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Signal Transduction of hCG Induces Decidualization and Uterine Receptivity

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ABSTRACT

All independent experimental data on epithelial and glandular cells lines of human endometrium support the evidence for a rapid production of eicosanoids from the LH/hCG receptors when exposed to the hCG hormone. Prostaglandins rapidly act on the surrounding endometrial stromal cells throughout the adenylyl cyclase enzyme leading to very large amounts of cAMP and angiogenic factors (VEGF) production. The cAMP is the most important intracellular second messenger and along with progesterone accomplishes the full process of decidualization and acquisition of receptivity after estrogenic priming of the endometrium. The status of uterine receptivity lasts few days only and timing for successful embryo-signal transduction system activation by the endometrium is probably short. In absence of in vivo embryonic signals it is impossible to predict, on individual bases, how the intensity of all the complex interlinked molecular changes of decidualization might ever be in case of exposure to native hCG. In other terms, amount of prostaglandins and cAMP produced in response to variably glycosylated hCG are all, in vivo, not measurable variables and should be viewed as a "wave" of biochemical chain reactions. Embryonic hCG is secreted in form of multiple isomers having an unpredictable variable level of glycosylation and control of this variable remains elusive. During cycles of ovarian stimulation many drugs (FSH, LH, HCG) interact with different G-protein coupled receptors (GPCRs) making it possible to alter the prostaglandins-mediated decidualization process ready to be elicited only by hCG of pregnancy. Since the molecules (cAMP and progesterone) controlling endometrial stromal cells differentiation into decidual cells are critical for successful implantation and placenta formation, the evidence of fast eicosanoids production associated with endometrial LH/hCG receptors exposure to hCG and the potential by human endometrium to produce, in response, very large amounts of cAMP has biological and clinical relevance.

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Introduction

The estimated natural fecundity rate of the general population is about 20% per month, and estimated rates of conceiving naturally are 45%, 65%, and 85% after 3, 6, and 12 months, respectively [1]. Optimal estimates - representing the likelihood of a live birth when there are no barriers to treatment continuation – support the hypothesis that similar rates can be achieved by means of assisted reproduction technology, in the context of favorable patient characteristics such as uterine environment, embryo quality, treatment method, with cumulative live-births rates from two attempts shown to be greater than 70% [1]. This notwithstanding, there is still a 30% of couples who will never get pregnant and among those who become pregnant an unpredictable and interposed number of pregnancy losses will sooner or later occurs before a successful live birth [2].

We don't know why in humans, as compared to most other mammalians, the process of embryo implantation is so an inefficient phenomenon associating with so high degree of pre-clinical and clinical abortions. Studies outcome after IVF, based on HCG measurement in serially daily urine samples collected from 9 to 19 days after oocyte retrieval, show that implantation is totally absent in almost half of the cases (48.6%) and when implantation occurs (51%) the rates observed for preclinical pregnancy loss, biochemical pregnancy, clinical miscarriage, and ongoing pregnancy are 33.7%, 3.7%, 14.9% and 47.7%, respectively [3].

The low monthly conception rates observed in humans (20%), irrespective of the maternal age and type of conception (natural or IVF), is in most cases the natural consequence of the extremely high prevalence of gross chromosomal instability present in human embryos due to mitotic and meiotic errors [4]. Part of the losses however are subclinical, occurring early during the pre-implantation period, in absence of embryonic chromosomal imbalances and regardless how good looking embryos or blastocysts may appear. These implantation failures probably deal with the uterine side of the problem.

It is not our intention here to enter into the updated information today available in the field of human embryo implantation [5,6,7,8,9,10]. Among the number of possible embryo-derived signals known to regulate the endometrial response to the pre-implanting embryo (PIF, EPF, EGF, interleukins, hCG) [11,12], we will recapitulate here some of the recent insights about hCG and signal transduction systems of uterine receptivity. A possible interfering action on this signal transduction is played by gonadotropins used for controlled ovarian hyper-stimulation (COH). This and other clinical implications will be also here addressed.

The ligand: hCG is not one but many Different Signals at the Same Time

Human chorionic gonadotropin (hCG) is classically regarded as signal for maternal recognition of pregnancy affecting the corpus luteum to prevent luteolysis and stimulating the ovary to produce progesterone. Intact hCG is the predominant form of hCG produced by the trophoblast during pregnancy and is a hetero-dimer of hCGα and hCGβ subunits. It is often erroneously referred to as hCGβ but β subunit of hCG is other thing (freeβ) [13]. Either high or diminished concentrations of intact hCG have been associated with maternal or embryo-placenta abnormalities. Its measurements becomes a useful method to detect higher risk of miscarriage, ectopic pregnancy, predict pre-eclampsia, intrauterine fetal growth restriction, fetal hydrops or identify trisomy [14]. Recently an electro-chemiluminescence (ECL) method has been able to detect intact hCG secreted by embryos in spent culture media of day 1, day 3 and day 5 [15]. The embryonic hCG secretion was found to be associated with morphological grading in blastocyst stage and embryos having strong implantation potential both on day 3 and

