



## Recent Advances In Physiology Of Implantation: Potential Role Of Heparin In Assisted Conception

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## Introduction

Despite many advances in assisted reproductive technologies, implantation rates are still low after controlled ovarian hyperstimulation and IVF(1). Implantation failure is thought to result from impairment of embryonic development and/or from abnormal uterine receptivity. The endometrium is receptive to blastocyst implantation during a spatially and temporally restricted window called the "implantation window"(2). In humans this period begins 6-10 days after the LH surge and lasts- 48hrs(3).

Embryo implantation is a complex process requiring synchronized endometrial receptivity and blastocyst competence. The initial apposition, attachment and adhesion of the blastocyst to the maternal endometrium is achieved via a bidirectional coordinated dialogue of molecules including cytokines, adhesion molecules such as integrins and extracellular matrix(ECM) molecules(4).

Endometrial cytokines and chemokines regulate the complex events of embryo implantation. In part they are directly modulated by early embryonic signals such as human chorionic gonadotrophin(Hcg), interleukin-1 $\beta$ (IL-1 $\beta$ ) or insulin like growth factor II(IGF II)(5). Besides that, the apoptotic molecule tumour necrosis factor related apoptosis-inducing ligand(TRAIL) regulates IL-6, IL-8, leukemia inhibitory factor(LIF) and monocyte chemoattractant protein(MCP)-1 in human endometrial stromal cells(HESC's) in a nonapoptotic manner(6). Members of the IL-6 family are known to play an important role in the feto-maternal interface(5,7). Disturbance of their expression or action may be involved in failure of implantation or abnormal implantation(5). Whereas the distribution of numerous cell adhesion and extracellular molecules such as integrins had been extensively studied in endometrium, functional significance of the changing distribution of IL-11 and leukemia inhibitory factor(LIF), two IL-6 family cytokines produced by the endometrium and absolutely required for implantation in mice was provided by Merwood et al to regulate the adhesion of endometrial epithelial cells in humans and suggested targets for regulating fertility by enhancing/blocking implantation(8). The chemokines IL-8, MCP-1, and

RANTES(Regulation on Activation Normal T Cell Expressed and Secreted) are produced by the human endometrium and have an influence on endometrial leukocyte recruitment(9). Fas is a member of the death receptor family. Fas activation by Fas ligand(Fas L) typically triggers apoptosis in susceptible cells by activation of caspases(10). The Fas/FasL system is one of the major effectors of cytotoxic T cells and natural killer cells and seems to play an important role in the immune privilege in specialized organs like eye, testis or placenta. Fas is expressed by human ESC's and Fas is secreted by the embryo at the feto-maternal interface(11,12). Human ESC's are usually resistant to Fas mediated apoptosis(13). Since the implanting embryo secreted FasL, Fluhr et al found LIF and IL-11 in undifferentiated and IL-8 in decidualized ESC's were upregulated by nonapoptotic Fas signaling. In contrast IL-6, MCP-1 and RANTES were not regulated by Fas. Caspases were activated upon Fas stimulation and Fas mediated effects on LIF, IL-11 and IL-8 were reversed by caspase inhibition. The transcription factor NF- $\kappa$ B was not activated in ESC's after stimulation of Fas. These results suggest a differentially regulatory role of caspase dependent Fas signaling in the fetomaternal interface during early implantation. This typical death machinery mediates nonapoptotic effects in the human endometrium rather than inducing apoptosis(14).

## Role of Endocannabinoid System(ECS)

Anandamide(N-Arachidonoyl-ethanolamine(AEA)) plays an important role in the local regulation of implantation in the uterus(15). High levels of NAPE-PLD(N-arachidonoyl phosphatidyl ethanolamine-phospholipase D) and consequently low levels of FAAH(fatty acid amide hydrolase) are present in the interimplantation site of mouse uterus on day5-7 whereas high levels of FAAH and low levels of NAPE-PLD and consequently low AEA levels are found at the implantation site (16,17). Implantation is associated with a four fold reduction in AEA levels in the implantation site and an increase in FAAH activity(18). The implanting blastocyst can also regulate uterine AEA levels by an inhibitory effect of uterine NAPE-PLD (19) as well as through the release

