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Regulation and roles of the hyaluronan system in mammalian reproduction

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Abstract

Hyaluronan (HA) is a non-sulfated gylcosaminglycan naturally occurring polymer found in tissues and fluids of mammals including the reproductive system. Its biosynthesis by HA synthasese (HAS1-3) and catabolism by hyaluronidases (HYALs) is regulated by ovarian steroid hormones. Depending on its molecular size, HA functions both as a structural component of tissues in the form of high molecular weight HA, or a signalling molecule in the form of small HA molecules or HA fragments which is mediated through interaction with its specific cell membrane receptors. HA is produced in the oocytes and embryos and in various segments of the reproductive system. This review provides information about expression and function of members of the HA system, including HAS, HYALs and HA receptors in various processes from folliculogenesis to oocyte maturation fertilisation and early stage embryo development to pregnancy, and its application in assisted reproduction technologies. Particular emphasis has been made on the role of the HA system in preimplantation embryo development and embryo implantation, and a hypothetical sequential model is proposed.

Introduction

Hyaluronan (HA), also known as hyaluronic acid or hyaluronate, is a high molecular weight anionic member of a group of macromolecules called glycosaminoglycans (GAGs) that constitute components of the extracellular matrix (ECM) in all animal tissues. Other GAGs include heparin sulphate, dermatan sulphate, keratin sulphate and chondroitin sulphate. HA is the simplest of all the GAGs and has a number of unique properties that distinguish it from other GAGs. (i) It is non-sulphated, (ii) it is a linear polysaccharide of thousands of repeated units of alternating D- glucuronic acid and N-acetylglucosamine (Weissmann, et al. 1954), (iii) it is synthesised at the plasma membrane rather than in the Golgi apparatus (Prehm 1984) and (iv) it is extruded into ECM via the cell surface as it is synthesised (Tammi, et al. 2002), and finally (v) HA is not restricted to the ECM, rather, its intracellular localisation has also been reported (Contreras-Ruiz, et al. 2011).

The concentrations of HA within the reproductive tract vary from one mammalian species to another. Some examples are provided in Table 1. HA is present in the oviduct, uterus and cervix (Afify et al. 2006, human; Perry et al. 2012, sheep; Raheem et al. 2013, sheep) and also produced by the cumulus and granulosa cells of ovarian follicles (Kimura et al. 2002, pig; Schoenfelder & Einspanier 2003, cow; Chavoshinejad et al. 2014, sheep). The role of HA in reproductive biology and clinical applications is gaining increasing recognition. HA's expansion of cumulus cells at ovulation (Salustri et al. 1989; mouse) and induction of cervical ripening during parturition (El Maradny et al. 1997, rabbit; Straach et al. 2005, mouse) are well documented. Treatment of ovariectomised mice with progesterone increased uterine HA concentration (Maioral et al. 2016). We and others have shown that the expression of HA synthases is influenced by ovarian steroid hormones having a differential effect on the expression of specific HAS and production of different size HAs during reproductive cycle and at parturition (Afify et al. 2006, Teixeira Gomes et al. 2009; mouse, Raheem et al. 2013). In addition, a range of growth factors, such as epidermal growth factor (Pienimaki et al. 2001) and transforming growth factor-β (Pasonen-Seppanen et al. 2003), and cytokines, such as interleukin 1-β (Oguchi & Ishiguro 2004) and interferon gamma (Campo et al. 2006), as well as local mediators such as prostaglandins (Sussmann et al. 2004) affect HAS expression. The actions of HA are mediated through its cell surface receptors CD-44 and RHAMM involving MAP kinases and Akt signalling (Straach et al. 2005, Kultti et al. 2010). Moreover, HA is expressed at different stages of pre-implantation embryo development (Marei et al. 2013, cow). Recently, HA has attracted more interest because its addition to embryo culture media seems to benefit in vitro fertilisation (IVF) and embryo transfer (Palasz et al. 1993; cow, mouse, Palasz et al. 2006; cow, Choudhary et al. 2007; mouse, Dattena et al. 2007; sheep, Hazlett et al. 2008; human, Hambiliki et al. 2010; human, Nakagawa et al. 2012; human).

The HA system includes hyaluronan synthases (HAS), HA-degrading enzymes (hyaluronidases; HYALs) and HA receptors. In this review, we shall explain the roles and regulation of the HA system in mammalian reproduction with particular emphasis on pre-implantation embryo development and embryo implantation.

Hyaluronan biosynthesis

HA is synthesised by three different but related trans-membrane enzymes named hyaluronan synthases (HAS1–3) (Prehm 1984), which produce different size HAs with diverse biological functions (Itano et al. 1999, Stern et al. 2006). The HAS genes have promoters reacting to common transcriptional signals in addition to their own specific responses (reviewed in Tammi et al. 2011).

