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Blastocyst Hatching in Humans

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Abstract—The human oocyte is surrounded by the zona pellucida—an elastic, transparent extracellular matrix consisting of specific glycoproteins. The zona pellucida is preserved after fertilization and surrounds the developing human embryo for a few days. The embryo needs to get out of the zona pellucida before implantation to establish cell contacts between the trophectoderm and endometrial epithelium. The release of the embryo from the zona pellucida is carried out at the stage of the blastocyst and called zona hatching. During zona hatching the blastocyst breaks the zona pellucida and performs active movements to escape through a gap formed in the zona. While microscopic description of zone hatching is well known, biochemical and cytological basis of zone hatching remains poorly understood. The break of the zona pellucida occurs under the influence of two forces: mechanical pressure of the growing blastocyst on the zone and chemical dissolution of the zone material with secreted lytic enzymes. There is only one paper (Sathananthan et al., 2003), which describes the specialized cells in the trophectoderm that locally dissolve the zona pellucida, promoting the emergence of the hole for blastocyst release. Taking into account the singleness of the paper and the absence of further development of this subject by the authors in the following decade, the existence of specialized cells for zone hatching should be assumed with great care. Lytic enzymes, secreted by cells of the trophectoderm for dissolving the zona pellucida, are different. Depending on the species of the mammal, different classes of proteases participate in the zone hatching process: serine proteases, cysteine proteases, metalloproteinases. Proteases, secreted by human trophectoderm, are not described. The mechanisms of the active movement during blastocyst hatching are investigated to a lesser degree. Only the involvement of the cytoskeleton of trophectoderm cells in the mechanism of blastocyst compression was shown, and the participation of desmosomes in the coordinated change in the form of trophectoderm cells during compression is suggested. This review summarizes literature data on the possible mechanisms of zone hatching in the development of human embryos, obtained in experiments in vitro, as well as in animal models.

Keywords: zona hatching, zona pellucida, strypsin, cathepsin L, ovastacin, SK3, embryo, in vitro fertilization

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INTRODUCTION

Human oocyte (as other mammals) is surrounded by a layer of extracellular matrix called the zona pellucida. In Russian literature, such terms as "shiny membrane" or, less frequently, "pellucid membrane" are traditionally used.

The membrane of the human oocyte is composed of glycoproteins; it is elastic, transparent, and has a thickness of 14–18 μm (Balakier et al., 2012). The zona pellucida appears in oocytes during growth, at the stage of preantral follicles, approximately 3 months prior to ovulation (Odor and Blandau, 1969; Dietl, 1989; Jovine at al., 2007; Wassarman, 2008), but it plays the main role in the process of fertilization and embryo development before implantation. Three functions of the zona pellucida are reliably known: recognition of the oocyte by the spermatozoon and acrosome reaction triggering (Clark, 2013), polyspermy blocking (the zona pellucida loses the recognition profile by the spermatozoon after fertilization) (Sun, 2003), and retention of blastomeres together during the division period (Bronson and McLaren, 1970; Suzuki et al., 1995). In scientific literature, other functions are also suggested, such as participation in the normal course of folliculogenesis and oogenesis (Wassarman and Litscher, 2012). After oocyte fertilization, the zona pellucida remains, thus forming embryo develops inside the zona pellucida until it reaches the blastocyst stage, at which the release from the zona pellucida is performed.

Before implantation, the embryo needs to get rid of the zona pellucida to perform a direct cell contact with endometrial epithelium. The process of embryo release from the zona pellucida is called zona hatching. Zona hatching is accompanied by a partial rupture

of the zona pellucida and subsequent dynamic movement of the embryo into the external environment. The hatching process occurs at the blastocyst stage approximately 5–6 days after fertilization and takes several hours (Gogolevskii, 1998; Sathananthan et al., 2003; Seshagiri et al., 2009). Only after release from the zona pellucida the blastocyst is able to carry out the implantation into uterine endometrium and to continue its development.

Not all blastocysts manage to realize hatching. According to the data obtained in vitro, up to 75% of morphologically normal human blastocysts are incapable of independent release from the zona pellucida (Thurin et al., 1998). Hatching failure may be one of the important causes of common implantation failure in humans (Seshagiri et al., 2009).

Despite the large number of studies in animal models and human embryos in vitro, biomechanical and molecular mechanisms of zone hatching are not fully understood and require further study. The study on release mechanisms of the blastocyst from the zone, as well as on the structure and functioning of the zona pellucida in general, is the most complex due to variety of these processes in different animal species. In this regard, it is sufficiently difficult to parallelize the data on zona hatching in humans and laboratory animals.

STRUCTURE OF ZONA PELLUCIDA

The zona pellucida of mammals represents a kind of yolk membrane of the oocyte in vertebrates. The analysis of the structure of genes encoding proteins of the zona pellucida in mammals and genes of yolk membranes in other classes of vertebrates shows the evolutionary relationship of these structures and common origin of all yolk membranes of vertebrates (Goudet et al., 2008).

