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An unexpected role for anticoagulant heparan sulfate proteoglycans in reproduction

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Summary

Major tissue remodelling occurs in hormone-responsive tissues of the female genital tract, at ovulation and during gestation, involving proteolysis and inflammation. Disorders of tissue remodelling events are associated with infertility in women with luteinised unruptured follicle syndrome and with gestational pathologies as pre-eclampsia.

Ovulation impairment is an important factor of infertility and a major concern in reproductive medicine. The gonadotrophin discharge inducing ovulation triggers proteolytic activities involved in the breakdown of the follicular wall and elicits an acute inflammatory reaction in the ovary. Tight control of these reactions is required to allow successful ovulation while avoiding excessive tissue damage.

Anticoagulant heparan sulfate proteoglycans (aHSPG), like heparin, possess a pentasaccharide sequence which binds and activates antithrombin III. These proteoglycans are produced by endothelial cells and are thought to endow the vascular wall with antithrombotic properties. aHSPG are also present in the reproductive tract; in the ovary they are strongly expressed in granulosa cells of preovulatory follicles and they are co-localised with serine protease inhibitors involved in the control of proteolytic activities at ovulation. The presence of aHSPG in the oviduct, in the uterus and in human follicular fluid, suggests that they could play additional distal roles in gestation.

The *Hs3st1*$^{-/-}$ mice are deficient in aHSPG and their phenotypes indicate that aHSPG exert an important function in reproduction. These mice did not show a procoagulant phenotype but instead they had severely compromised fertility. *Hs3st1*$^{-/-}$ females exhibited impaired ovarian function as well as intrauterine growth restriction linked to delayed placenta development. In these mice, the placenta is challenged by inflammation at mid-gestation, occasionally resulting in miscarriage and maternal death. Collectively, these observations suggest that aHSPG are involved in the control of inflammatory events occurring during tissue remodelling in hormone-responsive tissues. Further studies are needed to identify the inflammation mediators involved in this process.

Key words: heparan sulfate; reproduction; ovulation; coagulation; serpin

Introduction

Major tissue remodelling occurs in hormone-responsive tissues of the female genital tract, at ovulation and during gestation, involving proteolysis, fibrin deposition and tightly controlled inflammation. Anticoagulant heparan sulfate proteoglycans (aHSPG) bind and activate the haemostatic protease inhibitor antithrombin III (AT). The expression of aHSPG in extravascular compartments of the female genital tract and the reproductive defects of knockout mice deficient in aHSPG outline the emerging role of aHSPG in the control of tissue remodelling in reproduction.

Plasticity of the ovary: hormone-driven tissue remodelling requires inflammation and proteolysis

The development of the ovarian follicle, its rupture at ovulation and the subsequent formation of corpus luteum constitute one of the most striking examples of tissue remodelling in adult mam-

Financial support for this work: Swiss National Foundation grant N°3200B0-102148/1.

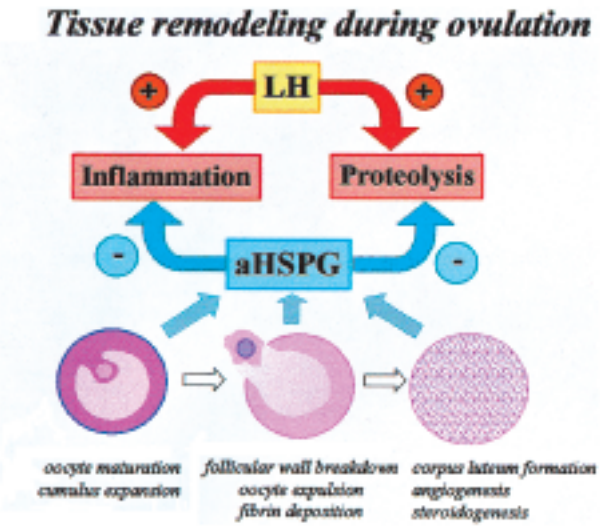

Figure 1

Diagram of ovulation related tissue remodelling, with implication of aHSPG from follicular GC to control the extent of inflammation and proteolysis elicited by the ovulatory LH surge

