukemia cells, downregulation of Notch1 during differentiation abrogates differentiation, and results in subsequent apoptosis [50]. Activation of Notch1 provides a temporary survival signal during the differentiation of many cells, including thymocytes [30], murine erythroleukemia cells [50], murine and human keratinocytes [51].

Notch1 has both dose- and time-dependent effects on the levels of apoptotic inhibitor Bcl-xL and cell cycle regulators p21 (cip1/waf1), p27 (kip1), and Rb [52, 53]. Additionally, Notch1 activation by a cell-associated ligand is accompanied by a rapid and transient induction of NFκB DNA binding activity, which is known to regulate anti-apoptotic genes [54, 55]. The relative effects of Notch1 signaling on these pathways depend on the levels of Notch1 expression, the mechanism of activation, and the timing of activation.

Transforming the uterus to a receptive uterine stromal environment from the pre-menses state involves the synchronized regulation of CG, ovarian hormones and Notch1. Work in our laboratory has shown that (h)CG and ovarian steroids induce Notch1 expression in preventing stromal cell apoptosis and regression that occurs in the non-pregnant woman at the onset of the menstrual cycle. Subsequently, Notch1 expression is downregulated to allow stromal cell differentiation to make way for the decidualization phenotype.

In summary, our data show that low expression of Notch1 can be correlated to shedding of the uterine lining and an inability of the uterus to accept an implanting embryo, making it tempting to speculate that the ability levels of Notch1 can be used as an early infertility marker in women undergoing multiple miscarriages.

Role of the cytoskeleton

A coordinated process of programmed cell death is involved in both embryonic development and tissue homeostasis in the adult and as such is an important regulator of endometrial function. Apoptosis was first described in the human endometrium in 1975 [56] and subsequently shown to be rare in the proliferative endometrium, whereas levels of apoptosis increase in secretory tissue and peaks during the menstrual phase [57, 58]. Treatment with (h)CG or progesterone in the mid-secretory phase inhibits apoptosis in late secretory phase, implying a role for embryonic signals in inhibiting apoptosis [59].

The cytoskeleton plays a decisive role in mitosis, cell growth, cell motility and inhibition of apoptosis [60, 61]. Studies from our laboratory indicate that the primary effect of (h)CG on stromal fibroblasts is the induction of αSMA, which is a consequence of integrins on the stromal cell membrane binding to secreted extracellular matrix proteins [16, 62–64]. We have demonstrated that the induction of αSMA by (h)CG may be essential in order to decrease the progesterone-regulated proliferation in these cells, and consequently initiating differentiation and a qualification to inhibit stromal cell apoptosis [65]. This inhibition of proliferation in response to (h)CG has also been documented in human breast epithelial cells and breast cancer cells in culture [66]. In addition, co-expression of both the (h)CG receptor and COX-2 in stromal cells at the implantation site during early pregnancy is in agreement with previous in vitro studies that demonstrated a direct effect of (h)CG on COX-2 gene expression and the induction of decidualization in human stromal cells cultured in vitro [67].

Noseda et al. [68, 69] have demonstrated the requirement of Notch activation on the regulation of αSMA expression levels, which are coordinately regulated in decidualization. Notch was shown to directly regulate expression of the mesenchymal and smooth muscle cell marker αSMA by activating a *cis* element consensus sequence on a transcriptional effector that is required for
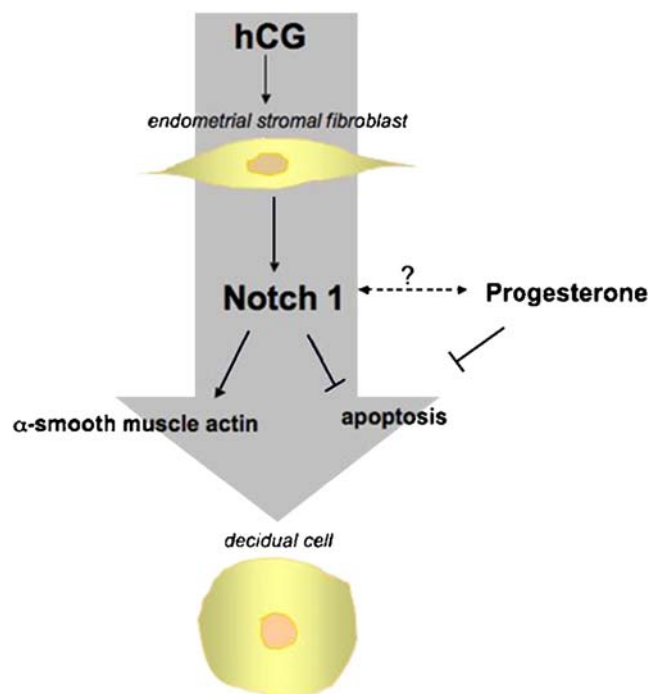