HAS2 synthesises HA of higher molecular weight than HAS1, in the range of >2 × 106 Da (Itano et al. 1999), whereas HAS3 synthesises HA of low molecular weight (1 × 105–1 × 106 Da) and represents the most active isoform of HAS. Normally, HA turnover in the body is quite constant and consistently rapid. One-third of the 15 g of HA in the human is replaced on a daily basis (Stern 2004). As formulated by Stern (2003, 2004), a sequence of enzymatic reactions by HYALs cleave high-molecular-weight HA at the β-N-acteyl linkage, progressively degrading HA by generating smaller fragments. There are six HYAL isoforms in the human genome, HYAL1, HYAL2, HYAL3, HYAL4, HYALP1, and sperm adhesion molecule 1 (SPAM1) (also known as PH20) (Csoka et al. 1999). HYAL1 and 2 are the most important isoforms involved in HA degradation and catabolism in somatic cells (Bastow et al. 2008). HYAL2 is a glycosylphosphatidylinositol-anchored enzyme attached to the external surface of the plasma membrane and expressed in many tissues (Lepperdinger et al. 2001). It has a specific binding capacity for the high-molecular-weight HA, cleaving it to fragments of ~20 kDa (about 50 disaccharides) (Stern et al. 2006). HYAL1 utilises HA of any size as a substrate to generate tetrasaccharides (4–8 saccharides in size) (Frost et al. 1997). HYAL3 and HYAL4 lack hyaluronidase activity and seem to play a non-significant role in constitutive HA degradation (Harada & Takahashi 2007, Kaneiwa et al. 2012). Similarly, HYALP1 that is present in mouse testis does not degrade HA (Reitinger et al. 2007). The role of SPAM1 is discribed later in fertilisation paragraph.

HA interacts with cells through its receptors, which include cluster domain 44 (Aruffo et al. 1990, CD44) and receptor for HA-mediated motility (Turley et al.2002, RHAMM). CD44 has been detected in various segments of the reproductive tract in mouse (Kennel et al. 1993), cow (Bergqvist et al. 2005a), sheep (Perry et al. 2010a), mare (Rodriguez et al. 2011) and human (López et al. 2013) under normal physiological conditions. It has also been detected in cow (Furnus et al. 2003), mouse (Matsumoto et al. 2004) and human (Campbell et al. 1995) embryos. Interaction between HYAL2 and CD44 facilitates the endocytosis of HA, which undergoes further degradation by lysosomal HYAL1 into smaller HA fragments (Lepperdinger et al. 2001).

In addition to its function as an adhesion molecule, there is evidence showing that CD44 is a potent signalling molecule. Many studies have shown that HA–CD44 interaction can initiate several signalling events under physiological or pathological conditions such as oocyte maturation and cancer pathogenesis (Schoenfelder & Einspanier 2003, Kimura et al. 2007, Toole 2009, Yokoo et al. 2010, Marei et al. 2012, Bourguignon & Bikle 2015, Misra et al. 2015). HA-mediated cell surface signalling through CD44 is usually initiated by low-molecular-weight HA or HA-oligosaccharides resulting in cell migration or cell proliferation (Lee & Spicer 2000). HA–CD44 interaction may also stimulate intracellular signalling through extracellular regulated kinase (ERK), phosphoinositide 3-kinase (P13K), Rac and Ras in various cell types (Kothapalli et al. 2008, Pure & Assoian 2009). Although many studies on HA–CD44 signalling focus on cancer, HA–CD44 signalling is also observed under physiological conditions. A study from our laboratory showed that small HA fragments of 20 kDa produced by treatment of bovine embryos with HYAL2 caused increased phosphorylation of mitogen-activated protein kinase MAPK1/3 signalling, resulting in increased blastocyst formation and quality, characterised by higher cell numbers. This effect was abrogated with the inhibition of CD44 (Marei et al. 2013). Another study also showed signalling by HA in human placenta through MAPK1/3 and PI3K pathways, which enhanced trophoblast growth and invasion and possibly placenta angiogenesis (Zhu et al. 2013a). Even though this study did not show that the signalling was through HA binding to CD44, it is likely to be through HA–CD44 because CD44 is the major receptor for HA, and earlier studies have shown the expression of CD44 in the human trophectoderm (Campbell et al. 1995) and trophoblast (Goshen et al. 1996), where it was proposed to play a significant role in placenta angiogenesis.

