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Regulation and roles of the hyaluronan system in mammalian reproduction

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1 **Abstract**

2 Hyaluronan (HA) is a non-sulfated gylcosaminglycan naturally occurring polymer found in tissues and

3 fluids of mammals including the reproductive system. Its biosynthesis by HA synthasese (HAS1-3) and

4 catabolism by hyaluronidases (HYALs) is regulated by ovarian steroid hormones. Depending on its

5 molecular size, HA functions both as a structural component of tissues in the form of high molecular

6 weight HA, or a signalling molecule in the form of small HA molecules or HA fragments which is

7 mediated through interaction with its specific cell membrane receptors. HA is produced in the oocytes

8 and embryos and in various segments of the reproductive system. This review provides information

9 about expression and function of members of the HA system, including HAS, HYALs and HA receptors

10 in various processes from folliculogenesis to oocyte maturation fertilisation and early stage embryo

11 development to pregnancy, and its application in assisted reproduction technologies. Particular

12 emphasis has been made on the role of the HA system in preimplantation embryo development and

13 embryo implantation, and a hypothetical sequential model is proposed.

14 **Introduction**

15 Hyaluronan (HA), also known as hyaluronic acid or hyaluronate, is a high molecular weight anionic

16 member of a group of macromolecules called glycosaminoglycans (GAGs) that constitute components

17 of the extracellular matrix (ECM) in all animal tissues. Other GAGs include heparin sulphate, dermatan

18 sulphate, keratin sulphate and chondroitin sulphate. HA is the simplest of all the GAGs and has a

19 number of unique properties that distinguish it from other GAGs. (i) It is non-sulphated, (ii) it is a linear

20 polysaccharide of thousands of repeated units of alternating D- glucuronic acid and N-

21 acetylglucosamine (Weissmann, et al. 1954), (iii) it is synthesised at the plasma membrane rather than

22 in the Golgi apparatus (Prehm 1984) and (iv) it is extruded into ECM via the cell surface as it is

23 synthesised (Tammi, et al. 2002), and finally (v) HA is not restricted to the ECM, rather, its intracellular

24 localisation has also been reported (Contreras-Ruiz, et al. 2011).

25 The concentrations of HA within the reproductive tract vary from one mammalian species to another.

26 Some examples are provided in Table 1. HA is present in the oviduct, uterus and cervix (Afify et al.

27 2006, human; Perry et al. 2012, sheep; Raheem et al. 2013, sheep) and also produced by the cumulus

28 and granulosa cells of ovarian follicles (Kimura et al. 2002, pig; Schoenfelder & Einspanier 2003, cow;

29 Chavoshinejad et al. 2014, sheep). The role of HA in reproductive biology and clinical applications is

30 gaining increasing recognition. HA's expansion of cumulus cells at ovulation (Salustri et al. 1989;

31 mouse) and induction of cervical ripening during parturition (El Maradny et al. 1997, rabbit; Straach

32 et al. 2005, mouse) are well documented. Treatment of ovariectomised mice with progesterone

33 increased uterine HA concentration (Maioral et al. 2016). We and others have shown that the

34 expression of HA synthases is influenced by ovarian steroid hormones having a differential effect on

35 the expression of specific HAS and production of different size HAs during reproductive cycle and at

36 parturition (Afify et al. 2006, Teixeira Gomes et al. 2009; mouse, Raheem et al. 2013). In addition, a

37 range of growth factors, such as epidermal growth factor (Pienimaki et al. 2001) and transforming

38 growth factor-β (Pasonen-Seppanen et al. 2003), and cytokines, such as interleukin 1-β (Oguchi &

39 Ishiguro 2004) and interferon gamma (Campo et al. 2006), as well as local mediators such as

40 prostaglandins (Sussmann et al. 2004) affect HAS expression. The actions of HA are mediated through

41 its cell surface receptors CD-44 and RHAMM involving MAP kinases and Akt signalling (Straach et al.

42 2005, Kultti et al. 2010). Moreover, HA is expressed at different stages of pre-implantation embryo

43 development (Marei et al. 2013, cow). Recently, HA has attracted more interest because its addition

44 to embryo culture media seems to benefit in vitro fertilisation (IVF) and embryo transfer (Palasz et al.

45 1993; cow, mouse, Palasz et al. 2006; cow, Choudhary et al. 2007; mouse, Dattena et al. 2007; sheep,

46 Hazlett et al. 2008; human, Hambiliki et al. 2010; human, Nakagawa et al. 2012; human).

47 The HA system includes hyaluronan synthases (HAS), HA-degrading enzymes (hyaluronidases; HYALs)

48 and HA receptors. In this review, we shall explain the roles and regulation of the HA system in

49 mammalian reproduction with particular emphasis on pre-implantation embryo development and

50 embryo implantation.

51 **Hyaluronan biosynthesis**

52 HA is synthesised by three different but related trans-membrane enzymes named hyaluronan

53 synthases (HAS1–3) (Prehm 1984), which produce different size HAs with diverse biological functions

54 (Itano et al. 1999, Stern et al. 2006). The HAS genes have promoters reacting to common

55 transcriptional signals in addition to their own specific responses (reviewed in Tammi et al. 2011).

56 HAS2 synthesises HA of higher molecular weight than HAS1, in the range of >2 × 106 Da (Itano et al.

57 1999), whereas HAS3 synthesises HA of low molecular weight (1 × 105–1 × 106 Da) and represents the

58 most active isoform of HAS. Normally, HA turnover in the body is quite constant and consistently rapid.

59 One-third of the 15 g of HA in the human is replaced on a daily basis (Stern 2004). As formulated by

60 Stern (2003, 2004), a sequence of enzymatic reactions by HYALs cleave high-molecular-weight HA at

61 the β-N-acteyl linkage, progressively degrading HA by generating smaller fragments. There are six

62 HYAL isoforms in the human genome, HYAL1, HYAL2, HYAL3, HYAL4, HYALP1, and sperm adhesion

63 molecule 1 (SPAM1) (also known as PH20) (Csoka et al. 1999). HYAL1 and 2 are the most important

64 isoforms involved in HA degradation and catabolism in somatic cells (Bastow et al. 2008). HYAL2 is a

65 glycosylphosphatidylinositol-anchored enzyme attached to the external surface of the plasma

66 membrane and expressed in many tissues (Lepperdinger et al. 2001). It has a specific binding capacity

67 for the high-molecular-weight HA, cleaving it to fragments of ~20 kDa (about 50 disaccharides) (Stern

68 et al. 2006). HYAL1 utilises HA of any size as a substrate to generate tetrasaccharides (4–8 saccharides

69 in size) (Frost et al. 1997). HYAL3 and HYAL4 lack hyaluronidase activity and seem to play a non-

70 significant role in constitutive HA degradation (Harada & Takahashi 2007, Kaneiwa et al. 2012).