day 5 suggesting that morphological scoring system combined with embryonic hCG concentration may be a reasonable way for selecting competent human embryos in clinical IVF reproductive medicine. The hCG molecule has several isoforms attributed to varying degrees of glycosylation and stages of metabolic degradation some of which have specific functions different from that of classically recognized. Studies on the qualitative hCG output of developing embryos in culture as analyzed by hCG isoforms expressed in the secretome shows that hCGβ (intact hCG) is detectable in media from 2 pronuclear (2PN) stage embryos through to the blastocyst stage and absent in 1PN and arrested embryos [16]. Prior to hatching, hyper-glycosylated hCG (hCGh) is observed selectively in 3PN embryos, but after hatching becomes along with intact hCG the dominant hCG molecule. Therefore hCG isoforms have potential roles as biomarkers of embryo viability and indicative of potential implantation success. This is of matter particularly considering the number of paracrine actions played by hCG around the time of implantation [17,18,19]. In fact there is growing in vitro and in vivo evidence that the hCG initially produced by the implanting embryo regulates the endometrial receptivity over the role played by the ovarian steroid hormones [18,20,21,22,23,24]. In the baboon, as long as adequate ovarian steroids priming of the endometrium is provided, the intrauterine addition of recombinant-hCG (rhCG) exerts an independent and additional modulation of the endometrial function [17,21]. Infusion of interleukin 1B (IL1B) at the time of implantation into the non-pregnant baboon treated with rhCG synergizes with hCG and mimics the early endometrial events associated with the presence of an embryo [22]. Modulation of endometrial cell receptivity via rhCG is not limited to IL1 receptor 'agonists and antagonists but also extends to other IL1 family members which, again, regulate embryo attachment, endometrial remodeling, angiogenesis and immune responses around the implanting blastocyst [19,23,24]. Overall these data suggest that important biological functions are regulated in endometrium by signals from the implanting primate embryo to enhance endometrial receptivity and support embryo implantation [17,18,20,21,22,23,24]. In vitro studies, using urinary-hCG (uhCG) and rhCG, show additional effects on embryo attachment [19] and rapid biosynthesis of prostaglandins by epithelial-glandular cells, respectively [25,26]. In humans, the intrauterine injection of hCG (500 UI) during the secretive phase of the cycle resulted to be associated with increased secretion of cytokines (PRL, LIF, VEGF, MMP-9) important for endometrial decidualization and vascularization [20]. Actions on stromal cells are probably secondary to eicosanoids produced by endometrial epithelial-glandular cells as first response to hCG [27]. Finally, hCG not only acts on the endometrium but probably also on the entire fetus-placental unit establishing the way for the initial embryo-maternal dialogue and placenta development as shown by other studies on trophoblast differentiation, tissue remodeling and trophoblast invasion [20,28,29].

The hCG molecule responsible for early modulation of uterine receptivity, embryo attachment and then embryo/decidua invasion might not be the same molecule. In fact in the first 3 or 4 weeks of pregnancy the predominant form of hCG is the hCGh but it is soon replaced with native hCG during a few weeks after implantation when it declines from more than 80% to 50% of total hCG forms. This shift in the glycosylation pattern results from production initially by cytotrophoblast, specifically extravillous endovascular trophoblast cells, gradually shifting to that by syncytiotrophoblast [14]. hCGh and not intact hCG has an autocrine rather endocrine function and directly modulates cell growth and cytotrophoblast invasion in early pregnancy. Recent data, obtained by using highly sensitive urine hCG tests, show that on the day of implantation pregnancies destined to fail as spontaneous abortions or biochemical pregnancies are marked by low production of hCGh [30]. This suggests that failure to

produce appropriately glycosylated-hCG most probably translates into invasion process abnormalities. hCGh is made exclusively by cytotrophoblast (extra villous trophoblast cells) and is the primary component of blastocyst invasion. This is in line with the finding that invading trophoblast from hydatiform mole and chorioncarcinoma secretes very high amounts of mostly hCGh [31]. In addition, these tumors over express hCG receptors compared to normal trophoblast suggesting that hCG has a role in trophoblast transformation, growth, and invasion in gestational trophoblastic neoplasms [32,33]. It is noteworthy, that grade of trophoblast invasion, amount of hCG receptors-expression and hCGh are all dependent on the percentage of paternal-ploidy inherited at fertilization. The invasive characteristics are made possible by concomitant HCG-dependent actions on maternal immunotolerance and endometrial apoptosis by regulating Fas-Fas ligand system [34].