mals. This process is hormonally orchestrated and involves important proteolysis. The inner follicle, constituted by the oocyte and granulosa cells (GC), is separated from outer theca layers by a basement membrane and remains avascular until ovulation. As ovulation approaches, the basement membrane of preovulatory follicles is progressively permeabilised and plasma proteins accumulate in follicular fluid [1]. The ovulatory surge of luteinising hormone (LH) triggers a cascade of events resulting in follicular rupture and oocyte expulsion. Proteases from the plasmin generating system and collagenases are activated and acute inflammation takes place, involving vascular permeabilisation, oedema, extravasation of plasma proteins and fibrin deposition [2, 3]. As a result, a fibrin clot forms in the follicular cavity that will serve as provisional matrix to fill the former antral cavity with highly vascularised luteal tissue in corpus luteum [4]. Proteolysis during the ovarian cycle involves serine proteases of the plasminogen activator and coagulation cascades. Heparan sulfate proteoglycans (HSPG) are widely expressed in ovarian GC and their turnover, synthesis, internalisation and release is regulated by gonadotrophins [5].

Anticoagulant heparan sulfate proteoglycans and their interactions with antithrombin III

Heparan sulfate proteoglycans

HSPG are ubiquitously distributed on the surface of animal cells and in extracellular matrix and have numerous important biological activities through interactions with diverse proteins. HSPG are composed of a core protein with covalently attached HS chains formed by repetitive sulfated disaccharides of uronic acid and glucosamine. The different length and variable sequence of sulfated disaccharides generates the structural diversity required to form specific oligosaccharide binding sites for proteins such as growth factors, protease inhibitors or cell adhesion molecules. Binding of proteins to HS affects their reactivity and in the case of heparin and anticoagulant HS (aHS) they accelerate the rate at which AT inhibits serine proteases in the blood clotting cascade [6].

Anticoagulant HSPG

The AT-binding pentasaccharide of heparin and aHS is the best characterised biologically active structure of HS. It specifically binds and activates AT by inducing a conformational change in the inhibitor that stabilises its active conformer. This pentasaccharide bears a cardinal 3-O-sulfated glucosamine essential for AT binding [7, 8]. 3-O-sulfates are added late in the biosynthetic pathway of HS by hs-3-O-sulfotransferases. Six different isoforms of 3-O-sulfotransferases have been identified, with different tissue expression pattern and acceptor substrate specificities. The hs-3-O-sulfotransferase-1 (3-OST-1) is the predominant form producing AT-binding sites in aHS particularly in vascular endothelial cells [9]. In addition, the pentasaccharide is a specific co-receptor promoting FGF-7 interactions with its epithelial cell receptor FGFR2IIIb and other 3-O-sulfated HS bind a viral envelope protein mediating the entry into cells of Herpex Simplex virus [10–12]. These data suggest that aHSPG might have additional co-receptor activities toward certain protein ligands such as cytokines.

aHSPG have first been characterised in endothelial cells [13–16], they accumulate in their basolateral basement membrane, and they can be released in soluble form by secretion or shedding of their extracellular domain [14, 17]. Besides endothelial cells, aHSPG are synthesised selectively in extravascular cell types such as the parietal endoderm lining Reichert's embryonic membrane and ovarian GC, but their function outside the vascular bed is unknown [18–21].

The localisation of aHSPG underneath the vascular endothelium suggests that they could limit fibrin deposition in case of increased vascular permeability. Along this line of thinking, the synthesis of aHSPG by non-vascular cells is evocative of a role for aHSPG in the control of proteolysis and fibrin deposition in tissue remodelling.

aHSPG in the ovarian follicle

Ovarian granulosa cell aHSPG

Purification from rat ovarian GC has shown that GC aHSPG are similar in composition and in amounts to endothelial cell aHSPG. In response to gonadotrophins, GC increase their aHSPG output and liberate cell-bound aHSPG in soluble form, according to the modulation of their expression during follicular development [21, 22]. In cultured rat GC and in rat follicular fluid soluble aHSPG have been observed, suggesting that in addition to their function in the follicle before ovulation, aHSPG might be released with follicular fluid at ovulation and play a distal role in the female genital tract, in the oviduct at fertilisation or in the uterus at implantation. This assumption is supported by the detection of a strong signal for aHSPG in human follicular fluid (unpublished observations).

HSPG core proteins in granulosa cells

Biologically active HSPG are bound to the cell surface through their core protein (syndecans and glypicans), integrated in extracellular matrix and basement membranes (perlecan), released in soluble form by shedding or degraded by heparanase. Rodent GC, express the four members of the syndecan family, glypican-1 and perlecan [22, 23] and perlecan is present in human follicular fluid [24, 25]. HS chains can be attached to all core proteins synthesised suggesting that aHS is associated with both membrane-bound and secreted HSPG. The expression levels of HSPG core proteins in the ovary remain stable during the cycle, indicating that the modulation observed in aHSPG levels affects the aHS biosynthesis, likely involving 3-OST-1, the limiting factor in the biosynthetic pathway [22].