71 Similarly, HYALP1 that is present in mouse testis does not degrade HA (Reitinger et al. 2007). The role

72 of SPAM1 is discribed later in fertilisation paragraph.

73 HA interacts with cells through its receptors, which include cluster domain 44 (Aruffo et al. 1990,

74 CD44) and receptor for HA-mediated motility (Turley et al.2002, RHAMM). CD44 has been detected in

75 various segments of the reproductive tract in mouse (Kennel et al. 1993), cow (Bergqvist et al. 2005a),

76 sheep (Perry et al. 2010a), mare (Rodriguez et al. 2011) and human (López et al. 2013) under normal

77 physiological conditions. It has also been detected in cow (Furnus et al. 2003), mouse (Matsumoto et

78 al. 2004) and human (Campbell et al. 1995) embryos. Interaction between HYAL2 and CD44 facilitates

79 the endocytosis of HA, which undergoes further degradation by lysosomal HYAL1 into smaller HA

80 fragments (Lepperdinger et al. 2001).

81 In addition to its function as an adhesion molecule, there is evidence showing that CD44 is a potent

82 signalling molecule. Many studies have shown that HA–CD44 interaction can initiate several signalling

83 events under physiological or pathological conditions such as oocyte maturation and cancer

84 pathogenesis (Schoenfelder & Einspanier 2003, Kimura et al. 2007, Toole 2009, Yokoo et al. 2010,

85 Marei et al. 2012, Bourguignon & Bikle 2015, Misra et al. 2015). HA-mediated cell surface signalling

86 through CD44 is usually initiated by low-molecular-weight HA or HA-oligosaccharides resulting in cell

87 migration or cell proliferation (Lee & Spicer 2000). HA–CD44 interaction may also stimulate

88 intracellular signalling through extracellular regulated kinase (ERK), phosphoinositide 3-kinase (P13K),

89 Rac and Ras in various cell types (Kothapalli et al. 2008, Pure & Assoian 2009). Although many studies

90 on HA–CD44 signalling focus on cancer, HA–CD44 signalling is also observed under physiological

91 conditions. A study from our laboratory showed that small HA fragments of 20 kDa produced by

92 treatment of bovine embryos with HYAL2 caused increased phosphorylation of mitogen-activated

93 protein kinase MAPK1/3 signalling, resulting in increased blastocyst formation and quality,

94 characterised by higher cell numbers. This effect was abrogated with the inhibition of CD44 (Marei et

95 al. 2013). Another study also showed signalling by HA in human placenta through MAPK1/3 and PI3K

96 pathways, which enhanced trophoblast growth and invasion and possibly placenta angiogenesis (Zhu

97 et al. 2013a). Even though this study did not show that the signalling was through HA binding to CD44,

98 it is likely to be through HA–CD44 because CD44 is the major receptor for HA, and earlier studies have

99 shown the expression of CD44 in the human trophectoderm (Campbell et al. 1995) and trophoblast

100 (Goshen et al. 1996), where it was proposed to play a significant role in placenta angiogenesis.

101 RHAMM (otherwise known as CD168) is alternatively spliced; hence, different isoforms of the protein

102 were found both on the cell surface and intracellularly (cytoplasm, cytoskeleton, mitochondria,

103 nucleus and nucleolus) (Turley et al. 2002). Intracellular RHAMM interacts with several signalling and

104 cytoskeletal proteins, including Src through its interaction with microtubules and actin filaments

105 (Assmann et al. 1999). Although RHAMM is not essential for embryo viability (Tolg et al. 2003), it has

106 been found to play a profound role in several relevant cellular events, such as mitosis, cell proliferation

107 and migration (Turley et al. 2002). RHAMM is highly expressed in the G2/M phase of the cell cycle,

108 thus controlling mitosis (Mohapatra et al. 1996, Assmann et al. 1999). Deletion of the RHAMM C-

109 terminus results in impaired spindle orientation in the dividing granulosa cells, folliculogenesis defects

110 and subsequent female hypofertility in mice (Li et al. 2015). RHAMM knockdown results in the

111 downregulation of several pluripotency markers in hESC, induction of early extraembryonic lineages,

112 loss of cell viability and changes in hESC cycle suggesting its major roles in the maintenance of human

113 embryonic stem cell pluripotency and cell viability (Choudhary et al. 2007). RHAMM protein and mRNA

114 are expressed at all stages of human pre-implantation embryo development from 2-cell to blastocyst

115 (Choudhary et al. 2007). The relative expression of RHAMM increased transiently from 4-cell to 8- to

116 12-cell stage embryos and then remained static in morula and early blastocyst, but significantly

117 increased in expanded blastocysts (Choudhary et al. 2007). The same was confirmed in bovine

118 embryos where mRNA for RHAMM/IHABP (intracellular HA binding protein) where the highest

119 expression was seen in the expanded blastocyst (Stojkovic et al. 2003). Moreover, Ozbilgin and

120 coworkers reported spatiotemporal expression of RHAMM protein in mouse endometrium during the

121 oestrous cycle and peri-implantation period, suggesting its possible role in endometrial receptivity

122 (Ozbilgin et al. 2012). Inhibition of RHAMM signalling by culture of sheep embryos in the presence of

123 anti-RHAMM antibody resulted in the arrest of the embryo development at the 6- to 8-cell stage

124 (unpublished data). Considering the co-presence of HA, CD44 and RHAMM in the reproductive system,

125 it is highly likely that they work together to support mitotic activity in the developing embryos ensuring

126 the development of blastocysts with high cell numbers.