RHAMM (otherwise known as CD168) is alternatively spliced; hence, different isoforms of the protein were found both on the cell surface and intracellularly (cytoplasm, cytoskeleton, mitochondria, nucleus and nucleolus) (Turley et al. 2002). Intracellular RHAMM interacts with several signalling and cytoskeletal proteins, including Src through its interaction with microtubules and actin filaments (Assmann et al. 1999). Although RHAMM is not essential for embryo viability (Tolg et al. 2003), it has been found to play a profound role in several relevant cellular events, such as mitosis, cell proliferation and migration (Turley et al. 2002). RHAMM is highly expressed in the G2/M phase of the cell cycle, thus controlling mitosis (Mohapatra et al. 1996, Assmann et al. 1999). Deletion of the RHAMM C-terminus results in impaired spindle orientation in the dividing granulosa cells, folliculogenesis defects and subsequent female hypofertility in mice (Li et al. 2015). RHAMM knockdown results in the downregulation of several pluripotency markers in hESC, induction of early extraembryonic lineages, loss of cell viability and changes in hESC cycle suggesting its major roles in the maintenance of human embryonic stem cell pluripotency and cell viability (Choudhary et al. 2007). RHAMM protein and mRNA are expressed at all stages of human pre-implantation embryo development from 2-cell to blastocyst (Choudhary et al. 2007). The relative expression of RHAMM increased transiently from 4-cell to 8- to 12-cell stage embryos and then remained static in morula and early blastocyst, but significantly increased in expanded blastocysts (Choudhary et al. 2007). The same was confirmed in bovine embryos where mRNA for RHAMM/IHABP (intracellular HA binding protein) where the highest expression was seen in the expanded blastocyst (Stojkovic et al. 2003). Moreover, Ozbilgin and coworkers reported spatiotemporal expression of RHAMM protein in mouse endometrium during the oestrous cycle and peri-implantation period, suggesting its possible role in endometrial receptivity (Ozbilgin et al. 2012). Inhibition of RHAMM signalling by culture of sheep embryos in the presence of anti-RHAMM antibody resulted in the arrest of the embryo development at the 6- to 8-cell stage (unpublished data). Considering the co-presence of HA, CD44 and RHAMM in the reproductive system, it is highly likely that they work together to support mitotic activity in the developing embryos ensuring the development of blastocysts with high cell numbers.

Hyaluronan in the ovarian follicle

A significant portion of the ECM of the ovarian follicles consists of HA (Irving-Rodgers & Rodgers 2005). HA serves both as a structural component of ovarian follicles and in signalling cascades leading to oocyte maturation and ovulation (Rodgers et al. 2003, Kimura et al. 2007). In mice, both oocytes and cumulus cells produce HA during folliculogenesis (Salustri et al. 1992, Ueno et al. 2009). Indeed, denuded oocytes produce increasing amount of HA during culture, which was suggested to be involved in the enlargement of the perivitelline space in mouse oocytes (Ueno et al. 2009). The granulosa cell layer of the mouse antral follicle is capable of HA synthesis (Salustri et al. 1992). HA was also detected in the extracellular matrix of rat granulosa and theca cell layers of primary and more advanced follicles (Takahashi et al. 2014). HAS1 is the dominant HAS protein in theca cells of swine ovaries and may be responsible for an increase in the HA concentration of follicular fluids in atretic follicles (Miyake et al. 2009) containing macrophages expressing CD44 as a phagocytic receptor involved in phagocytosis of the apoptotic granulosa cells (Miyake et al. 2006). In sheep ovaries, we recently reported the expression of HAS and CD44, which were mainly localised in the granulosa cells (GCs) (Chavoshinejad et al. 2014). Large-size HA produced by the follicular cells contributes to the osmotic gradient of the antral follicle resulting in the accumulation of the follicular fluid and antrum formation (Clarke et al. 2006; cow, Rodgers & Irving-Rodgers 2010). This osmotic gradient across the basal lamina restricts the movement of molecules above 100 kDa from the theca capillaries into the follicular fluid in healthy follicles (Irving-Rodgers et al. 2002; cow, Rodgers & Irving-Rodgers 2010). It was reported that the LH surge permeabilises the blood barrier of the follicle, and serum glycoproteins in the inter-α-inhibitor family (IαI) can then enter the antral cavity (Hess et al. 1999; mouse, Rodgers et al. 2003; cow). However, it is now evident that the family of IαI molecules can freely cross the blood–follicle barrier; follicular fluid collected at any stage of folliculogenesis can be successfully used instead of serum to form expanded cumulus ECM in pig (Nagyova 2015); and covalent binding between hyaluronan and heavy chains of IαI is essential for the expansion of the cumulus cell mass before ovulation (Chen et al. 1996; mouse, Nagyova et al. 2004; pig). Using cultures of sheep granulosa cells, we have shown that reproductive hormones differentially regulate HAS2, HAS3 and CD44 in ovaries (Chavoshinejad et al. 2014). Oestradiol, when combined with IGF-1, insulin and FSH, stimulated HAS2 mRNA expression, which is essential for cumulus cell expansion prior to ovulation.

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

Cumulus cell expansion and oocyte maturation

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

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

The preovulatory surge of LH activates HAS2 expression leading to the production of high-molecular-weight HA by the cumulus cells; water absorbed by the HA results in the expansion of the COC (Saito et al. 2000, Stock et al. 2002). HA secreted by the mouse cumulus oophorus is detectable between 2 h and 18 h, peaking at 4–10 h after the LH surge (Tirone et al. 1997, Zhuo & Kimata 2001). In mice, this HA-rich matrix is organised into a cross-linked network through the cooperative action of IαI, pentraxin-3 and TSG-6 (Sato et al. 2001, Fulop et al. 2003, Salustri et al. 2004) to gain a stabilised viscoelastic state that is required to facilitate the transfer of the oocyte to the oviduct for fertilisation (Salustri et al. 1999). However, a recent report showed that binding of TSG-6 to HA does not play a major role in the stabilisation of the cumulus cell matrix in mice (Briggs et al. 2015).