Localisation of aHSPG and of serine protease inhibitors in the ovulating follicle

The expression of aHSPG oscillates in ovarian follicles during the reproductive cycle [22, 26]. In primordial follicles, aHSPG are present in the basement membrane surrounding the single GC layer and might protect them from the active proteolysis occurring in nearby developing follicles. At the onset of follicular development in response to oestrogen, aHSPG disappear from actively growing follicles until the early antral stage and during follicle stimulating hormone–induced maturation of preovulatory follicles they are strongly expressed. At ovulation, GC aHSPG are transiently decreased during the remodelling of the follicular wall to form the corpus luteum. In mature corpus luteum, a very intense aHSPG labelling is restored, in luteal cells and in capillary endothelial cells.

The strong expression of aHSPG in ovulatory follicles endows them with anticoagulant properties, probably required to maintain the fluidity of the oocyte environment despite the influx of procoagulant proteins due to inflammatory vascular permeabilisation at ovulation [27]. After ovulation, the decrease in aHSPG corresponds to a change to a more procoagulant environment, allowing the formation of a fibrin clot in the antral cavity.

The proteases of the plasminogen activation and coagulation cascades induced in response to the LH surge, are controlled by serine protease inhibitors (serpins) that are often modulated by heparin. aHSPG could be involved in the control of the proteolytic breakdown of the follicular wall, through interactions with heparin-activated inhibitors such as protease nexin-1 (PN-1) and plasminogen activator inhibitor-1 (PAI-1).

The serpins PAI-1, PN-1 and AT are present in the ovary [28–31]. AT is synthesised in the liver and it binds to vascular wall aHSPG. In the ovary, AT leaks from permeabilised vessels in preovulatory follicles and concentrates on GC aHSPG. PN-1 is found on GC of developing follicles, where it accumulates until the onset of ovulation to quickly disappear thereafter. PAI-1 is present in developing follicles, in basement membranes of follicular theca until ovulation and then in corpus luteum [26, 28, 29]. So, the localisation of the serpins AT, PN-1 and PAI-1 in the ovary overlaps during the follicular cycle.

To reveal the interactions connecting aHSPG to these serpins in the ovary they have been coordinately localised in the same follicles. aHSPGs are co-localised with PN-1 during follicular growth, and then in preovulatory stage, with both PN-1 and AT. After ovulation, aHSPG coincide with AT and PAI-1 during corpus luteum formation. Maximal labelling of aHSPG, AT and PN-1 on GC is observed shortly before the oocyte expulsion, 6h after ovulation induction (figure 2). Thus, aHSPG are adequately positioned in the follicle in time and space to activate sequentially the three serpins PN-1, AT and PAI-1 [22, 26].

The serpins AT, PN-1 and PAI-1 share a common binding site for heparin on their D helix [32]. As the structural requirements on heparin for activation of PAI-1 and PN-1 are less restrictive than for AT, their binding sites on aHS might overlap and aHSPG could exert variable effects on these serpins during the reproductive cycle [33–35].

PN-1 is a potent inhibitor of thrombin and of plasminogen activators, its reactivity toward these