Variant molecular forms of hCG exist each one having different plasma life, receptor binding affinity and bioactivity. We refer to the extensive review studies on this topic [13,14]. Briefly, the cell type (cytotrophoblast, synciotrophoblast, pituitary, tumor cells) determines the sort of oligosaccharide processing on α and β subunits but it is the α-β combination which modulates the extension of this processing. The set of enzymes contained in the secretory cell determines the nature of the processing and composition of the oligosaccharides added to both subunits. The metabolic sequence, cleavage of signal peptides, assembly of the native hCG, sequential posttranslational glycosylation and formation of the disulfide bonds happens at the same time that the hCG molecules are translocated from the place of synthesis up to the cell surface [14]. Fine tuning knowledge of how the carbohydrate units affect in vivo bioactivity in the clinical setting is, however, still missing.

The ligand: impact of hCG-isoforms with different Glycosylation on Signal Transduction

In vitro studies on the angiogenic and immunological actions played by hCG confirm how variability present in glycosylation rates among the various hCG molecules used (urinary-, recombinant- or hyper-glycosylated-hCG), is important [19]. Recombinant hCG (rhCG) has a direct angiogenic function on uterine endothelial cells via a G protein coupled receptors to PLC/IP3 –DAG/Ca2+/ protein kinase C [35]. Studies on endothelial cells recruitment and vessel formation in co-cultures of umbilical vein endothelial cells (HUVECs) and aortic smooth muscle cells (AoSMCs) in presence of rhCG showed involvement of different signal transduction systems for different actions (PKA and PKC pathways) [36]. Besides a direct effect on endothelial cells proliferation, an indirect angiogenic action is possible through a hCG-mediated release of prostaglandins from epithelial and glandular endometrial cells and subsequent prostaglandins-stimulation of important angiopoietins (VEGF, BFGF, PLGF) by stromal cells [37,38]. In experiments where hCGh rather than rhCG has been used, different receptor coupling and internal pathway were found [39]. The hCGh molecule has large carbohydrate side chains structure similar to that found in transforming growth factor β (TGF β) and because of this, it does compete with the TGFβ for binding to appropriate receptors [30]. In experimental model, using endothelial and mural cells of the aortic ring assay, it has been shown that the potent angiostimulation effect played by hCGh was independent of the classic signaling pathway and an alternative TGFβ-receptor II was identified as the only receptor for the hCGh [39]. Similar observations are valid for the hCGh-mediated actions on uterine natural killer cell proliferation (representing 70% of the endometrial leukocytes), uterine macrophages activation and interleukin 8 production by blood-circulating monocytes: in all these cases the HCG signal was not mediated by classical pathways but via the mannose receptor, a member of the c-type lectin receptor family that binds glycoproteins with N-linked carbohydrate side chains [40,41]. These last data

suggest that the embryo may transmit the information about its presence to maternal immune systems also using hCGh which mimics some products released in bacterial infection. Such an immune-endocrine network involving hCG and peripheral blood immune cells might be compromised by infections and explain recurrent pregnancy losses following Chlamydia Trachomatis infection [40].

The receptor: binding sites undergo variable and Dynamic Transcription during Cycle

The human LH/hCG receptor is encoded by a single gene located in the short arm of chromosome 2. The gene is 80kb and consists of 10 introns and 11 exons. While the extra cellular domain arises from splicing of exons 1-10, the entire serpentine and C-terminal domains are encoded in exon 11 [42]. Transcription of the LH/hCG receptor gene gives rise to multiple mRNA species and the relative abundance of each six transcripts differs between gonadal and endometrial tissue. Mammalian cells transfected with the cDNAs for the rat and human LH/hCG receptor display three distinct species with molecular masses of 65-75 kDa, 85-95 kDa and 165-200 kDa [42]. The 85-95 kDa band is the mature LH/hCG receptor present at the cell surface while the 65-75 kDa form of the LH/hCG receptor is a precursor of the cell surface receptor. This second form is located intracellularly because it is insensitive to surface proteolysis. Although the precursor of the LH/hCG receptor has a conformation that permits hormone binding this conformation is not the same as that of the mature form of the LH/hCG receptor [42]. In endometrium at least three different protein-bands migrating at 90 kDa, 68 kDa and even lower (35 kDa) molecular weight have been reported [43,44] and their functional properties have been the subject of debate in original studies. Presence and function of extra-gonadal LH/hCG receptors in a number of different human tissues (including the endometrium) have been largely described by Rao et coll [32,33,43,45,46]. These investigators would have provided in vitro experimental data suggesting for a direct coupling of the endometrial hCG receptors to the adenylyl cyclase enzyme with a cAMP dependent eicosanoids production. On the other side, the existence of full receptor expression in human endometrium has been questioned by others and identical functional results were obtained also by incubating endometrial cells with other hormones such as TSH, FSH and alpha subunit [47,48]. Because no known receptor could transduce the effects of all these hormones the existence of a unique uterine receptor has been speculated. Accordingly, it has been found that compared to gonadal receptors, the endometrial LH/hCG receptor genes express mRNA for the highly conserved transmembrane portion of the hCG receptor but not for the extra cellular ligand-binding portion that recognizes and specifically binds LH/hCG [47,49].