127 **Hyaluronan in the ovarian follicle**

128 A significant portion of the ECM of the ovarian follicles consists of HA (Irving-Rodgers & Rodgers 2005).

129 HA serves both as a structural component of ovarian follicles and in signalling cascades leading to

130 oocyte maturation and ovulation (Rodgers et al. 2003, Kimura et al. 2007). In mice, both oocytes and

131 cumulus cells produce HA during folliculogenesis (Salustri et al. 1992, Ueno et al. 2009). Indeed,

132 denuded oocytes produce increasing amount of HA during culture, which was suggested to be

133 involved in the enlargement of the perivitelline space in mouse oocytes (Ueno et al. 2009). The

134 granulosa cell layer of the mouse antral follicle is capable of HA synthesis (Salustri et al. 1992). HA was

135 also detected in the extracellular matrix of rat granulosa and theca cell layers of primary and more

136 advanced follicles (Takahashi et al. 2014). HAS1 is the dominant HAS protein in theca cells of swine

137 ovaries and may be responsible for an increase in the HA concentration of follicular fluids in atretic

138 follicles (Miyake et al. 2009) containing macrophages expressing CD44 as a phagocytic receptor

139 involved in phagocytosis of the apoptotic granulosa cells (Miyake et al. 2006). In sheep ovaries, we

140 recently reported the expression of HAS and CD44, which were mainly localised in the granulosa cells

141 (GCs) (Chavoshinejad et al. 2014). Large-size HA produced by the follicular cells contributes to the

142 osmotic gradient of the antral follicle resulting in the accumulation of the follicular fluid and antrum

143 formation (Clarke et al. 2006; cow, Rodgers & Irving-Rodgers 2010). This osmotic gradient across the

144 basal lamina restricts the movement of molecules above 100 kDa from the theca capillaries into the

145 follicular fluid in healthy follicles (Irving-Rodgers et al. 2002; cow, Rodgers & Irving-Rodgers 2010). It

146 was reported that the LH surge permeabilises the blood barrier of the follicle, and serum glycoproteins

147 in the inter-α-inhibitor family (IαI) can then enter the antral cavity (Hess et al. 1999; mouse, Rodgers

148 et al. 2003; cow). However, it is now evident that the family of IαI molecules can freely cross the

149 blood–follicle barrier; follicular fluid collected at any stage of folliculogenesis can be successfully used

150 instead of serum to form expanded cumulus ECM in pig (Nagyova 2015); and covalent binding

151 between hyaluronan and heavy chains of IαI is essential for the expansion of the cumulus cell mass

152 before ovulation (Chen et al. 1996; mouse, Nagyova et al. 2004; pig). Using cultures of sheep granulosa

153 cells, we have shown that reproductive hormones differentially regulate HAS2, HAS3 and CD44 in

154 ovaries (Chavoshinejad et al. 2014). Oestradiol, when combined with IGF-1, insulin and FSH,

155 stimulated HAS2 mRNA expression, which is essential for cumulus cell expansion prior to ovulation.

156 Oestradiol and LH had complementary effects in increasing HAS3 and CD44 mRNA expression in the
157 granulosa cells, an event that occurs during ovulation. Interestingly, high HAS3 and CD44 were
158 detected in the corpus luteum, indicating a pattern of expression in the ovaries during the oestrous
159 cycle. This may suggest a shift from production of large-size HA during follicular maturation and
160 cumulus cell expansion (stimulated by E2, IGF-1 and FSH) to a smaller-size HA produced by HAS3 after
161 the LH surge. Low-molecular-weight HA molecules have been linked with inflammatory processes and
162 angiogenesis (Collins et al. 2011, Rayahin et al. 2015), which are characteristic of the follicles during
163 ovulation (Richards et al. 2002, Blundell et al. 2003) and corpus luteum formation (Skarzynski et al.
164 2013, Berisha et al. 2015).

165 **Cumulus cell expansion and oocyte maturation**

166 Mammalian oocytes are surrounded by multiple layers of cumulus cells, together known as the
167 cumulus-oocyte complex (COC). The cumulus oophorus supports oocyte maturation, ovulation and
168 fertilisation (Magier et al. 1990, Tanghe et al. 2002). Before ovulation, the cumulus oophorus
169 contributes to the control of cytoplasmic maturation and meiotic arrest (El-Hayek & Clarke 2016,
170 Macaulay et al. 2016). During ovulation, it facilitates oocyte movement into the oviduct (Akison et al.
171 2012, mouse) and shortly after ovulation, it participates in the complex mechanisms controlling the
172 access of spermatozoa to the oocyte (Russell et al. 2016).

173 It has been demonstrated that cumulus cell expansion is a prerequisite for ovulation and may also
174 reflect the competence of such oocytes after fertilisation (Chen et al. 1993). Many related studies
175 showed HA to be the main component of cumulus expansion in the COCs (reviewed by Nagyova 2015).
176 Cumulus expansion leads to the detachment of the oocyte from the follicular wall and interruption of
177 the gap junctions between the cumulus cells and the oocyte (Sela-Abramovich et al. 2005). Reduced
178 cGMP transfer from the cumulus cells to the oocyte leads to a decline in cAMP concentrations in the
179 oocyte and resumption of oocyte nuclear maturation (Sanchez & Smitz 2012). cGMP inhibits
180 phosphodiesterase 3A, which maintains a high cAMP concentration in the immature oocyte during
181 follicular growth (Norris et al. 2009), which is essential for maintaining arrest at the prophase of the
182 first meiotic division until the preovulatory LH surge (Downs et al. 1989).

183 The preovulatory surge of LH activates HAS2 expression leading to the production of high-molecular-
184 weight HA by the cumulus cells; water absorbed by the HA results in the expansion of the COC (Saito
185 et al. 2000, Stock et al. 2002). HA secreted by the mouse cumulus oophorus is detectable between 2 h
186 and 18 h, peaking at 4–10 h after the LH surge (Tirone et al. 1997, Zhuo & Kimata 2001). In mice, this
187 HA-rich matrix is organised into a cross-linked network through the cooperative action of IαI,

188 pentraxin-3 and TSG-6 (Sato et al. 2001, Fulop et al. 2003, Salustri et al. 2004) to gain a stabilised

189 viscoelastic state that is required to facilitate the transfer of the oocyte to the oviduct for fertilisation

190 (Salustri et al. 1999). However, a recent report showed that binding of TSG-6 to HA does not play a

191 major role in the stabilisation of the cumulus cell matrix in mice (Briggs et al. 2015).

192 In pigs, COCs cultured in the presence of an HA synthesis inhibitor (6-diazo-5-oxo-1-norleucine) or

193 HYAL failed to expand at all (Yokoo et al. 2010). Our studies in sheep also revealed that the formation

194 of large-molecular-weight HA is essential for cumulus cell expansion (Marei et al. 2012). HAS2 and

195 CD44 expression in bovine cumulus cells were found to be potential markers of oocyte competence

196 (Assidi et al. 2008), and increased CD44 in follicular fluid was associated with good-quality oocytes

197 (Ohta et al. 2001). The localisation of CD44, the major cell surface receptor for HA in cumulus cells

198 (Kimura et al. 2002), suggests that HA–CD44 interaction may also be a likely player in oocyte

199 maturation. HA–CD44 interaction regulates the tyrosine phosphorylation of Connexin 43 (the major

200 gap junction protein found in the COCs), which leads to the closure of the gap junction and subsequent

201 activation of maturation promotion factor (MPF) activity (Sato & Yokoo 2005). The latter brings about

202 resumption of meiosis in oocytes that have been arrested in meiotic prophase I until shortly before

203 ovulation. Apparently, this activation occurs regardless of the structural expansion of cumulus cells as

204 inhibition of cumulus cell expansion by HYAL2 did not affect further fertilisation and embryo

205 development (Marei et al. 2012). On the other hand, inhibition of HA synthesis by 4-

206 methylumbelliferone during in vitro maturation completely inhibited the development to the

207 blastocyst stage, an effect which was partially alleviated by the addition of exogenous HA (Marei et al.