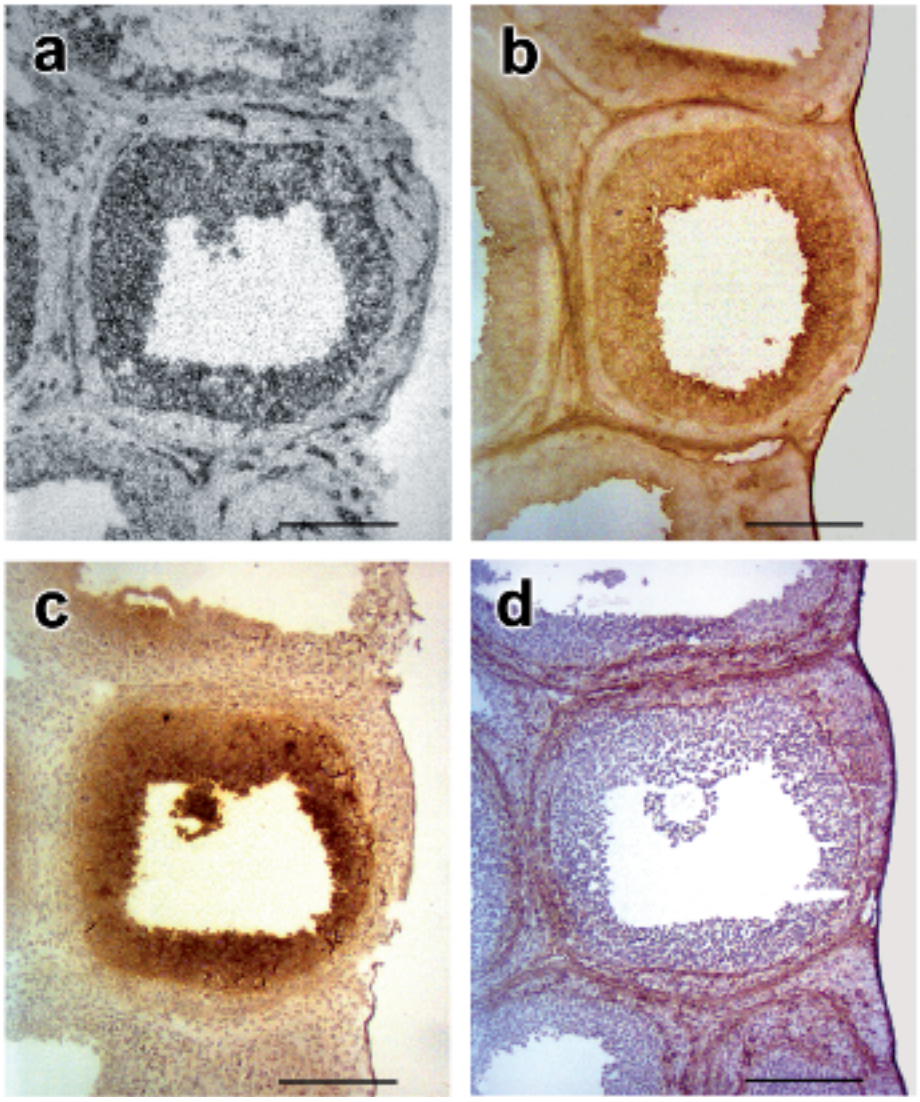

Figure 2

Co-localisation of aHSPG and of serpins in rat ovarian follicles Serial cryosections stained for aHSPG by ^{125}I-AT-binding revealed by autoradiography (a), and for AT (b), PN-1 (c) and PAI-1 (d) by immunohistochemistry. Gonadotrophin-induced cycle, preovulatory follicle 6h after ovulation induction. Shortly before ovulation, aHSPGs, AT and PN-1 labelling is maximal showing that AT and PN-1 are exactly co-localised with aHSPGs on the whole surface of the GC layer (a, b, c), while PAI-1 is strictly restricted to basement membranes (d). Scale bar 200 μm [26].

enzymes is increased by heparin [36] and it could therefore increase as aHSPG appear on GC of preovulatory follicles. PAI-1 targets are the plasminogen activators, but in the presence of heparin its reaction rate toward thrombin is markedly increased [37], suggesting that when co-localised with aHSPG, PAI-1 could control thrombin activity. AT activation by aHSPG increases its reactivity toward Factor Xa and thrombin [14] and could prevent fibrin clotting until oocyte expulsion. Thus, AT is critically located on GC aHSPG to control thrombin activity in the follicle while PN-1 and PAI-1 could play dual roles by controlling plasminogen activators activity, until their interaction with aHSPGs redirects them toward the control of thrombin activity. Altogether, the thrombin-inhibitory potential successively present in the follicle along the cycle is impressive, with the redundant presence of three potent thrombin inhibitors AT, PN-1 and PAI-1, that are likely activated by aHSPG.

Effects of aHSPG removal

Mobilisation of aHSPG by an inactive variant of AT: impact on ovulation

The role of aHSPG in the regulation of serpin activity in the ovary was shown by sequestration of their AT-binding sites with an inactive form of AT, that lacks thrombin inhibitory activity but retains normal heparin binding [38]. R393C-AT injection decreased the number of ovulated oocytes and increased fibrin deposition within follicles. In addition, luteinised follicles were frequently observed with the oocyte trapped inside, similar to the human luteinised unruptured follicle syndrome (LUF) [39]. These data confirm that aHSPG promote thrombin inhibition in the follicle to maintain the fluidity of the oocyte environment at ovulation.