of a putative lipid 'FAAH activator'(20). The FAAH activator produced by both the ICM and trophectoderm upregulates FAAH in the uterine cavity which then reduces AEA levels, incidentally a FAAH activator has recently been demonstrated in mouse endometrial stromal cells (ESC's) suggesting that such an entity may be instrumental in regulating FAAH beyond the reproductive events(21). Progesterone(P) stimulates the release of LIF through IL-4 which also has been demonstrated to promote implantation and pregnancy continuation(20). P upregulates lymphocytic FAAH activity through the transcriptional factor ikaros but has minimal effects on EMT/NAPE-PLD/CB1 expression in lymphocytes(20,21,22,23). P and Oestrogen(E2) have been shown to downregulate uterine NAPE-PLD expression in mice, possibly leading to a decrease in AEA levels. ECS have a role in immune regulation of human fertility. In this context, it has been found that FAAH expression is regulated by the Th1 and Th2 cytokines; IL4 and IL10 enhance FAAH activity, whereas IL-2 and INF- $\gamma$  decrease FAAH expression(20). In addition IL-2 inhibits the release of LIF and IL-4 stimulates it (20,24,25,26). AEA reduces the release of LIF from T cells via a CB1 receptor dependent mechanism (20,27,28) and thereby carries out its anti fertility action. Leptin, in human studies has been shown to upregulate the promoter region of the FAAH gene through STAT 3 signalling (22,23) and concomitantly reduces AEA levels in T cells. Consequently inhibition of LIF release by AEA is reduced(24,25) and embryo implantation is impaired(29).

## Role of Prokineticins(PROK1)

PROK1 is a multifunctional secreted protein which signals via the G protein coupled receptor PROKR1. Evans et al showed that PROK1-PROKR1 signalling regulates numerous genes important for establishment of early pregnancy which include LIF, COX-2, IL-8 and IL-11(30,31). IL-11 is known to be essential for successful decidualization and implantation. In human ESC's IL-11 has been shown to advance P induced decidualization implying a role for IL-11 in preparing the endometrium for implantation(32). Furthermore relaxin and prostaglandin E2 (PGE2) have both been shown to increase IL-11 mRNA and protein secretion in decidualized ESC's (5,33). Cook et al characterized a novel pathway that regulates IL-11 secretion via PROK1-PROKR1 in human endometrium in first trimester decidua via the calcineurin signaling pathway in a guanine nucleotide binding protein(Gq/11), calcium and extracellular signal regulated

kinase(ERK), calcineurin-nuclear factor of activated T cells (NFAT) dependent manner in human endometrium and first trimester deciduas and overexpression of regulator of calcineurin 1 isoform 4(RCAN1-4), a negative regulator of calcineurin signaling leads to a reduction in PROK1 induced IL-11 indicating that RCAN 1-4 is acting as a negative regulator in the signaling pathway(34). Wnt signaling has been demonstrated to be an important regulator of proliferation, whereas switching off wnt signaling permits the occurrence of cellular differentiation(35). Wnt signaling has been suggested to contribute to the regulation of endometrial development and differentiation during the normal menstrual cycle(36) and to the events of normal pregnancy(37). Wnt signaling mediates endometrial proliferation during the proliferative phase(38). Inhibition of the same by upregulation of Dickkopf1(DKK1) during midsecretory phase may allow differentiation of the stroma(39). McDonald et al showed PROK1 induced DKK1 expression in the non pregnant endometrium and first trimester deciduas via a Gq-Ca-calcineurin/NFAT mediated pathway. Endometrial epithelial cell proliferation is negatively regulated by PROK-PROKR1 signalling. They demonstrated that the effect on cell proliferation occurs via DKK1 expression(40)

## Role of MAST cells in Implantation

Uterine derived histamine has been known to be a key regulator in implantation because of its ability of altering uterine vasculature permeability and inducing stromal decidualization(41). Histamine is produced mainly by mast cells(MC's) that are present in both the uterus and placenta. Human preimplantation embryo induced MC's to release histamine by secreting histamine releasing factor(42). Also the potential of MC's on mediating ECM degradation through the activation and production of matrix metalloproteinases(MMP's)(43). MC tryptases and chymases have been shown to activate the precursor of MMP2(44). MMP9(45) collagenase and stromelysin(46). Furthermore, it is known that heparin is essential by controlling the levels of specific granules(47). The primary function of heparin in normal individuals is to bind to mast cell proteases(48). Moreover MC degranulation correlates with angiogenesis during pregnancy. The importance of phase dependent oscillations of MC numbers during hormone regulated menstrual cycle has been well known since 60's-70's because of their secreting substances that promote tissue remodeling and

invasion. This concept was given up due to normal pregnancy outcome in mouse deficient in MC. But again now MC'S as novel mediators of implantation has recently been described. Jensen et al showed that MC migration from the periphery to the uterus and feto-maternal-interface, upon upregulation of the expression of chemokine receptors(CCR4 and CCR5 in humans) which was modulated by estradiol and progesterone(49). The degranulation of these MC's may prepare uterus for implantation by release of pivotal factors eg histamine, tryptase, MMP's, VEGF(41,50,51,52) factors which are crucial for extra cellular matrix degradation and neovascularization. These processes are required for correct embryo attachment to the uterus and subsequent development of embryo. MMP's produced by cytotrophoblasts cells regulate the invasive behavior. Zhang et al showed that human uterine MC's are able to induce the expression of MMP's both in endometrial stromal and decidual cells(53).