To ascertain whether human uterine receptor is identical to the cloned gonadal receptor, a further study utilizing reverse transcription, selective pre-amplification of full-length hCG/LH receptor mRNA + several shorter fragments of the receptor gene, and nested PCR with internal primers (followed by DNA sequencing) has been attempted [50]. The results of this study, once for all, could substantiate the view that human endometrium expresses the classical hCG/LH receptor. This study also showed that alternative splicing is a regulatory mechanism by which the endometrial tissue controls the expression of full-length and functional receptors. Down regulation of full-length hCG/LH receptor mRNA appears to take place during the late secretory phase and early pregnancy decidua whereas up to the mid-secretory phase full-length receptor mRNA is expressed in all samples. Pattern of receptor mRNA expression reported in this study for human endometrium supports the concept of a possible direct role for ovulatory LH as well as embryonic hCG during the early secretory changes of endometrium and pre-implanting embryo arrival into the uterine cavity.

We have characterized in vivo the HCG receptors, G proteins and the adenylyl cyclase enzyme in membranes from human endometrium [27,44,51]. Using polyclonal LH/hCG receptors antibodies raised against a synthetic N -terminus amino acid sequence of 15-38 (these antibodies recognize also the truncated receptor protein) we firstly found that in human endometrial cell membranes a single band migrating at 68 kDa was present. Secondly despite the abundance of G proteins and adenylyl cyclase enzyme this system was not, in endometrium, coupled to the LH/hCG receptors when saturating concentrations of uhCG were added. On the contrary, the endometrial adenylyl cyclase enzyme was well responsive in vivo to PGE2. In internal control luteal membranes multiple LH/hCG receptor-bands migrating at higher molecular weight were found and the addition of the ligand (uhCG) did, as expected, stimulate the adenylyl cyclase enzyme activity. We also noted that after few days of progesterone the Gs protein expression was significantly increased and the adenylyl cyclase resulted to be maximally responsive to its classical stimulators. We concluded that the endometrial LH/hCG receptors are not active throughout the classical signal pathway described for gonadal tissue. Considering however that many independent in vitro studies all reported a rapid synthesis of prostaglandins after endometrial cells exposure to the hCG hormone we argued that the endometrial LH/hCG receptor must somehow be functional even though probably by means of alternative pathways.

Our data agree with more recent in vitro demonstration that when endometrial epithelial cells from both the baboon and humans are stimulated by rhCG the LH/hCG receptors activate a cAMP-independent MAPK pathway leading to PGE2 synthesis [25]. Recombinant hCG acts via its G protein coupled receptor phosphorylating protein kinase B (PKB), c-Raf and ERK1/2 in a PI3K dependent manner. ERK1/2 phosphorylation was independent of the signaling paradigms of Gs and Gi transactivation typical of gonadal cells indicating alternative signaling pattern in endometrium [25,26]. Blocking the LH/CG receptor expression by small interfering RNA (siRMA) transfection, in both endometrial epithelial and stromal cells, directly inhibits the signal transduction cascade that is activated by CG in these cells [52]. The internal coupling of the endometrial LH/hCG receptors to this particular pathway (PKB, c-Raf, ERK1/2) has been confirmed also by others [18]. According to this last study, the endometrial LH/ hCG receptors have multiple functional proprieties in response to acute doses of hCG while prolonged low-dose pre-exposition to the ligand blunts any effect. This is what may happen in the clinical setting of ovarian stimulation cycles where injection of exogenous gonadotropins would become detrimental on the endometrial capacity to transduce incoming signals of native embryonic-hCG.

The receptor: G Proteins Coupled Receptors (GPCRs) in Endometrium

The above mentioned PKB mediated prostaglandins release by CG in endometrium is a novel finding and adds to the information already available for other internal pathways (PKA and PKC) better known as active pathways in gonads as well as animal uteri [53,54,55,56]. All these studies demonstrate that, in endometrium, many hormones (LH, FSH, GnRh, Relaxin, CRF, and Oxytocin) bind to their cognate receptors and through different G proteins activate either the adenylyl cyclase or phospholipase C. These receptors couple to Gi or Gq proteins to activate PLC or Gs to activate adenylyl cyclase. Activation of PKC by Ca2+ and DAG and activation of PKA by cAMP then act independently or synergically to promote an increase in COX2 and prostaglandins. Since all the signal transduction systems present in endometrium may converge to produce large amounts of prostaglandins it is difficult to get a complete picture of the in vivo intracellular dynamics and to consider the independent action of HCG separately from