Knockout mouse model of aHS deficiency: the *Hs3st1*$^{-/-}$ mice

Endothelial cell production of aHS is controlled by the *Hs3st1* gene, which encodes the rate limiting enzyme 3-OST-1. Generation of *Hs3st1*$^{-/-}$ knockout mice, deficient in aHS, allowed to evaluate their physiological function. The most prominent phenotypes of these animals were perinatal lethality and intrauterine growth restriction (IUGR), while adult animals did not show obvious procoagulant phenotypes or increased fibrin deposition in tissues. These unanticipated phenotypes suggest that aHS or additional 3-OST-1- derived structures may serve alternate biologic roles [40].

In the normal female genital tract, aHSPG were found in epithelial basement membranes of the uterus, in endothelial cells and in GC while they were absent in *Hs3st1*$^{-/-}$ females [17, 22]. The physiological importance of aHSPG in the reproductive tract was revealed by the observation that *Hs3st1*$^{-/-}$ mice suffer from markedly decreased fertility.

Defective reproduction of *Hs3st1*$^{-/-}$ females

We have examined the reproductive performance of female *Hs3st1*$^{-/-}$ mice and observed multiple defects at different levels (unpublished observations).

Despite the fact that young *Hs3st1*$^{-/-}$ females undergo normal puberty and cycle normally, they have a decreased litter number and their litter size is significantly smaller as compared to wild-type littermates. The average number of pups born is decreased by half and live born pups suffer from increased perinatal mortality. Moreover, the pups born from *Hs3st1*$^{-/-}$ mothers have a significantly reduced body weight at birth, followed by catch-up growth, a pattern typical for IUGR due to placental insufficiency during gestation. In addition, *Hs3st1*$^{-/-}$ females produce a significantly decreased number of pre-implantation embryos (E 2.5) as compared to wild type controls and they have a shortened reproductive period, with altered oestrous cyclicity at 7 months of age. These data indicate that ovulation is impaired in aHSPG deficient females contributing to the smaller litter size observed at birth. This is concordant with the demonstration in the rat that mobilisation of aHSPG with inactive AT variant compromises ovulation [26].

Gestation defects in *Hs3st1*$^{-/-}$ mice

Gestating females have a high incidence (about 50%) of mortality around mid-gestation with abrupt death due to massive haemorrhage and inflammation of the genital tract. To document the events leading to maternal death in gestating *Hs3st1*$^{-/-}$ females, we did histological analysis of implantation sites at mid-gestation (E9.5–E11.5) when placentation occured.

Structure and development of the murine and human placenta

The placenta has major endocrine functions that help to promote growth and survival of the embryo, it promotes the growth of maternal blood vessels to the implantation site and their dilation, and suppresses the local immune system. Failure in these functions are associated with complications of human pregnancy, missed abortion, miscarriage, IUGR and pre-eclampsia [41].

Comparisons between the human and murine placenta allows to draw parallels between cell types and tissue organisation. The overall structure of the murine placenta can be compared to that of a single lobe of the human placenta. In the mouse, trophoblast giant cells (the equivalent of extravillous cytotrophoblasts in humans) are involved in placentation, a process taking place at gestation days 8.5 to 10.5 (E 8.5–E 10.5) that creates an intimate encounter with the maternal cells of the uterine wall, culminating with the establishment of the maternal-fetal circulation in the labyrinth (corresponding to human chorionic villi) [42].

Acute inflammation at the foetal-maternal interface in gestating *Hs3st1*$^{-/-}$ mice

The histology of implantation sites at E 9.5 to E 11.5 was compared for *Hs3st1*$^{-/-}$ mice and for their wild-type littermates (unpublished results) [43]. At day 9.5, acute local inflammation was present in *Hs3st1*$^{-/-}$ females with strong infiltration of polymorphonuclear leukocytes (PMN) and extended fibrin deposition around the placenta, in close proximity to the maternal-foetal interface. At day 10.5, the placenta developing in *Hs3st1*$^{-/-}$ maternal uterus displayed markedly delayed development, with typically increased numbers of trophoblast giant cells, a rudimentary labyrinth, and no patent maternal-fetal circulation.

These observations suggest the following scenario to explain the occurrence of IUGR and maternal mortality in *Hs3st1*$^{-/-}$ mice. The deficiency in aHSPG of *Hs3st1*$^{-/-}$ mothers appears to trigger an acute inflammatory reaction at the maternal-foetal interface during placentation. This inflammation is later controlled and the placenta recovers but its development is delayed, leading to the IUGR observed at birth. Occasionally, the inflammation overspills, eventually resulting in miscarriage and maternal death. These data strongly indicate the involvement of aHSPG in the control of inflammation in this system.