In pigs, COCs cultured in the presence of an HA synthesis inhibitor (6-diazo-5-oxo-1-norleucine) or HYAL failed to expand at all (Yokoo et al. 2010). Our studies in sheep also revealed that the formation of large-molecular-weight HA is essential for cumulus cell expansion (Marei et al. 2012). HAS2 and CD44 expression in bovine cumulus cells were found to be potential markers of oocyte competence (Assidi et al. 2008), and increased CD44 in follicular fluid was associated with good-quality oocytes (Ohta et al. 2001). The localisation of CD44, the major cell surface receptor for HA in cumulus cells (Kimura et al. 2002), suggests that HA–CD44 interaction may also be a likely player in oocyte maturation. HA–CD44 interaction regulates the tyrosine phosphorylation of Connexin 43 (the major gap junction protein found in the COCs), which leads to the closure of the gap junction and subsequent activation of maturation promotion factor (MPF) activity (Sato & Yokoo 2005). The latter brings about resumption of meiosis in oocytes that have been arrested in meiotic prophase I until shortly before ovulation. Apparently, this activation occurs regardless of the structural expansion of cumulus cells as inhibition of cumulus cell expansion by HYAL2 did not affect further fertilisation and embryo development (Marei et al. 2012). On the other hand, inhibition of HA synthesis by 4-methylumbelliferone during in vitro maturation completely inhibited the development to the blastocyst stage, an effect which was partially alleviated by the addition of exogenous HA (Marei et al. 2012). This further emphasises the importance of HA signalling during oocyte maturation.

Sperm-related functions

HA is expressed in various segments of the male reproductive tract, including the epididymis, seminal vesicles, prostate and Cowper's gland and with traces in the testes (Tammi et al. 1994). The accessory sex glands provide the fluid medium necessary for nourishment and transportation of spermatozoa through the reproductive tract. HA is a component of the seminal plasma in ram and alpaca (Kershaw-Young et al. 2012) and may be responsible for the viscosity of the seminal plasma as observed in llama and alpaca (Bravo et al. 2000). Sakairi and coworkers (2007) reported the presence of HA in the seminal vesicles of immature pigs, without investigating further its particular roles. However, they speculated that it may contribute to the regulation of homeostasis rather than sperm functioning. Studies in mice suggest HA involvement in spermatogenesis (Thakur et al. 2006), even though the mechanism still remains to be clarified. HA induces sperm capacitation (Tienthai et al. 2004, Tienthai 2015) by the activation of membrane-associated adenylate cyclase (Fernandez & Cordoba 2014), and it also enhances the acrosome reaction in bovine (Gutnisky et al. 2007), and porcine (Suzuki et al. 2002) without necessarily modifying the sperm nuclear condensation and morphology, possibly by decreasing the formation of vacuoles in the sperm head (Montjean et al. 2012). In dog spermatozoa, HA accelerates the calcium influx into the sperm cytoplasm and increases lactate dehydrogenase activity and cAMP production, provoking capacitation (Kawakami et al. 2006). HA may also help to prevent polyspermy during in vitro fertilisation as well as supporting blastocyst development (Kano et al. 1998) and quality by reducing apoptosis (Opiela et al. 2014). Supplementation of HA to human sperm in the swim-up procedure increased the sperm motility and reduced the number of sperm with DNA damage (Saylan & Duman 2016).

One of the criteria by which spermatozoa are assessed is their progressive motility. In artificial insemination where semen is frozen and stored for future use, the viability of spermatozoa is greatly affected by the reduction in motility and membrane stability during cryopreservation (Critser et al. 1988). However, this impairment could be overcome by the addition of HA to the semen diluent. HA supplementation of the diluent helps to preserve post-thaw viability of boar spermatozoa in vitro and maintains the membrane stability after cryopreservation (Pena et al. 2004, Qian et al. 2016). Similar results were found in dogs (Prinosilova et al. 2009). Likewise in human, HA has been proposed to enhance sperm motility (Ghosh et al. 2002) through phosphorylation of proteins that include HA-binding protein (Ranganathan et al. 1995).

Hyaluronan-binding protein 1 (HABP1), a 68 kDa glycoprotein, was detected on spermatozoa of cattle, buffalo, rat and human (Ranganathan et al. 1994, Bharadwaj et al. 2002, Ghosh et al. 2002, Ghosh & Datta 2003). It participates in sperm–oocyte interaction (Ghosh et al. 2007) through its mannose residues (Ghosh & Datta 2003). A reduction in the level of HABP1 is associated with loss of sperm motility (Ghosh et al. 2002), the mechanism that may be attributed to the ability of HABP1 to modulate sperm–oocyte interaction even in sub-fertile spermatozoa (Ghosh et al. 2007). The number of spermatozoa bound to an oocyte was reduced significantly in the presence of D-mannosylated albumin, the universal blocker of sperm–oocyte interaction, and this effect could be reversed by the addition of purified recombinant HABP1 (Ghosh et al. 2007).