that of other hormones probably actively present at the time of implantation. This is particularly true during cycles of ovarian stimulation when many different GPCRs may concomitantly be activated. The fact that a single ligand elicits changes in more than one second messenger system is not unprecedented as it is well known that on luteal cells Gs and Gi proteins are involved in dual coupling of the LH/HCG receptor to adenylyl cyclase and phospholipase C [42,57]. It is now generally accepted that a given GPCR can independently activate more than one subfamily of G proteins and these may not be the only mediators of GPCR signaling [42,58]. Activation of adenylyl cyclase, phospholipase C, PI3K and MAPK by G proteins coupled receptors has been interpreted either in terms of coupling to two different G proteins (Gs and Gq/11) or in terms of generation of GTP-bound α and βγ dimers derived from Gs [57,58].

The receptor: the Catalytic compound Adenylyl Cyclase (AC) and Decidualization

While investigating the signal transduction system possibly linked to endometrial hCG receptors we discovered an incredible amount of highly functioning adenylyl cyclase enzyme in human endometrium cell membranes as compared with luteal cell membranes [51]. The enzymatic activity found in cell membranes from hormone replacement therapy (HRT) cycles was much higher than that present in cell membranes from controlled ovarian stimulation (COH) cycles. Within the HRT cycles this enzyme resulted at its maximum level of activity on day plus 3 rather than on days plus 6 or 9 of progesterone supplementation. Against our expectations this transduction signaling resulted not to be classically coupled with the endometrial hCG receptors [27]. The biological significance however of so abundant availability of adenylyl cyclase enzyme in human endometrium was novel and remained to be interpreted. In our opinion, the abundance of this enzyme in human endometrium has probably to do with the well-known potent cAMP-action on the process of stromal decidualization independently of and in addition to that one played by progesterone [59].

Decidualization is first apparent in the stromal cells surrounding the terminal spiral arteries of the superficial layer around days 23 of a 28-day cycle. It marks the end of the limited period of endometrial receptivity during which embryo attachment takes place. The mutual potentiation of cAMP and progesterone (PR) in stimulated effects on cultured endometrial stromal cells has been clearly demonstrated, however, what correlation is in vivo for a convergence of cAMP and PR signaling is still unknown [59,60]. As the secretory phase progresses circulating LH and FSH levels fall but initial local arrival of hCG could indirectly sustain the cAMP signaling throughout a large and rapid production of eicosanoids (PGE2) which are known to act, in endometrium, by coupling with the adenylyl cyclase enzyme. Numerous experimental data show that decidual process is primarily dependent on elevated levels of the second messenger cAMP (not progesterone) which is produced in response to factors activating the adenylyl cyclase enzyme (relaxin, CRF, PGE2) and simultaneous down regulation of phosphodiesterases (converting cAMP into AMP) during the early-mid secretory phase of the cycle [59,60,61]. Upon continuous cAMP stimulation and protein kinase A activation, endometrial stromal cells become first responsive and then dependent on steroid hormones (progesterone and androgens). cAMP and progesterone interact and cooperate. After estradiol priming short time of progesterone supplementation induces a significant increase of Gs protein which constitutes part of the enzyme (adenylyl cyclase) producing cAMP. This second intracellular mediator in turn induces over-expression of progesterone receptors on stromal cells membranes and it is a well-known potent stimulator of embryonic hCG secretion [14].

Recent studies on endometrial stromal cells undergoing differentiation into specialized decidual cells in presence of culture-media products from growing and arrested blastocysts have opened a new way of interpreting implantation [62,63,64]. These studies suggest that the molecular changes occurring in endometrial stromal cells during early-mid secretory phase of the cycle are fundamental not only because correspond to the "uterine receptivity" but also because they play an active role in the ability to recognize, respond to and eliminate compromised implanting embryos –"window of natural embryo selection" [63]. Accordingly, decidual cells behave as embryo quality-biosensors and in case of poor or arresting embryo-cleavage, a specific decidual phenotype is derived which translates into late implantation, missed embryo quality control and early pregnancy failure. It is the decidualization process to be critical for the development of a normally functioning placenta. This agrees with in vivo data showing that successful implantation in natural cycles develops within a short time period, 8-10 days after ovulation meanwhile the risk of early pregnancy loss increases with later implantation being 13%, 26%, 52% and 82% for implantation days 9, 10, 11 and 12, respectively [65]. Up to date it is unknown whether the enigmatic signal "biosensoring" decidual cells in presence of arrested embryos may be that one represented by abnormal output of hCG molecule.