Fig. 1 The induction of Notch1 in stromal fibroblasts in response to hCG and progesterone inhibits apoptosis induces α-smooth muscle actin and enables the stromal cells to proliferate and differentiate into the decidualized phenotype

Notch-mediated αSMA induction. Furthermore, evidence in our baboon model indicates that coincident with the induction of αSMA, Notch1 and its ligand Jagged1 are also expressed in stromal fibroblasts.

As a consequence of implantation, apoptosis is avoided, and so are the subsequent sloughing off of endometrial tissue and disruption of spiral artery integrity that are hallmarks of menstruation. Studies in human endometrial fibroblasts with an apoptosis-inducing agent which disrupts the actin microfilaments, cytochalasin D indicate that (h) CG can significantly inhibit cytochalasin D-induced apoptosis of stromal cells, and this effect is enhanced in the presence of estrogen and progesterone [70]. Studies in human endometrial tissue suggest that ovarian steroids regulate three apoptotic related proteins (Bcl-2, Bcl-X and Bax) [59, 71, 72]. Thus, (h)CG and progesterone significantly reduce apoptosis in the endometrium. Furthermore, in vivo treatment of women with (h)CG or progesterone significantly increased Bcl-2 in the late luteal phase. Bcl-X levels were higher in the (h)CG treated group compared to the progesterone treated group, while Bax was inhibited by progesterone primarily [59]. Notch1 has also been shown to up regulate the anti-apoptotic protein, Bcl-X$_L$ [52], suggesting that it may participate in this effect of (h)CG.

These observations suggest that (h)CG induces αSMA and Notch1, which act synergistically on stromal fibroblasts during the window of uterine receptivity to inhibit apoptosis and enhance differentiation by upregulating anti-apoptotic genes [73, 74] (Fig. 1).

Conclusions

Human reproduction, though fundamental to our species survival, remains strikingly inefficient. Reproduction of an organism is viewed as if it is something that organisms have full control of, as if an organism makes copies of itself, by itself. Strictly speaking, such language is appropriate only to asexually reproducing populations since, as sexually reproducing organisms neither produce clones, nor produce other organisms by themselves. The probability of conception during one menstrual cycle is approximately 30% [75]. Accordingly, a better understanding of the molecular mechanisms responsible for implantation will improve treatment for infertility and early pregnancy loss in women.

Differentiation of a stromal fibroblast to a decidualized cell is essential for the maintenance of pregnancy in the primate. As the primate endometrium undergoes cyclical changes during menstrual cycle in preparation for implantation, an embryonic "signal" is responsible for rescuing stromal fibroblasts from apoptosis and normal regression at the end of each non-pregnant ovarian cycle. Notch

signaling dictates cell fate and critically influences cell proliferation, differentiation, and apoptosis. Seemingly, the co-expression of αSMA and Notch1, both arising from CG signaling, inhibits apoptosis of stromal cells during the establishment of pregnancy. Low expression of Notch1 can be correlated to shedding of the uterine lining and the inability of the uterus to accept an embryo. Supplementation with (h)CG or progesterone will induce Notch1 expression to mediate the survival of the uterine lining.

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