The correlation of HABP1 with sperm motility initiated the development and use of sperm HA-binding assay (sHABA) in assessing the sperm viability in fertility clinics (Huszar et al. 2003). sHABA has proved useful in selecting spermatozoa with a high DNA integrity and morphology and may sometimes be used as a screening test for sperm quality before IVF (Worrilow et al. 2013). However, its use remains controversial as sHABA does not predict freeze-thawing sperm survival (Boynukalin et al. 2012), and it does not predict the pregnancy rates either in intrauterine insemination (Yogev et al. 2010) or IVF (Ye et al. 2006, Boynukalin et al. 2012).

Intracytoplasmic sperm injection (ICSI) is used in clinical IVF to bypass the physiological barriers of the cumulus oophorus and the zona pellucida in the treatment of severe male infertility due to low sperm numbers or function. The selection of the sperm for injection may perhaps be promoted by HA binding as a screening technique, given that HA-bound sperm in general are fully matured and have better morphology with a reduced risk of aneuploidy or fragmented DNA (Pregl Breznik et al. 2013), which has been reported as associated with increased pregnancy and implantation rates (Worrilow et al. 2013). However, it is not a reliable test for the prediction of sperm intracellular reactive oxygen species, DNA fragmentation and DNA maturity and mitochondrial membrane potential risks and healthy spermatozoa selection (Rashki Ghaleno et al. 2016), and the result of a recent meta-analysis 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

Isoforms of HYAL found in sperm are SPAM1 and HYAL5. These unique hyaluronidases are located in the testis or epididymis and have been detected in mouse (Zhang & Martin-DeLeon 2001, Chen et al. 2006), pig (Day et al. 2002) and human (Evans et al. 2003). It is secreted and located on the sperm surface during epididymal maturation (Deng et al. 2000, Day et al. 2002, Evans et al. 2003, Chen et al. 2006, Martin-DeLeon 2006). SPAM1 is a GPI-anchored hyaluronidase (also known as PH20), which depolymerises HA into tetrasaccharide and hexasaccharide products (Kim et al. 2005, Hofinger et al. 2008, Thompson et al. 2010). It is unique among hyaluronidases, in that it shows enzyme activity at both acidic and neutral pH, activities that appear to involve two different domains in the protein (Gmachl & Kreil 1993, Cherr et al. 2001). Several studies have confirmed that SPAM1 is the only hyaluronidase identified to date in mammalian sperm, including the sperm of guinea pigs, rats, macaques and humans (Cherr et al. 2001, Zheng et al. 2001). It is also present in the lysosome-derived acrosome, where it is bound to the inner acrosomal membrane (Morin et al. 2010). SPAM1 is initially synthesised as a polypeptide with an apparent molecular weight of 64 kDa. During the course of sperm maturation, part of SPAM1 is processed into two fragments that are linked through disulphide bridges, such as at the N-terminal domain of 41–48 kDa and at the C-terminal domain of 27 kDa.

Hyal5 is exclusively expressed in the testis and the plasma and acrosomal membranes of rodent sperm (Kim et al. 2005). It is enzymatically active in the pH range 5–7 and inactive at pH 3 and 4. Both Hyal5-enriched SPAM1-free soluble protein extracts and SPAM1-deficient mouse sperm were capable of dispersing cumulus cells, which was inhibited by the presence of a hyaluronidase inhibitor, apigenin.

These results suggest that in the mouse, Hyal5 may function principally as a 'cumulus matrix depolymerase' in the sperm penetration through the cumulus mass (Kim et al. 2005).

The concentration of HA in follicular fluid has been used to estimate the viability of oocytes for fertilisation with concentrations as high as 50 ng/mL (Saito et al. 2000) to 239.3 ng/mL (Babayan et al. 2008) being associated with fertilisation of the oocyte and embryo implantation in human.