Micro array studies of human endometrial stromal cells decidualized in vitro in response to progesterone and cAMP have demonstrated that of the 588 genes screened marked up regulation was observed of cytokines, growth factors, nuclear transcription factors, members of cyclin family, and mediators of the cAMP signal transduction pathway [66]. In addition unexpected mRNA up regulated by progesterone and cAMP included the insulin receptor, the FSH receptor, neurotransmitter receptors, inhibin/activins and TNF-related apoptosis inducing ligand (TRAIL). This demonstrates that decidualization consists in sequential reprogramming of functionally related families of genes involved in extra cellular matrix organization, cell adhesion, cytoskeleton organization, signal transduction, metabolism, differentiation and apoptosis [59,66]. Upon biochemical reprogramming the endometrial stromal cells acquire many new functions that critically govern successful trophoblast invasion and placenta formation. The increased in CG receptors expression around the spiral arteries in early pregnancy probably reflects a maternal adaptation to ensure adequate blood supply to the developing embryo [67]. During early pregnancy cAMP up-regulates the secretion of hCGh from cytotrophoblast to favor invasion and down-regulates the expression of classical LH/CG receptors of decidua [14,50,67]. Notably, the extra villous trophoblast cells, which invade the decidua and the inner third of myometrium and then intravasate the terminal spiral arteries, are also particularly rich of LH/hCG receptors [33,68]. It remains not clear why in pregnant decidua the LH/hCG receptors undergo down regulation by cAMP [48]. It is possible that during the early phases of placentation, in order to comply with an extraordinary angiostimulation request, these receptors undergo transcription and conformational changes (kind of TGF β -receptors) in order to better transduce signals of the special hCGh produced by the extra villous trophoblast cells. The highly invasive nature of the extra villous trophoblast cells (attributed to paternally derived factors) must be tightly controlled in a temporal and spatial fashion to accommodate the need of a growing embryo while safeguarding the maternal host. Despite their extremely high proliferative and invasive nature their malignant transformation is rare. These cells disseminate into the maternal vasculature and the amazing precision of invasion inside the spiral arteries relies on an intricate communication with the maternal decidua [68].

Clinical Implications

1) In endometrium hCG binds to LH/hCG receptors different from those present in gonads and mainly expressed in the form of post-transcription variants. These receptors act, in endometrial epithelium and vascular endothelium, by coupling to a special internal pathways (PKB, PKC) and their exposure to hCG rapidly associates with endometrial production of eicosanoids. Since prostaglandins work by coupling to adenylyl cyclase and this enzyme is overabundant in human endometrium it is reasonable to imagine that huge amount of cAMP and angiopoietins (VEGF, PLGF) are indirectly produced in response to hCG throughout a previous production of prostaglandins. Additional amounts of cAMP are also directly produced in response to other signals deriving from the embryo (i.e., interleukin 1), corpus luteum (relaxin) and endometrium (oxytocin). The overwhelming availability of endometrial cAMP acts on implanting blastocyst further increasing the output of both hCG and hCGh by trophoblast cells and, at the same time, potentiates the progesterone receptors expression on the endometrial stromal cells. Both the molecules, progesterone and cAMP, altogether accomplish a functional reprogramming of the stromal cells undergoing process of decidualization. During the early secretory phase of the cycle an increased expression of constitutive parts of signal transduction systems (G proteins, adenylyl cyclase and hCG receptors) occurs as for preparing maximal endometrial response to embryonic signals: if the implanting embryo secretes enough amounts of glycosylated-hCG, the endometrial response in terms of prostaglandins and thus cAMP and VEGF is optimal and implantation successfully develops (early implanting embryos), otherwise if embryo cleavage goes wrong and/or secretion of hCG is abnormal or lacking then the transduction system might not be elicited adequately. The deriving low availability of cAMP and angiopoietins then would translate into aberrant vascularization and immunologic response leading to abnormally delayed implantation, early abortion or abnormal placentation with subsequent obstetric complications (late implanting embryos).

The capacity to harvest co-culture systems for reproducing, ideally, in vitro models to study all the complex molecular interactions between epithelial-glandular cells and underlying stromal cells in presence of cleaving embryos remains, at the moment, a major experimental challenge [10]. New immune-assays today available to detect specific types of circulating hCG (hCGh, free-β, intact hCG) should help clinical studies aimed to better understand early events of implantation and other obstetric disorders [13,14].

2) Based on the above mentioned biological actions played by hCG on embryo attachment and decidua function, an increasing number of studies have recently been attempted to evaluate, in terms of implantation and pregnancy rates, the intrauterine effect of few hundreds units of rhCG injected before the transfer of cleaving embryos [69,70,71,72,73,74]. These studies however are heterogeneous under a number of reasons including type of endometrial preparation (COH vs HRT cycles), type, doses and timing of hCG used and number and quality of embryos transferred. Results from preliminary prospective studies would suggest higher pregnancy rates (not implantation rates) for cleavage stage embryos (day 3) transfers. No differences in implantation and pregnancy rates were noted, in comparison with controls, for blastocysts stage transfers (day 5). This was true even if the rhCG injection was started on day 3 for delayed transfers on day 5. It is well known that synchrony between embryo developmental stages and endometrial maturity is critical for implantation and embryos transferred at two- to 12-cell stage successfully implant only between days 17 and 19 of a histologically defined 28 days cycle (corresponding to days plus 3 - 5 of progesterone supplementation) [75]. It is logical to presume that the effect of local delivery of rhCG induces only a temporary availability of endometrial molecules