The relevance of MC's may be just as those of uterine NK cells (54,55) reg Tcells(56), dendritic cells(57) for the fundamental of a successful pregnancy outcome in mammals in the maternal tolerance of the semiallogenic fetus, which is based on a well orchestrated modulation of the maternal immune system and the functionality of the hormonal system. High amounts of MC's were detected in the uterus during pregnancy(58) with the levels of MC'S being much higher than nonpregnant uterus(59). The cytokine stem cell factor(SCF) which binds the receptor c-kit(CD117) is required for MC development and proliferation and promotes MC survival through inhibiting apoptosis. The interplay between the receptor tyrosine kinase c-kit and its ligand SCF is essential for various processes like regulation of proliferation, differentiation and survival of haematopoietic germ cells and melanocytes(60). In a c-kit deficiency model Kit<sup>wsh/sh</sup>mice with MC deficiency based on restriction of c-Kit gene expression, exhibited several impaired implantation which could be completely rescued by systemic or local transfer of wild type bone marrow derived MC's. This was due to insufficient placentation and remodelling of spiral arteries in Wsh mice. The embryo itself could act as the stimulus for the implantation process. Here the embryo derived histamine releasing factor(EHRF) might be one of the first signals from the embryo to the uterus. The EHRH produced produced local secretion of histamine by UMC's could play a role in preventing maternal immune rejection at the implantation stage(42). In the Wsh model woidacki et al found that MC'S were involved in the interplay between CTGF and TGFβ1(61). CTGF has been

implicated in matrix production, during the menstrual cycle, uterine cell growth, implantation and differentiation of the embryo, ECM synthesis and angiogenesis. Also MC's contributed to trophoblast survival, placentation and fetal growth through secretion of glycan binding protein galectin1. The earlier scepticism was due to the usage of syngeneic matings in mouse models although most of the expected increase in procreation is by allogeneic to maintain genetic variability of a species(62).

## Role of Heparin

Heparin can alter the haemostatic response to controlled ovarian hyperstimulation(COH) and modify the risk of thrombosis. It can also modulate many of fundamental physiological processes required for blastocyst apposition, adhesion and implantation, as well as trophoblast differentiation and invasion due to its similarities with heparin sulphate and has the potential to improve pregnancy rates and outcomes(63). Both unfractionated and LMWH are able to modulate the decidualization of human ESC's in vitro and therefore might be useful to control endometrial differentiation and receptivity in ART(64). Furthermore the molecular charges and size of heparins determine their impact on decidualization of human ESC'S as determined by induction of same effect of IGF1, PRL and IGFBP1 as heparin by dextran sulphate, a polysaccharide of similar size and charges as heparin but without anticoagulant properties. LMWH with same anti factor Xa binding activity as heparin showed less pronounced effect on ESC's than heparin, whereas very short pentasaccharide fentaparinux(17Kda) had barely any effect which supports that decidualization of human ESC's seems to be independent of its anticoagulant activity(65).

LMWH at therapeutic doses has been shown to induce trophoblast invasiveness by expression of matrix specific proteases such as matrix metalloproteinases(MMP2 and MMP9) transcription and protein expression in vitro with a concomitant reduction in TIMP1 and TIMP2(66). Hills et al 2006 demonstrated that heparin abrogates apoptosis of primary first trimester villous trophoblast in response to treatment with proinflammatory cytokines such as interferon (IFN)γ and tumour necrosis factor (TNF)α (67). Heparin suppresses invasiveness of hepatocyte growth factor stimulated trophoblast cell line culture. Heparin and LMWH increase free IGF1 in a dose dependent manner without altering total IGF1 or IGFBP levels(68). As IGF 1 promotes trophoblast cell migration in vitro, it is possible that localized elevations

in free IGF1 with concomitant reductions in TGF $\beta$  due to LMWH administration may promote trophoblast invasion(63).