208 2012). This further emphasises the importance of HA signalling during oocyte maturation.

209 **Sperm-related functions**

210 HA is expressed in various segments of the male reproductive tract, including the epididymis, seminal

211 vesicles, prostate and Cowper's gland and with traces in the testes (Tammi et al. 1994). The accessory

212 sex glands provide the fluid medium necessary for nourishment and transportation of spermatozoa

213 through the reproductive tract. HA is a component of the seminal plasma in ram and alpaca (Kershaw-

214 Young et al. 2012) and may be responsible for the viscosity of the seminal plasma as observed in llama

215 and alpaca (Bravo et al. 2000). Sakairi and coworkers (2007) reported the presence of HA in the

216 seminal vesicles of immature pigs, without investigating further its particular roles. However, they

217 speculated that it may contribute to the regulation of homeostasis rather than sperm functioning.

218 Studies in mice suggest HA involvement in spermatogenesis (Thakur et al. 2006), even though the

219 mechanism still remains to be clarified. HA induces sperm capacitation (Tienthai et al. 2004, Tienthai

220 2015) by the activation of membrane-associated adenylate cyclase (Fernandez & Cordoba 2014), and

221   it also enhances the acrosome reaction in bovine (Gutnisky et al. 2007), and porcine (Suzuki et al.

222   2002) without necessarily modifying the sperm nuclear condensation and morphology, possibly by

223   decreasing the formation of vacuoles in the sperm head (Montjean et al. 2012). In dog spermatozoa,

224   HA accelerates the calcium influx into the sperm cytoplasm and increases lactate dehydrogenase

225   activity and cAMP production, provoking capacitation (Kawakami et al. 2006). HA may also help to

226   prevent polyspermy during in vitro fertilisation as well as supporting blastocyst development (Kano et

227   al. 1998) and quality by reducing apoptosis (Opiela et al. 2014). Supplementation of HA to human

228   sperm in the swim-up procedure increased the sperm motility and reduced the number of sperm with

229   DNA damage (Saylan & Duman 2016).

230   One of the criteria by which spermatozoa are assessed is their progressive motility. In artificial

231   insemination where semen is frozen and stored for future use, the viability of spermatozoa is greatly

232   affected by the reduction in motility and membrane stability during cryopreservation (Critser et al.

233   1988). However, this impairment could be overcome by the addition of HA to the semen diluent. HA

234   supplementation of the diluent helps to preserve post-thaw viability of boar spermatozoa in vitro and

235   maintains the membrane stability after cryopreservation (Pena et al. 2004, Qian et al. 2016). Similar

236   results were found in dogs (Prinosilova et al. 2009). Likewise in human, HA has been proposed to

237   enhance sperm motility (Ghosh et al. 2002) through phosphorylation of proteins that include HA-

238   binding protein (Ranganathan et al. 1995).

239   Hyaluronan-binding protein 1 (HABP1), a 68 kDa glycoprotein, was detected on spermatozoa of cattle,

240   buffalo, rat and human (Ranganathan et al. 1994, Bharadwaj et al. 2002, Ghosh et al. 2002, Ghosh &

241   Datta 2003). It participates in sperm–oocyte interaction (Ghosh et al. 2007) through its mannose

242   residues (Ghosh & Datta 2003). A reduction in the level of HABP1 is associated with loss of sperm

243   motility (Ghosh et al. 2002), the mechanism that may be attributed to the ability of HABP1 to modulate

244   sperm–oocyte interaction even in sub-fertile spermatozoa (Ghosh et al. 2007). The number of

245   spermatozoa bound to an oocyte was reduced significantly in the presence of D-mannosylated

246   albumin, the universal blocker of sperm–oocyte interaction, and this effect could be reversed by the

247   addition of purified recombinant HABP1 (Ghosh et al. 2007).

248   The correlation of HABP1 with sperm motility initiated the development and use of sperm HA-binding

249   assay (sHABA) in assessing the sperm viability in fertility clinics (Huszar et al. 2003). sHABA has proved

250   useful in selecting spermatozoa with a high DNA integrity and morphology and may sometimes be

251   used as a screening test for sperm quality before IVF (Worrilow et al. 2013). However, its use remains

252   controversial as sHABA does not predict freeze-thawing sperm survival (Boynukalin et al. 2012), and

253   it does not predict the pregnancy rates either in intrauterine insemination (Yogev et al. 2010) or IVF

254   (Ye et al. 2006, Boynukalin et al. 2012).

255   Intracytoplasmic sperm injection (ICSI) is used in clinical IVF to bypass the physiological barriers of the

256   cumulus oophorus and the zona pellucida in the treatment of severe male infertility due to low sperm

257   numbers or function. The selection of the sperm for injection may perhaps be promoted by HA binding

258   as a screening technique, given that HA-bound sperm in general are fully matured and have better

259   morphology with a reduced risk of aneuploidy or fragmented DNA (Pregl Breznik et al. 2013), which

260   has been reported as associated with increased pregnancy and implantation rates (Worrilow et al.

261   2013). However, it is not a reliable test for the prediction of sperm intracellular reactive oxygen

262   species, DNA fragmentation and DNA maturity and mitochondrial membrane potential risks and

263   healthy spermatozoa selection (Rashki Ghaleno et al. 2016), and the result of a recent meta-analysis

264   study has not supported its use in human ICSI cycles (Beck-Fruchter et al. 2016).

Sperm hyaluronidases and the role of HA system in fertilisation

266   Isoforms of HYAL found in sperm are SPAM1 and HYAL5. These unique hyaluronidases are located in

267   the testis or epididymis and have been detected in mouse (Zhang & Martin-DeLeon 2001, Chen et al.

268   2006), pig (Day et al. 2002) and human (Evans et al. 2003). It is secreted and located on the sperm

269   surface during epididymal maturation (Deng et al. 2000, Day et al. 2002, Evans et al. 2003, Chen et al.

270   2006, Martin-DeLeon 2006). SPAM1 is a GPI-anchored hyaluronidase (also known as PH20), which

271   depolymerises HA into tetrasaccharide and hexasaccharide products (Kim et al. 2005, Hofinger et al.