Despite the presence of HYAL in mouse, its role in fertilisation remains uncertain. Kimura and coworkers (2009) showed SPAM1 to be required for sperm penetration through the cumulus matrix for fertilisation in mice. It was also reported to be involved in sperm-ZP binding (Myles & Primakoff 1997, Cherr et al. 2001) and induction of the acrosome reaction (Overstreet et al. 1995, Sabeur et al. 1998). Reddy and coworkers (1980) used a hyaluronidase inhibitor in mice to clarify HYAL function in fertilisation. In their study, myochrysine, a natural inhibitor of HYAL with no effect on the acrosome reaction, inhibited fertilisation due to reduced breakdown of the COC. However, a similar effect was not observed when using oocytes devoid of follicular cells. Another study using a double knockout model confirmed that sperm serine proteases, ACR (acrosin) and/or PRSS21 (testisin), function cooperatively with SPAM1 in cumulus penetration in mice (Zhou et al. 2012). In addition, HA fragments generated by SPAM1 stimulate cytokine/chemokine production via the TLR2 and TLR4 pathways in cumulus cells of ovulated COCs, which may enhance fertilisation (Shimada et al. 2008). However, mice lacking SPAM1 and HYAL5 are fertile, indicating that the HA-degrading ability of HYAL in mouse sperm is not essential for fertilisation (Kang et al. 2010). It is also possible that SPAM-1 secreted by the oestrous uterus and oviduct, with the potential to bind to sperm during capacitation (Zhang & Martin-DeLeon 2003, Griffiths et al. 2008) might have compensated for its absence in the sperm itself in the knockout model. In addition, the detection of functionally active HYAL5 on the surface of SPAM1-deficient spermatozoa confirmed that compensation was possibly occurring by this HYAL (Zhang et al. 2005). Moreover, HYAL2 that was reported to be present in mouse sperm (Modelski et al. 2014) may have contributed to this functional redundancy.

Pre-implantation embryo development

In cattle, HAS2 and HAS3 are expressed at all stages of early embryo development from 2-cell to blastocyst (Marei et al. 2013). We found that HAS2 mRNA expression tended to decrease with the progression to the blastocyst stage, whereas HAS3 expression was maintained. Moreover, HA receptors CD44 and RHAMM were also expressed at all stages (Furnus et al. 2003, Palasz et al. 2006, 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, we recently reported that infusion of Hyalovet (500–750 kDa HA) into sheep oviduct on day 2 after mating significantly reduced the incidence of blastocyst formation by day 7 and decreased insulin-like growth factors IGF2 and IGFBP2 expression in the oviduct epithelial cells. In contrast, HYAL-2 infusion increased blastocyst formation, quality and the number of hatched blastocysts and increased HSP70 expression in oviductal epithelial cells (Marei et al. 2016a). Similar opposing effects of Hyalovet and HYAL-2 were observed in in vitro-produced sheep embryos (Marei et al. 2016a). Small-sized HA has been shown to regulate the expression of IGFs (Homandberg et al. 2004) and heat shock proteins (Xu et al. 2002), which are important for early embryo development in the oviduct (Aviles et al. 2010). We concluded that the presence of large-size HA in the vicinity of developing embryos disturbs the oviductal environment and embryo development. Interestingly, HYAL-2 mRNA is expressed in sheep embryos starting from the morula stage (Marei et al. 2013). HYAL2 is also expressed in the oviduct with significantly higher levels in the isthmus as compared to the ampulla (Marei et al. 2013). We hypothesise that the small-sized HA produced by oviductal HYAL-2 supports embryo development until the morula stage as cleavage-stage embryos do not express HYAL-2 (Marei et al. 2013).

Embryo implantation-contrasting data

Synthesis of HA is increased significantly in the uterus of mice on the day of implantation (Carson et al. 1987), and HA differential expression in the human endometrium during the menstrual cycle implies its involvement in implantation. In the human uterus, peak expression of HAS and CD44 is in the mid-secretory stage (Afify et al. 2006). There is a plethora of data suggesting the beneficial roles for HA in human embryo implantation (Urman et al. 2008, Hambiliki et al. 2010, Nakagawa et al. 2012). It is thought that implantation failure could be reduced by providing a ‘sticky’ matrix for the embryos to attach and for this reason HA (which is also called ‘magic glue’ (Girish & Kemparaju 2007), or EmbryoGlue (Hazlett et al. 2008)) is often used as a supplement in human embryo transfer medium. The presence of HA in mouse embryo transfer medium resulted in higher implantation and live birth rates (Gardner et al. 1999). Similarly, a Cochrane meta-analysis of clinical trials concluded that HA inclusion in embryo transfer media significantly increases clinical pregnancy rates and live birth rates (Bontekoe et al. 2014). In an attempt to develop human embryo culture media free from blood-derived additives, HA was successfully used to replace albumin as a sole macromolecule in a human embryo transfer medium and resulted in high pregnancy and implantation rates (Simon 2003). In addition, the use of HA in transfer media for human frozen embryos significantly increased the implantation rate without increasing the delivery rate (Hambiliki et al. 2010). The mechanism through which HA promotes implantation still remains uncertain. It is generally attributed to facilitating apposition and attachment of the trophectoderm to the maternal endometrium during the early stages of implantation. The role of CD44 at the blastocyst–endometrial interface during implantation was stressed in the study of Illera and coworkers (2004) in rabbits, where intrauterine infusion of anti-CD44 hindered implantation, whereas intra-peritoneal infusion of the same antibodies in the control rabbits had no effect on implantation.