## Role of HB-EGF in endometrial decidualization and role of heparin

Heparin binding epidermal growth factor(EGF) like growth factor(HB-EGF) a member of the EGF family, functions as a mitogen and potent survival factor during stress. They reduce apoptosis induced either by transforming growth factor (TGF) $\beta$  or TNF $\alpha$  in human endometrial stromal cells(HESC)(69). It is synthesized as a transmembrane protein of 208 amino acids. A small part of the membrane anchored HB-EGF form, or pro-HB-EGF gets cleaved from the cell surface resulting in a soluble growth factor of 75-85 aa's, while most of the molecule remains uncleaved on the cell surface. Besides that pro-HB-EGF is also biologically active molecule which is complexed with CD9 and  $\alpha 3 \beta$  integrin. Thompson et al 1994 characterized the sequences in the N terminal portion of the EGF-like domain which mediated interaction with heparin and could modulate EGF like biologic activity(70). HB-EGF mRNA expression in isolated endometrial and stromal cells is regulated by estrogen and P(71). Exogenous HB-EGF stimulates the expression of IL-11(72). Recent studies by DiSimone et al 2012 revealed LMWH(tinzaparin and enoxaparin) significantly increased decidual HB-EGF expression along with reducing TNF $\alpha$  induced apoptosis(73). Particularly tinzaparin(Tz) significantly increased HB-EGF expression and secretion in a dose dependent manner at concentrations that are reached in vivo at therapeutic doses (1 and 10iu/ml). Tz's manufacture by enzymatic degradation in contrast to chemical depolymerization of unfractionated heparin(74) gives a higher mean molecular weight(6500 Da as compared to 4300 Da for enoxaprim(En)). It is also more heavily sulphated than other LMWH's(75). This effect of LMWH's on HB-EGF decidual; production is independent of their anticoagulant function but depends on the charge and size of these polysulphated glycosaminoglycans(65). TNF $\alpha$  induced apoptosis by the activation of upstream aspartate specific cysteine proteases(caspases), namely caspase 8 and caspase 3 which have been shown to be necessary in determining the nuclear alteration of apoptosis(76). In vitro studies suggest heparin suppresses natural killer toxicity(77,78 ) LMWH (En) prevents leukocyte adhesion by reducing

monocyte adhesion, ICAM 1 and P-Selectin expression and decreased nuclear transcription factors (NF- $\kappa$ B and AP1 (79); prevents efflux by inhibiting CCL21 induced Tcell adhesion and migration(80), antagonizes IFN $\gamma$  signaling(81) and inhibits complement activation(82).

Furthermore LMWH's (En and Tn) increased extravillous trophoblast(EVT) invasiveness by enhancing MMP proteolytic activity and induces expression/secretion of HB-EGF and Cyr61 in EVT (83). This effect was mediated by an increasing DNA binding activity of activation protein 1(AP1). During implantation EVT invasion into maternal endometrium requires a complex series of steps, including the degradation of ECM and the establishment of cell matrix interactions. Thus LMWH might affect EVT invasion not only via increased MMP2 activity but also by promoting the HB-EGF and Cyr61 expression and secretion; through stimulated activity of the final common pathway represented by AP1. Cyr61 is a novel cysteine-rich matricellular protein (A member of connective tissue growth factor(CTGF) family (84,85). Since Cyr 61 does not increase cell proliferation on its own but only enhances DNA synthesis induced by other mitogens lead to the consideration of Cyr61 as a matricellular protein, as compared to a growth factor which is tightly associated with ECM and supports cell adhesion, an adhesive signaling through direct binding to integrin  $\alpha \beta 3$  receptors(86).

Heparins have also been shown to prevent adverse pregnancy outcome in women with recurrent miscarriages without apparent cause or with inherited thrombophilia(87). Although LMWH'S given in the luteal phase of the menstrual cycle seem to be beneficial in improving implantation rates, as well as live birth rate in women with recurrent implantation failure treated in an ICSI programme, Urman et al didn't get statistically significant results but advocated study of larger cohort of patients(88). Further heparin has been shown to inhibit TNF  $\alpha$  signaling in invitro model of HESC's by interaction with NF- $\kappa$ B signaling(89). With heparin being recommended for prevention of preeclampsia, abruptio placentae, IUGR, low birth weight, besides recurrent miscarriages in non thrombophilia patients Tersigni et al highlighted the invitro effects on embryo implantation and trophoblast invasion(90). Time has come for a double blind randomized trial to test the hypothesis of Nelson and Greer for routine use of LMWH in all cases of IVF to improve implantation and thus success rates after ruling out any endometrial pathology like tuberculosis etc(63).

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