272   2008, Thompson et al. 2010). It is unique among hyaluronidases, in that it shows enzyme activity at

273   both acidic and neutral pH, activities that appear to involve two different domains in the protein

274   (Gmachl & Kreil 1993, Cherr et al. 2001). Several studies have confirmed that SPAM1 is the only

275   hyaluronidase identified to date in mammalian sperm, including the sperm of guinea pigs, rats,

276   macaques and humans (Cherr et al. 2001, Zheng et al. 2001). It is also present in the lysosome-derived

277   acrosome, where it is bound to the inner acrosomal membrane (Morin et al. 2010). SPAM1 is initially

278   synthesised as a polypeptide with an apparent molecular weight of 64 kDa. During the course of sperm

279   maturation, part of SPAM1 is processed into two fragments that are linked through disulphide bridges,

280   such as at the N-terminal domain of 41–48 kDa and at the C-terminal domain of 27 kDa.

281   Hyal5 is exclusively expressed in the testis and the plasma and acrosomal membranes of rodent sperm

282   (Kim et al. 2005). It is enzymatically active in the pH range 5–7 and inactive at pH 3 and 4. Both Hyal5-

283   enriched SPAM1-free soluble protein extracts and SPAM1-deficient mouse sperm were capable of

284   dispersing cumulus cells, which was inhibited by the presence of a hyaluronidase inhibitor, apigenin.

285   These results suggest that in the mouse, Hyal5 may function principally as a 'cumulus matrix

286   depolymerase' in the sperm penetration through the cumulus mass (Kim et al. 2005).

287   The concentration of HA in follicular fluid has been used to estimate the viability of oocytes for

288   fertilisation with concentrations as high as 50 ng/mL (Saito et al. 2000) to 239.3 ng/mL (Babayan et al.

289   2008) being associated with fertilisation of the oocyte and embryo implantation in human.

290   Despite the presence of HYAL in mouse, its role in fertilisation remains uncertain. Kimura and

291   coworkers (2009) showed SPAM1 to be required for sperm penetration through the cumulus matrix

292   for fertilisation in mice. It was also reported to be involved in sperm-ZP binding (Myles & Primakoff

293   1997, Cherr et al. 2001) and induction of the acrosome reaction (Overstreet et al. 1995, Sabeur et al.

294   1998). Reddy and coworkers (1980) used a hyaluronidase inhibitor in mice to clarify HYAL function in

295   fertilisation. In their study, myochrysine, a natural inhibitor of HYAL with no effect on the acrosome

296   reaction, inhibited fertilisation due to reduced breakdown of the COC. However, a similar effect was

297   not observed when using oocytes devoid of follicular cells. Another study using a double knockout

298   model confirmed that sperm serine proteases, ACR (acrosin) and/or PRSS21 (testisin), function

299   cooperatively with SPAM1 in cumulus penetration in mice (Zhou et al. 2012). In addition, HA fragments

300   generated by SPAM1 stimulate cytokine/chemokine production via the TLR2 and TLR4 pathways in

301   cumulus cells of ovulated COCs, which may enhance fertilisation (Shimada et al. 2008). However, mice

302   lacking SPAM1 and HYAL5 are fertile, indicating that the HA-degrading ability of HYAL in mouse sperm

303   is not essential for fertilisation (Kang et al. 2010). It is also possible that SPAM-1 secreted by the

304   oestrous uterus and oviduct, with the potential to bind to sperm during capacitation (Zhang & Martin-

305   DeLeon 2003, Griffiths et al. 2008) might have compensated for its absence in the sperm itself in the

306   knockout model. In addition, the detection of functionally active HYAL5 on the surface of SPAM1-

307   deficient spermatozoa confirmed that compensation was possibly occurring by this HYAL (Zhang et al.

308   2005). Moreover, HYAL2 that was reported to be present in mouse sperm (Modelski et al. 2014) may

309   have contributed to this functional redundancy.

310   **Pre-implantation embryo development**

311   In cattle, HAS2 and HAS3 are expressed at all stages of early embryo development from 2-cell to

312   blastocyst (Marei et al. 2013). We found that HAS2 mRNA expression tended to decrease with the

313   progression to the blastocyst stage, whereas HAS3 expression was maintained. Moreover, HA

314   receptors CD44 and RHAMM were also expressed at all stages (Furnus et al. 2003, Palasz et al. 2006,

315   Choudhary et al. 2007).

Studies in murine, porcine and bovine have shown that HA supplementation of culture media improves embryo development, viability and blastocyst cell number in vitro (Furnus et al. 1998, Gardner et al. 1999, Jang et al. 2003, Lane et al. 2003, Toyokawa et al. 2005). HA has also been shown to improve the cryotolerance of blastocysts, which then leads to increased birth rates in cows (Lane et al. 2003), mice (Palasz et al. 1993) and ewes (Dattena et al. 2007). On the contrary, in a randomised clinical trial of human IVF, hyaluronan enrichment of the embryo transfer media did not have any beneficial effects on IVF outcome in terms of clinical pregnancy implantation and delivery rates, although higher birthweights occurred in the HA group (Fancsovits et al. 2015). However, the inhibition of HA synthesis by 4-methyumbelliferone (4-MU) suppressed blastocyst formation in sheep, (Marei et al. 2013) indicating the critical role of HA in embryo development in this species. 4-MU is a coumarin derivative that has been shown to supress HA synthesis in mammalian cell cultures (Nakamura et al. 1997). The effect seems to be reversible upon removal of 4-MU from the cell culture. The disruption of HA synthesis by 4-MU is both at the level of the substrates (UDP-GlcUA and UDP-GlcNAc) and HAS expression. 4-MU has affinity to conjugate with UDP-GlcUA, with reduction in the cellular pool of this substrate as well as causing downregulation of HAS2 and HAS3 (Kultti et al. 2009).

The effect of HA on embryo development seems to be HA-size dependent. HA fragments generated by HA depolymerisation by HYALs are biologically active molecules that have important functions (Stern et al. 2006). Most of these functions are receptor mediated and increase cell proliferation through binding to CD44 and RHAMM (Xu et al. 2002) incurring phosphorylation and activation of the MAPK pathway (Zhu et al. 2013a,b) and stimulation of mitosis. In cleavage-stage bovine embryos treated with HYAL2, we detected higher levels of MAPK1 and MAPK3, an increased incidence of blastocyst development and increased blastocyst quality as shown by higher total numbers of cells and trophectoderm cells (Marei et al. 2013). These effects were abrogated if CD44 was blocked (Marei et al. 2013). These data show the potential beneficial effects and importance of small-size HA in the development of pre-implantation embryos.