Collectively, the data obtained on *Hs3st1*$^{-/-}$ female reproduction indicate that in absence of aHSPG, mice present ovarian and gestational dysfunctions that affect ovulation and placentation. In these two systems, intense tissue remodelling occurs, driven by hormonal stimulations and involving a limited and reversible acute local inflammation that destabilises existing tissue to

allow its reorganisation. The hormonally controlled expression of aHSPG in the genital tract could serve to limit the extent of inflammation and proteolysis occurring during tissue remodelling.

Perspectives

The notion that dysregulation of tissue remodelling and associated inflammation underlies the defects in ovulation and placentation observed in mice deficient in aHSPG suggests that this mechanism might be involved in related human pathologies.

Tissue remodelling related disorders in human reproduction

The LUF syndrome is frequent in women with ovulation disorders where the oocyte remains entrapped in incompletely luteinised follicles that fail to rupture. The pathogenesis of LUF involves a defective stimulation of follicular cells by the ovulatory LH surge, with decreased inflammatory response. In women, indomethacin treatment was associated with delayed follicular rupture and decreased intrafollicular blood flow demonstrating the close link between LUF and defects in the inflammatory pulse required for follicular rupture [44, 45]. The insufficient response to LH is expected to result in persistent expression of aHSPG on luteinising GC that could contribute to impair the inflammation and proteolysis required for successful ovulation.

Poly-cystic ovary syndrome is associated with increased risk of cardiovascular disease and recently, it has been shown to be associated with a global decrease in fibrinolysis capacity, both in the ovary and in plasma [46]. These data suggest that the control of fibrin turnover is important for efficient ovulation, and malfunctioning aHSPG could have an impact on this mechanism.

Association between fibrin deposition and trophoblast apoptosis has been frequently reported in pathological human pregnancy [47, 48]. Moreover, women with heritable thrombophilia, such as AT deficiencies at are high risk of pregnancy complication including pre-eclampsia [49–52]. These data emphasise the importance of haemostasis in the utero-placental unit during gestation and suggest that impairment of anticoagulant mechanisms, such as aHSPG, could compromise this equilibrium.

Pre-eclampsia is a severe disorder of pregnancy associated with defective development of the haemochorial bed, with poor invasion of the maternal spiral arteries by extravillous cytotrophoblasts and above normal apoptosis in the trophoblast [53, 54]. Several cytokines have been found to be up-regulated in pre-eclampsia, suggesting that inflammatory mechanisms are involved in this pathology. Plasma interleukin-1 receptor antagonist (IL-1Ra) and leukocyte counts are elevated in plasma of women with pre-eclampsia suggesting PMN activation [53]. The heparin-binding cytokine colony stimulating factor-1 (CSF-1) is elevated 1000-fold in decidua as compared to non-pregnant uterus, it stimulates trophoblastic growth, differentiation and production of chorionic gonadotrophin and placental lactogen. CSF-1 was found to be abnormally elevated in pre-eclampsia, promoting compensatory hypertrophy with poorly differentiated trophoblasts [55, 56]. Similarly, we observed PMN activation and defective trophoblasts differentiation in the placenta of *Hs3st1*$^{-/-}$ mice, suggesting that aHSPG might be involved in the control of inflammation and of CSF-1 action in the utero-placental unit.

Conclusion

Ovulation disorders are a frequent cause of female infertility that often remains unexplained and the control of ovulation is a major concern in infertility treatments. Pre-eclampsia is a life-threatening complication of gestation that has no other cure than delivery of the foetus. Placental pathologies often involve inflammatory damage, associated with excessive fibrinoid deposition. The control of inflammation in ovulation and placentation is therefore a major priority in reproductive medicine and the involvement of aHSPG in this process may open new therapeutic perspectives in this field. HSPG are involved in inflammatory reactions at several levels, they bind and modulate the activity of inflammatory mediators, cytokines, chemokines and their receptors [57, 58]. Binding of aHSPG to AT can mediate the anti-inflammatory properties of AT, either in a thrombin-independent way through syndecan-4 signalling, or by promoting thrombin inhibition and affecting proinflammatory stimuli such as fibrin deposition and protease activated receptors signalling [59–61]. Further studies are needed to elucidate the functional link between aHSPG and tissue remodelling, fibrin turnover and inflammation in the female reproductive system.

Acknowledgements

Part of the work presented in this review has been done by Ghamartaj Hosseini, Marc Princivalle and Ji-Cui Dong for their Ph.D. thesis. The author is supported by the Swiss National Science Foundation.

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