useful perhaps to the attachment of an embryo [19]. After that if the invading embryo doesn't start to produce, by itself, the right amount and type of hCG, we doubt that implantation might really be enhanced. It is possible that on day-transfer 3 the intrauterine delivery of rhCG can act on early secretory changes of endometrium when it is still maximally responsive to growing embryo signals while on the contrary on day-transfer 5 the endometrium is already at advanced stage of maturation and additional locally delivered hCG doesn't influence it anymore. Very likely a blastocyst is a mature and healthy embryo producing, during apposition, enough amount of well glycosylated hCG-hormone conferring an independent invasive property regardless of the underlying endometrium maturation conditions. This could explain why reported clinical results are inconsistent. Perhaps for better evaluating the independent effect of locally injected rhCG on embryo attachment and thus implantation, a model using an artificial preparation of the endometrium combined with the use of GnRh-antagonist (to avoid LH interferences) and single embryo transfer (sET) should rather be used than fresh cycles. To date, definitive information on this topic awaits for results from prospective multicenter studies still ongoing (Dr Fazleabas NIH clin.gov. trial N.NCT01786252).

3) The suspect that during ovarian stimulation the endometrial receptivity might be reduced as compared to natural or hormone replacement cycles has been a concern since long time, subject of debate and classically attributed to the supra-physiological circulating levels of ovarian steroids [76,77,78,79,80,81,82,83]. In particular, a detrimental impact on endometrium due to increased circulating progesterone levels on the day of hCG injection and oocyte retrieval responsible for a dis-synchronic glandular-stromal maturation [84,85] and a premature appearance of endometrial pinopodes has been emphasized [86]. For this reason even the use of a progesterone receptor antagonist (RU486) or the use of assisted hatching to favor earlier embryo contact with an advanced endometrium have been prospected [87]. From 2006 up to date there has been a clear shift toward increased use of frozen-thawed cycles in USA coincident with greater implantation and pregnancy rates and reduced risks of low birth weight and prematurity [88,89,90]. Implantation patterns in cycles with and without ovarian stimulation have shown greater implantation rates of day-5 blastocysts when compared with day-6 blastocysts in cycles with ovarian stimulation exposure and greater implantation rates of day-6 blastocysts in freeze-thaw cycles than in fresh transfer after ovarian stimulation [88]. Results from all these studies confirm that ovarian stimulation exposure and the resulting altered hormone levels advance could alter the endometrial receptivity. This is particularly true for relatively slow cleaving embryos and may be exacerbated by premature progesterone elevation. The fact that the endometrium is less receptive in fresh transfers after ovarian stimulation than in frozen-thawed cycles and that certain perinatal risks are reduced suggests that not only the ability to implant but also the quality of implantation is affected. Nonetheless because the process of freezing and post-thawing-resumed development may itself be the cause of benefit, others authors, in order to avoid this limiting confounding factor, have looked at the pregnancy outcomes in fresh cycles donor oocyte recipients undergoing programmed endometrial preparation as compared to a matched group of autologous patients undergoing controlled ovarian hyperstimulation [91]. This retrospective study from SART registry is the largest one. It also is adjusted for important variables including the patient's age, the number of oocytes retrieved and the number of embryos transferred. Results from this study show implantation, clinical pregnancy and live birth rates in donor oocyte recipients 10% higher, on average, than that found in patients undergoing fresh autologous cycles. These results are confirmed also when comparisons are made

between oocyte recipients and a subgroup of autologous patients with a history of elective tubal ligation suggesting that differences reported are not biased by the fertility status. When results are analyzed in case of elective single blastocyst transfers cycles, differences among the two sub-groups of patients still remain. This last finding is consistent with the evidence suggesting that embryos become more vulnerable to hormonal dyssynchrony at later developmental stages. The statistically significant differences reported in this study in terms of implantation, clinical pregnancies and live births rates once again suggest that the consequences of not physiologic hormone levels around the time of implantation translate into a reduced potential of embryo invasion, a reduced clinical success rates, a higher risk of ectopic implantation, an abnormal placentation and even poorer neonatal outcomes [90,91]. On this regard, it has been repeatedly demonstrated that, in respect with natural or HRT cycles, during ovarian stimulation the endometrium undergoes indeed marked changes by histological as well as biochemical point of view [85,92,93,94,95].