In vivo, early stages of embryo development in most mammals happen in the isthmus compartment of the oviduct. HA was detected in oviductal fluids collected by catheterisation during the oestrous cycle in heifers and cows (Stojkovic et al. 2002) and was shown to be highest at ovulation (Bergqvist et al. 2005b). Transcripts for HAS2 and HAS3 have been found in the oviducts of several animal species (Tienthai et al. 2003, Ulbrich et al. 2004, Mohey-Elsaeed et al. 2015). It has been noted that HAS3 expression was higher in the isthmus compared to the ampulla (Ulbrich et al. 2004, Marei et al. 2013, Mohey-Elsaeed et al. 2015) suggesting that a gradient of decreasing molecular size of HA is experienced during embryo development and progression down the oviduct. In support of this idea,

349 we recently reported that infusion of Hyalovet (500–750 kDa HA) into sheep oviduct on day 2 after

350 mating significantly reduced the incidence of blastocyst formation by day 7 and decreased insulin-like

351 growth factors IGF2 and IGFBP2 expression in the oviduct epithelial cells. In contrast, HYAL-2 infusion

352 increased blastocyst formation, quality and the number of hatched blastocysts and increased HSP70

353 expression in oviductal epithelial cells (Marei et al. 2016a). Similar opposing effects of Hyalovet and

354 HYAL-2 were observed in in vitro-produced sheep embryos (Marei et al. 2016a). Small-sized HA has

355 been shown to regulate the expression of IGFs (Homandberg et al. 2004) and heat shock proteins (Xu

356 et al. 2002), which are important for early embryo development in the oviduct (Aviles et al. 2010). We

357 concluded that the presence of large-size HA in the vicinity of developing embryos disturbs the

358 oviductal environment and embryo development. Interestingly, HYAL-2 mRNA is expressed in sheep

359 embryos starting from the morula stage (Marei et al. 2013). HYAL2 is also expressed in the oviduct

360 with significantly higher levels in the isthmus as compared to the ampulla (Marei et al. 2013). We

361 hypothesise that the small-sized HA produced by oviductal HYAL-2 supports embryo development

362 until the morula stage as cleavage-stage embryos do not express HYAL-2 (Marei et al. 2013).

363 **Embryo implantation-contrasting data**

364 Synthesis of HA is increased significantly in the uterus of mice on the day of implantation (Carson et

365 al. 1987), and HA differential expression in the human endometrium during the menstrual cycle

366 implies its involvement in implantation. In the human uterus, peak expression of HAS and CD44 is in

367 the mid-secretory stage (Afify et al. 2006). There is a plethora of data suggesting the beneficial roles

368 for HA in human embryo implantation (Urman et al. 2008, Hambiliki et al. 2010, Nakagawa et al. 2012).

369 It is thought that implantation failure could be reduced by providing a 'sticky' matrix for the embryos

370 to attach and for this reason HA (which is also called 'magic glue' (Girish & Kemparaju 2007), or

371 EmbryoGlue (Hazlett et al. 2008)) is often used as a supplement in human embryo transfer medium.

372 The presence of HA in mouse embryo transfer medium resulted in higher implantation and live birth

373 rates (Gardner et al. 1999). Similarly, a Cochrane meta-analysis of clinical trials concluded that HA

374 inclusion in embryo transfer media significantly increases clinical pregnancy rates and live birth rates

375 (Bontekoe et al. 2014). In an attempt to develop human embryo culture media free from blood-

376 derived additives, HA was successfully used to replace albumin as a sole macromolecule in a human

377 embryo transfer medium and resulted in high pregnancy and implantation rates (Simon 2003). In

378 addition, the use of HA in transfer media for human frozen embryos significantly increased the

379 implantation rate without increasing the delivery rate (Hambiliki et al. 2010). The mechanism through

380 which HA promotes implantation still remains uncertain. It is generally attributed to facilitating

381 apposition and attachment of the trophectoderm to the maternal endometrium during the early

382 stages of implantation. The role of CD44 at the blastocyst–endometrial interface during implantation

383 was stressed in the study of Illera and coworkers (2004) in rabbits, where intrauterine infusion of anti-

384 CD44 hindered implantation, whereas intra-peritoneal infusion of the same antibodies in the control

385 rabbits had no effect on implantation.

386 On the other hand, some reports contradict the published beneficial effects of HA supplementation in

387 transfer media for embryo transfer (Loutradi et al. 2007, Hazlett et al. 2008, Check et al. 2010). In

388 women who failed to conceive despite at least 3 previous embryo transfers, a 25% clinical pregnancy

389 and 14.2% delivered pregnancy were achieved using EmbryoGlue (high-molecular-weight HA

390 produced by Vitrolife), when compared to women not using EmbryoGlue (39.2% and 39.2%

391 respectively) (Dietterich et al. 2007). Among 120 cases, no statistical difference was found between

392 clinical pregnancies in a control group compared to a test group using EmbryoGlue (38% vs 42%) (Chao

393 et al. 2008). Similar results were obtained by Marek and coworkers (2004) and Chun and coworkers

394 (2016). Routine use of EmbryoGlue in unselected patients did not significantly improve pregnancy or

395 implantation rates after embryo transfer on day 3 or day 5 compared with standard culture media

396 (Hazlett et al. 2008). A better understanding of the mechanism of HA's involvement in reproduction

397 and implantation in particular will improve the prospects for developing an effective clinical

398 intervention based upon this molecule.

399 Accumulation of HA resulting from the dysregulated expression of HASs or HYALs is associated with

400 the disease. For example, failure of HA turnover in HYAL2 knockout mice resulted in HA accumulation

401 and severe cardiopulmonary dysfunction (Chowdhury et al. 2013). Enhanced synthesis of HA by pro-

402 inflammatory cytokines has been associated with renal and rheumatoid diseases (Dahl et al. 1985,

403 Manicourt et al. 1993, Feusi et al. 1999). Similarly, dysregulation of HA metabolism is a typical feature

404 of diabetes (Nieuwdorp et al. 2007) or endometrial cancer (Afify et al. 2005, Nykopp et al. 2010). HA

405 dysregulation may be associated with unexplained infertility (Altmäe et al. 2010), and most relevant

406 here, HA accumulation in the uterus has been linked with early embryo loss including spontaneous

407 abortion (Camenisch et al. 2000, Cordo-Russo et al. 2009). Studies in pregnant mice reported the

408 disappearance of HA at the maternal–embryo interface at days 5–7 of pregnancy (Brown &

409 Papaioannou 1992, 1993, Martins et al. 2003). HYAL-2 is also expressed in trophoblast giant binucleate

410 cells and the multinucleated syncytia of sheep placentomes commencing on day 16 of gestation

411 (Dunlap et al. 2005), coinciding with the attachment and perhaps contributing to the clearance of HA

412 at the implantation sites. In line with these reports, inhibition of HA by the infusion of 4-MU into the

413 sheep uterus on day 14 after natural mating enhanced embryo implantation (Marei et al. 2016b).