On the other hand, some reports contradict the published beneficial effects of HA supplementation in transfer media for embryo transfer (Loutradi et al. 2007, Hazlett et al. 2008, Check et al. 2010). In women who failed to conceive despite at least 3 previous embryo transfers, a 25% clinical pregnancy and 14.2% delivered pregnancy were achieved using EmbryoGlue (high-molecular-weight HA produced by Vitrolife), when compared to women not using EmbryoGlue (39.2% and 39.2% respectively) (Dietterich et al. 2007). Among 120 cases, no statistical difference was found between clinical pregnancies in a control group compared to a test group using EmbryoGlue (38% vs 42%) (Chao et al. 2008). Similar results were obtained by Marek and coworkers (2004) and Chun and coworkers (2016). Routine use of EmbryoGlue in unselected patients did not significantly improve pregnancy or implantation rates after embryo transfer on day 3 or day 5 compared with standard culture media (Hazlett et al. 2008). A better understanding of the mechanism of HA's involvement in reproduction and implantation in particular will improve the prospects for developing an effective clinical intervention based upon this molecule.

Accumulation of HA resulting from the dysregulated expression of HASs or HYALs is associated with the disease. For example, failure of HA turnover in HYAL2 knockout mice resulted in HA accumulation and severe cardiopulmonary dysfunction (Chowdhury et al. 2013). Enhanced synthesis of HA by pro-inflammatory cytokines has been associated with renal and rheumatoid diseases (Dahl et al. 1985, Manicourt et al. 1993, Feusi et al. 1999). Similarly, dysregulation of HA metabolism is a typical feature of diabetes (Nieuwdorp et al. 2007) or endometrial cancer (Afify et al. 2005, Nykopp et al. 2010). HA dysregulation may be associated with unexplained infertility (Altmäe et al. 2010), and most relevant here, HA accumulation in the uterus has been linked with early embryo loss including spontaneous abortion (Camenisch et al. 2000, Cordo-Russo et al. 2009). Studies in pregnant mice reported the disappearance of HA at the maternal–embryo interface at days 5–7 of pregnancy (Brown & Papaioannou 1992, 1993, Martins et al. 2003). HYAL-2 is also expressed in trophoblast giant binucleate cells and the multinucleated syncytia of sheep placentomes commencing on day 16 of gestation (Dunlap et al. 2005), coinciding with the attachment and perhaps contributing to the clearance of HA at the implantation sites. In line with these reports, inhibition of HA by the infusion of 4-MU into the sheep uterus on day 14 after natural mating enhanced embryo implantation (Marei et al. 2016b). Therefore, further prospective randomised clinical trials are essential for a robust conclusion to be made concerning the potential beneficial effects of HA pathway manipulation for women undergoing embryo transfer (Loutradi et al. 2008).

Cervix ripening/relaxation

The cervix is the entrance to the uterus. In most species especially sheep, it forms a rigid and tightly closed non-distensible structure, which is necessary to prevent access of microorganisms into the uterus. However, a pathway through the cervix is essential under two conditions. One is for the passage of sperm after coitus and secondly at parturition. The cervical connective tissue is mainly composed of collagen, HA and proteoglycan (Leppert 1992). The HA content of the cervix varies with the stage of oestrus cycle, with the highest and lowest values during pre-LH surge and post-LH surge periods respectively, whereas the value in the luteal stage is intermediate (Perry et al. 2010a).

Cervical remodelling at parturition can be divided into cervical softening (a gradual process that occurs several days (gestation day 12 in the rat; Harkness & Harkness 1959) or weeks prior to parturition (during the second trimester of pregnancy in the human; Leppert 1995)) and cervical ripening phases. Cervical ripening, which occurs in the hours (rodents) and days (women) preceding parturition, is characterised by hydration and further growth, decreased tensile strength, increased cervical secretions and lubrication, disorganisation of collagen fibrils, further changes in the composition of GAGs and infiltration of inflammatory cells. These are influenced by the local endocrine milieu, as well as interactions and cross-talk between the cellular components (stroma and epithelium), inflammatory cells and extracellular matrix (Straach et al. 2005).

Regulation of HA synthesis in the cervix is a conserved process in mammalian species. Hyaluronan content of cervix increases markedly during late pregnancy in human, sheep, guinea pig, rabbit and rat (Downing & Sherwood 1986, Anderson et al. 1991, Rajabi et al. 1992, El Maradny et al. 1997). The HA level increases from 19% of total GAG in early pregnancy to 71% at term (Akgul et al. 2012) and the majority of cervical HA in mice is synthesised by HAS2 (Akgul et al. 2014). Uchiyama and coworkers (2005) reported peak levels of HAS1 and HAS2 mRNA expression in mouse cervix at delivery. HAS2 has also been identified to be specifically upregulated in women at labour relative to pregnant women not in labour (Straach et al. 2005). HAS2 produces high-molecular-weight HA, which may facilitate the ripening of the cervix by increasing the water content and cytokines (interleukin 8) of the cervix, possibly due to its hydrodynamic and viscoelastic properties (El Maradny et al. 1997). Despite this, more recent work by Akgul and coworkers on HAS knockout mice has revealed that HA is not necessary for the increased cervical distensibility during late gestation (Akgul et al. 2014).