4) Since functional receptors for gonadotropin hormones are present in human endometrium, the possibility of a direct influence on the endometrium by drugs used for ovarian stimulation is very likely [96,97]. This agrees with the observation that when the hCG injection is used during natural cycles for IUI or frozen-thawed cycles pregnancy rates are lower than those reported in similar cycles not employing the hCG injection [98,99]. Most probably the high dosage of injected HCG hormone interferes with the endometrial LH/hCG receptors reducing their subsequent activation by the natural hCG arriving from the embryo. A recent molecular study would support this and confirms the concept of closely restricted time frame, at the beginning of the secretory phase, for maximal response of the endometrial hCG receptors to embryonic signals [18]. This short time window of endometrial receptivity-potential disappears during the course of luteal phase and it is probably anticipated and disrupted whenever external stimulation of the receptors or enzymes is altered by the presence of exogenous gonadotropins (FSH, LH, hCG). It is likely that these drugs, overall, may start a premature prostaglandin-mediated release of endometrial cAMP with a subsequent acceleration of the process of stromal maturation and impairment of uterine receptivity. At the same time, the high circulating levels of estradiol during ovarian stimulation cycles increase the endometrial expression of progesterone receptors, accelerate the decidualization maturation thus contribute to abbreviate the window of implantation. The existence in human endometrium of receptors for FSH and GnRh hormones has been reported but their functional proprieties and eventual relationship with other GPCRs-related internal pathways still to be elucidated [55,56,93,95].

5) The biological significance of cAMP as strong inducer of decidualization has also additional clinical implications. Because the intracellular cAMP concentration is the result of an interplay between cAMP generation and hydrolysis by specific phosphodiesterases (PDE), the selective use of a PDE4 inhibitor (may be in conjunction with a relaxin agonist) has been proposed by some to support decidualization and local blood flow when suboptimal endometrial thickness is noted during ultrasound evaluation of HRT [60,100]. For the same reason the use of aspirin as anti-aggregating drug in reproduction appears questionable. Since the use of aspirin implies a reduced biosynthesis of prostaglandins (which act in endometrium by coupling to adenylyl cyclase and provoke rapid intracellular release of cAMP) a detrimental impact on decidualization cannot be excluded. Aspirin in fact is a strong inhibitor of COX1 and a weak inhibitor of COX2 but both these enzymes are expressed in the uterine epithelium at different times in early pregnancy and in particular the COX1 expression in the decidual lining of the uterus is

much greater than that of COX2 [61,101]. It is noteworthy that benefits and mechanisms supposed to be at the biological bases of the pro-inflammatory effect of the "endometrial scratching" involve an increased availability and production of factors (interleukins, eicosanoids, VEGF) known to be all induced and controlled also by hCG while acting on endometrium [102]. The ability of local tissue injury to modify subsequent decidual responses has been long recognized and increasingly exploited for clinical purposes [103]. Studies from patients suffering from recurrent pregnancy loss suggest that inflammatory signals are important epigenetic modifiers making possible that tissue trauma associated with menstrual events between pregnancies may provide cues that dynamically modulate subsequent decidual responses in the endometrium [103,104].

Summary and Conclusion

Within a limited temporal window, the endometrial lining, prepared by adequate ovarian steroids priming, must receive signals from the embryo to start a cascade of signal transduction pathways for completing decidualization and allow implantation. Since it reflects the endometrial capacity to transduce embryonic signals required to complete decidualization we believe that it is impossible to measure or predict it. Here we have indicated hCG as the main embryonic signal capable to drive all the biological cascade of the process. This molecule undergoes important changes in terms of glycosylated isoforms and amount produced from one embryo to another. Possible biomarker for decidual cells biosensor activity could be represented by the hCG signaling produced by normal cleaving and arresting embryos. Hyper-glycosylated hCG associates with deeper myometrial invasion and this might explain why sometimes ectopic embryos implant everywhere regardless of the basically absent "ectopic lining receptivity". Origin of hormone glycosylation and relative changes into signal transduction pathways of receptors are at the moment still enigmatic factors. It is likely that the paternal inheritance of imprinted genes may impact on many critical factors including the type of hCG molecule produced, the expression of LH/hCG receptors and the invasive potential of the extra villous trophoblast cells.

The importance of hCG glycosylation for adequate placenta invasion and formation has been speculated in terms of phylogenetic evolution of reproductive characteristics unique to human beings. According to this view, the low implantation rates observed in humans as compared to less evolved animal species are the price to pay for the evolutionary changes requested in brain mass development from early primates to humans [31]. A precise temporal association would exist, during evolution from early simians (primates) to human beings, between increased level of CG hormone glycosylation, deepness of myometrial invasion of hemochorial placenta and percentage of brain mass development. In humans, the highest level of CG molecule glycosylation would be associated with the deepest grade of myometrium invasion in order to obtain best hemochorial placenta for optimal nourishment of a single conceptus every time [31].

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