414 Therefore, further prospective randomised clinical trials are essential for a robust conclusion to be

415   made concerning the potential beneficial effects of HA pathway manipulation for women undergoing

416   embryo transfer (Loutradi et al. 2008).

417   **Cervix ripening/relaxation**

418   The cervix is the entrance to the uterus. In most species especially sheep, it forms a rigid and tightly

419   closed non-distensible structure, which is necessary to prevent access of microorganisms into the

420   uterus. However, a pathway through the cervix is essential under two conditions. One is for the

421   passage of sperm after coitus and secondly at parturition. The cervical connective tissue is mainly

422   composed of collagen, HA and proteoglycan (Leppert 1992). The HA content of the cervix varies with

423   the stage of oestrus cycle, with the highest and lowest values during pre-LH surge and post-LH surge

424   periods respectively, whereas the value in the luteal stage is intermediate (Perry et al. 2010a).

425   Cervical remodelling at parturition can be divided into cervical softening (a gradual process that occurs

426   several days (gestation day 12 in the rat; Harkness & Harkness 1959) or weeks prior to parturition

427   (during the second trimester of pregnancy in the human; Leppert 1995)) and cervical ripening phases.

428   Cervical ripening, which occurs in the hours (rodents) and days (women) preceding parturition, is

429   characterised by hydration and further growth, decreased tensile strength, increased cervical

430   secretions and lubrication, disorganisation of collagen fibrils, further changes in the composition of

431   GAGs and infiltration of inflammatory cells. These are influenced by the local endocrine milieu, as well

432   as interactions and cross-talk between the cellular components (stroma and epithelium),

433   inflammatory cells and extracellular matrix (Straach et al. 2005).

434   Regulation of HA synthesis in the cervix is a conserved process in mammalian species. Hyaluronan

435   content of cervix increases markedly during late pregnancy in human, sheep, guinea pig, rabbit and

436   rat (Downing & Sherwood 1986, Anderson et al. 1991, Rajabi et al. 1992, El Maradny et al. 1997). The

437   HA level increases from 19% of total GAG in early pregnancy to 71% at term (Akgul et al. 2012) and

438   the majority of cervical HA in mice is synthesised by HAS2 (Akgul et al. 2014). Uchiyama and coworkers

439   (2005) reported peak levels of HAS1 and HAS2 mRNA expression in mouse cervix at delivery. HAS2 has

440   also been identified to be specifically upregulated in women at labour relative to pregnant women not

441   in labour (Straach et al. 2005). HAS2 produces high-molecular-weight HA, which may facilitate the

442   ripening of the cervix by increasing the water content and cytokines (interleukin 8) of the cervix,

443   possibly due to its hydrodynamic and viscoelastic properties (El Maradny et al. 1997). Despite this,

444   more recent work by Akgul and coworkers on HAS knockout mice has revealed that HA is not necessary

445   for the increased cervical distensibility during late gestation (Akgul et al. 2014).

446

447 Artificial insemination (AI) is one of the greatest technologies devised for genetic improvement of
448 animals. The success of AI, however, depends greatly on the ease of introducing the prepared
449 spermatozoa through the cervix into the uterus with the aid of a catheter (Kaabi et al. 2006). As
450 intracervical application of HA has the potential to improve cervical dilation, there may also be a very
451 practical application for HA during artificial insemination in mammals (Perry et al. 2010b).

452 **Cryopreservation of embryos and in vitro embryo production**

453 In cattle, one of the major factors limiting the usefulness of IVF is the problem of cryopreservation of
454 bovine oocytes. This process is frequently accompanied by intracellular ice formation and generation
455 of reactive oxygen species that subsequently lead to degeneration during thawing, and hence, a high
456 chance of fertilisation failure. Addition of HA to the culture medium may perhaps alleviate this
457 problem, although it remains to be seen whether the observations of improvements in embryo
458 cryopreservation can be replicated in oocytes. HA-supplemented media enhances blastocyst yield,
459 improves survival after blastocyst vitrification and promotes post-transfer survival of fresh morula and
460 blastocyst stage embryos as compared to those in medium supplemented with bovine serum albumin
461 (Block et al. 2009). HA improves the developmental capacity of bovine embryos under in vitro
462 conditions and is warranted as a culture supplement for in vitro production of bovine embryos,
463 particularly if they are to be cryopreserved (Stojkovic et al. 2002). In humans, a high level of HA in the
464 embryo transfer medium was found to improve the clinical pregnancy rate and chances of attachment
465 of frozen-thawed embryos (Hambiliki et al. 2010), possibly by reducing apoptosis and induction of
466 heat shock protein. Small fragments of HA induce heat shock protein and suppress apoptosis in vitro
467 (Xu et al. 2002). Similar effects promoting cryosurvival have been reported in stem cells where both
468 post-thaw viability and phenotypic characteristics are improved by HA (Turner et al. 2012).

469 **Integral model to explain the reproductive functions of HA**

470 It is apparent that most of the signalling effects of HA have been attributed to low-molecular-weight
471 HA; however, it becomes a subject of contention whether low or high molecular weight HA is more
472 beneficial (Camenisch & McDonald 2000). HA's biological functions depend upon its molecular weight.
473 Interestingly, low- and high-molecular-weight HAs have opposing functions. The functions of high-
474 molecular-weight HA are premised on its physical properties of being hygroscopic, space filling,
475 antiangiogenic and immunosuppressive, impeding differentiation and causing cell cycle arrest (Fraser
476 et al. 1997, Necas et al. 2008). On the contrary, low-molecular-weight HA is associated with pro-
477 inflammatory, angiogenic and anti-apoptotic effects, facilitating cell-to-cell interaction, cell
478 proliferation and HA-receptor-mediated signalling (Toole 2004, Matou-Nasri et al. 2009).

479   As far as reproduction is concerned, we need to consider the anatomical component and physiological

480   status of the tissue in context. Clearly, high-molecular-weight HA may be required at a particular point

481   in time by a reproductive tissue, whereas the next phase of the same tissue's differentiation may

482   require low-molecular-weight HA. Low-molecular-weight HA may be produced directly by HAS3 or

483   through cleavage of high-molecular-weight HA by HYAL i. Therefore, we wish to emphasise that the

484   prediction of HA function resulting from HAS1, HAS2 or HAS3 is difficult without taking into

485   consideration the HA-degrading activity of HYALs. The functions of HA therefore depend not only upon

486   its intrinsic properties but also upon a complex balance of polymerisation by HASs, depolymerisation

487   by HYALs and interactions with HA receptors and HA-binding proteins as well as other intracellular

488   and extracellular components such as growth factors and cytokines.