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

Cryopreservation of embryos and in vitro embryo production

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

Integral model to explain the reproductive functions of HA

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

As far as reproduction is concerned, we need to consider the anatomical component and physiological status of the tissue in context. Clearly, high-molecular-weight HA may be required at a particular point in time by a reproductive tissue, whereas the next phase of the same tissue's differentiation may require low-molecular-weight HA. Low-molecular-weight HA may be produced directly by HAS3 or through cleavage of high-molecular-weight HA by HYAL i. Therefore, we wish to emphasise that the prediction of HA function resulting from HAS1, HAS2 or HAS3 is difficult without taking into consideration the HA-degrading activity of HYALs. The functions of HA therefore depend not only upon its intrinsic properties but also upon a complex balance of polymerisation by HASs, depolymerisation by HYALs and interactions with HA receptors and HA-binding proteins as well as other intracellular and extracellular components such as growth factors and cytokines.

Based upon our work and that of others, as outlined previously, we now propose a model that takes into account the integrated functions of HA according to size, the location of HA in different places throughout the reproductive tract and the timing of its presence, relative to female reproductive cycles and the prevailing hormonal environment at any given moment (Fig. 1). Such model is primarily applicable to ungulate species such as sheep and cow. Nevertheless, the three genes encoding hyaluronan synthases are highly conserved in vertebrates, and the simple structure of HA is conserved throughout all mammals. This implies that a similar pattern of expression and regulation may be generalised to other mammals.

In conclusion, we have presented evidence from a range of mammalian species for the central role of HA in key events in reproduction. HA is ubiquitous; however, its actions at different locations within the reproductive tract depend critically upon its size, which is controlled by the balance of synthesis by one of three isoforms, degradation, which is undertaken principally by two hyaluronidase isoforms, together with a sperm-specific isoform around fertilisation, and its signalling pathways, which occur via CD44 and RHAMM. Superimposed upon these variables is the cyclicity inherent in female mammalian reproduction, with steroid hormones affecting the synthetic enzymes and thereby tilting the balance of small- or large-molecular-weight HA being predominant. A better understanding of how the different components are orchestrated will provide opportunities for correction of pathology and promotion of normal fertility or contraception in a range of situations and species. In particular, assisted conception in animal species, rare species preservation and human IVF will benefit from improved reagents and strategies to control implantation.

Declaration of interest

The authors declare that there is no financial a) or other potential conflict of interest; or (b) conflict of interest, that could be perceived as prejudicing the impartiality of the research reported in the review paper.

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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)	Human	Dahl et al., 1983
	5.1 ug/ml (week 12) 1.9 ug/ml (weeks 15-17)	Sheep	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

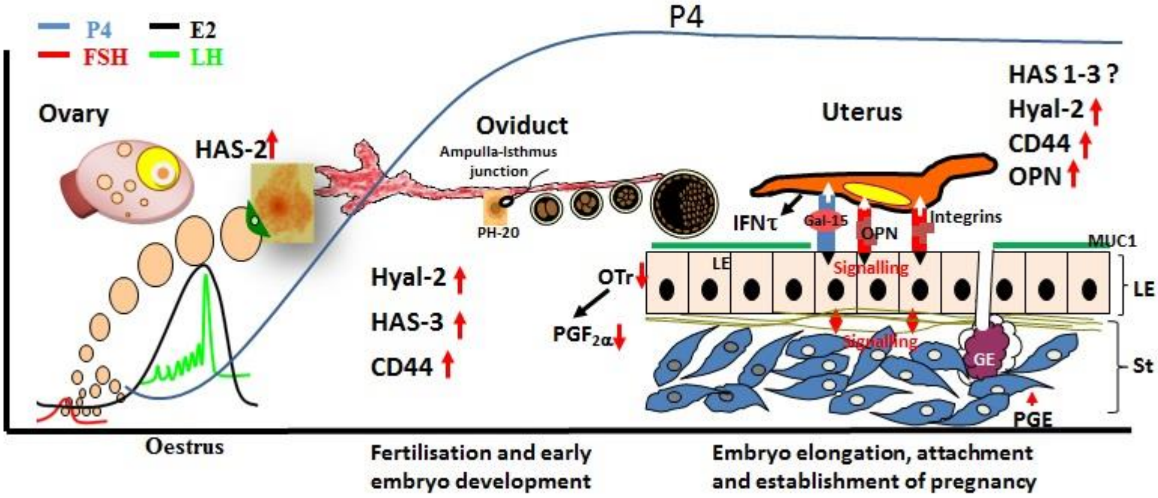

Figure 1. Model of the regulation of HA biosynthesis, degradation and function in the reproductive system. We hypothesise that at least in ungulates, steroid hormones orchestrate a sequential expression pattern for HA of different sizes in the reproductive system, with oestradiol (E2) inducing expression 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.