489   Based upon our work and that of others, as outlined previously, we now propose a model that takes

490   into account the integrated functions of HA according to size, the location of HA in different places

491   throughout the reproductive tract and the timing of its presence, relative to female reproductive

492   cycles and the prevailing hormonal environment at any given moment (Fig. 1). Such model is primarily

493   applicable to ungulate species such as sheep and cow. Nevertheless, the three genes encoding

494   hyaluronan synthases are highly conserved in vertebrates, and the simple structure of HA is conserved

495   throughout all mammals. This implies that a similar pattern of expression and regulation may be

496   generalised to other mammals.

497   In conclusion, we have presented evidence from a range of mammalian species for the central role of

498   HA in key events in reproduction. HA is ubiquitous; however, its actions at different locations within

499   the reproductive tract depend critically upon its size, which is controlled by the balance of synthesis

500   by one of three isoforms, degradation, which is undertaken principally by two hyaluronidase isoforms,

501   together with a sperm-specific isoform around fertilisation, and its signalling pathways, which occur

502   via CD44 and RHAMM. Superimposed upon these variables is the cyclicity inherent in female

503   mammalian reproduction, with steroid hormones affecting the synthetic enzymes and thereby tilting

504   the balance of small- or large-molecular-weight HA being predominant. A better understanding of how

505   the different components are orchestrated will provide opportunities for correction of pathology and

506   promotion of normal fertility or contraception in a range of situations and species. In particular,

507   assisted conception in animal species, rare species preservation and human IVF will benefit from

508   improved reagents and strategies to control implantation.

509   **Declaration of interest**

510   The authors declare that there is no financial a) or other potential conflict of interest; or (b) conflict

511   of interest, that could be perceived as prejudicing the impartiality of the research reported in the

512   review paper.

513

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Table 1. Concentration of hyaluronan in fluids and tissues of the reproductive system.

| Reproductive fluid/tissue	Concentration of HA	Species	Reference
Seminal plasma	3.4± 1.14 µg/ml 2.3 ± 0.72 µg/ml	Alpaca Ram	Kershaw-Young et al., 2012
Follicular fluid	50.0 ± 2.6 ng/ml fertilized oocyte 66.9 ± 5.9 ng/ml unfertilised oocyte	Human	Saito et al, 2000
Uterus	4053.0 ± 651.4 ng/g dry tissue during dioestrus.	Mouse	Teixeira Gomes et al., 2009
Oviductal fluid	3.9 mg/ml at metoestrus (minimum) 10.4 mg/ml proestrus (maximum)	Pig	Tienthai et al., 2000
Cervix	3.0 ± 0.4 ng/mg dry tissue at pre-LH 2.0 ± 0.2 ng/mg dry tissue at post LH 2.1 ± 0.2 ng/mg dry tissue	Sheep	Perry et al., 2010a
Amniotic fluid	20 µg/ml (weeks 16-20) 1 µg/ml (week 30 to week 30) 5.1 ug/ml (week 12) 1.9 ug/ml (weeks 15-17)	Human Sheep	Dahl et al., 1983 Dahl et al., 1989
Serum	11.4 ± 4.5 ng/ml (weeks 5-14) 13.6 ± 2.8 ng/ml (weeks 15-26) 46.9 ±7.9 ng/ml (weeks 38-40) 100.4 ± 11.3 ng/ml (labour)	Human	Kobayashi et al., 1999
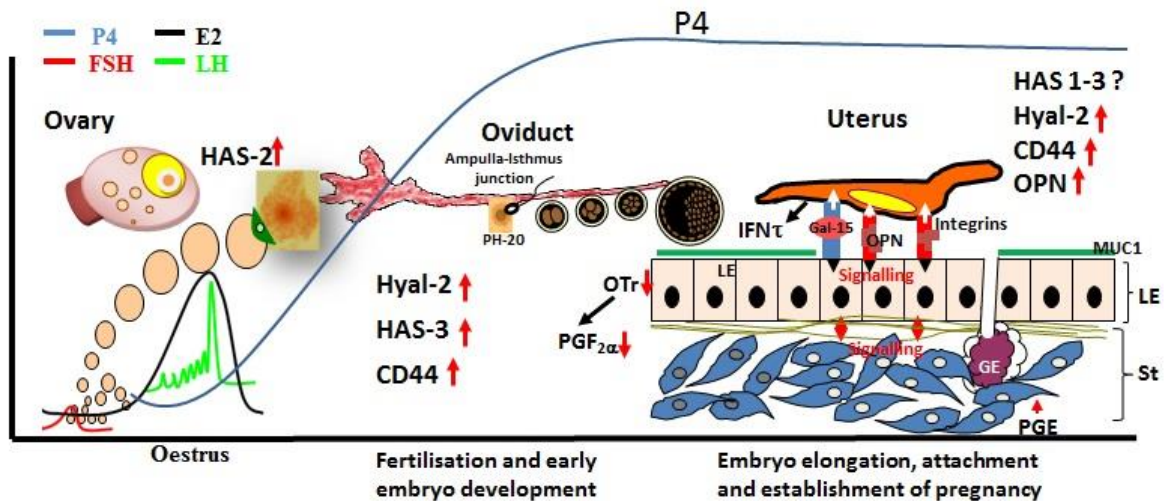
Figure 1. Model of the regulation of HA biosynthesis, degradation and function in the reproductive system. We hypothesise that at least in ungulates, steroid hormones orchestrate a sequential expression pattern for HA of different sizes in the reproductive system, with oestradiol (E2) inducing expression of HAS-2 resulting in the production of large-molecular-weight HA to support ovulation and fertilisation, followed by the progesterone (P4)-dominated phase, which upregulates CD44 expression and stimulates small-size HA production by HAS3 and HA fragments Hyal-2. Hyal-2 and HA fragments support early embryo development and induce the expression of adhesion molecules and signalling cascades required for the attachment of the blastocyst to the uterine luminal epithelium (LE) and establishment of pregnancy. FSH, follicle-stimulating hormone; GE, glandular epithelium; LH, luteinising hormone; IFNt, interferon tau; MUC1, mucin 1; OPN, osteopontin; OTr, oxytocin receptor; PG"2a, prostaglandin F2 alpha; PGE, prostaglandin E; St, uterine stroma cells.