All ZP proteins have a similar structure: N-terminal signal sequence conserved ZP domain, C-terminal sequence containing specific restriction site and transmembrane domain. After the cleavage of the ZP precursor protein by furin, the region containing the ZP domain loses contact with the plasma membrane and may interact with other ZP proteins by forming fibrils. The zona pellucida is formed by four types of glycoproteins, designated as ZP1, ZP2, ZP3, and ZP4, respectively (Lefièvre et al., 2004). Presumably, the evolution of mammalian zona pellucida went through the loss of a number of structural proteins (Meslin et al., 2012). Thus, the yolk membrane of fish (lat. *Zona radiata*) is formed by 6–7 types of proteins, the membrane of amphibian oocytes is formed by 4–5 types of proteins, and the yolk membrane of birds is formed by 5–6 types of proteins (Darie et al., 2004; Barisone et al., 2007; Goudet et al., 2008; Serizawa et al., 2011). The zona pellucida of mammals, including monotremes and marsupials (Meslin et al., 2012), typically consists of four types of proteins. All proteins of the oocyte membrane in vertebrates belong to four groups: ZPA (ZP2 in human), ZPV (ZP1 and ZP4 in human), ZPC (ZP3 in human), and minor group ZPX (is absent in human and other mammals). Several mammalian species (mouse, dog, cow and, probably, pig) have only three proteins in the composition of the zona pellucida. The loss of these proteins in different species of mammals occurred independently and, apparently, relatively recently. Thus, the zona pellucida of gray rat consists of four proteins—ZP1, ZP2, ZP3, and ZP4—and it consists of only three proteins—ZP1, ZP2, ZP3—in house mouse, an evolutionarily related species, while a pseudogene ZP4, which is damaged by microdeletion and does not produce the protein, is present in the mouse genome (Boja et al., 2005; Goudet et al., 2008). The zona pellucida in dog and cow is also constructed of three proteins, but it lacks the ZP1 protein; the presence of its pseudogenes was also shown for these species (Goudet et al., 2008). The composition of the zona pellucida for some mammal species is still under clarification. Thus, it was previously stated that the zona pellucida in cat consists of three proteins (Harris et al., 1994, Goudet et al., 2008), but the presence of all four ZP proteins was later clearly demonstrated for this species (Stetson et al., 2015).

The zona pellucida formation in pig has a special character. Formally, porcine zona pellucida consists of four ZP proteins, but they all are encoded by only three genes. The ZPA gene, similar to the ZP2 gene of other mammals produces a polypeptide chain, which is cleaved during posttranslational changes into two proteins that functionally correspond to ZP2 and ZP1 of other mammals. The ZP1 gene in the pig genome is not detected, and the ZP1 pseudogene was not found. This suggests that the ZP1 gene, which was lost during evolution, was compensated by the processing of the ZP2 protein, leading to the formation of protein similar to ZP1 (Hedrick and Wardrip, 1986; Goudet et al., 2008).

It was definitely shown that growing human oocyte synthesizes ZP proteins (Eberspaecher et al., 2001). However, there are papers indicating that ZP protein synthesis is also carried out by follicular cells. The localization of proteins ZP1, ZP2, and ZP3 in follicular cells of growing human follicles was demonstrated by the immunofluorescence method (Gook et al., 2008). The two-layer visualization of human zona pellucida in polarized light can serve as an indirect sign of its dual origin. Probably, this bilayer structure is associated with the fact that the zone inside accretes with glycoproteins synthesized in the oocyte, while it accretes with glycoproteins synthesized by theca cells outside (Keefe et al., 1997; Shen et al., 2005; Swiatecka et al., 2014).

For different species of mammals, there are enough contradictory data about what type of cell produces proteins of the zona pellucida: the oocyte itself or cells of the follicle, which surround it. In mouse, the syn-

thesis of all three glycoproteins is exclusively carried out by oocyte (Rankin et al., 2000), while zona pellucida proteins are present in the oocyte and follicular cells in rabbit, cow, pig, and common marmoset according to some data (Kolle et al., 1998; Lee, 2000; Sinowatz et al., 2001, Bogner et al., 2004). Only in dog is ZP1 excreted by the oocyte, while ZP2 and ZP3 are excreted by follicular cells surrounding the oocyte (Blackmore et al., 2004). In 2001, Jewgenow and Rudolph published a paper in which they argued that zona pellucida proteins in cats are synthesized only by thecal cells of the follicle, and the oocyte is not involved in the formation of the zona pellucida (Jewgenow and Rudolph, 2001). This contradicts the data presented earlier, according to which ZP proteins in cats are localized both in the oocyte and theca cells (Barber et al., 2001). The data that the proteins that comprise the zona pellucida in horse are exclusively of follicular origin were also obtained (Kolle et al., 2007).

Such disparate data require a critical assessment; it is necessary to understand which evidentiary methods were used by the authors. It is mainly immunohistochemical staining of tissue sections.

ZP proteins undergo posttranslational modification: a part of the polypeptide chain is cleaved, and modified proteins are excreted into extracellular environment by vesicular transport after glycosylation, where they form three-dimensional structure of the zona pellucida. The exact interposition of proteins ZP1, ZP2, ZP3, and ZP4 in the three-dimensional structure of the zona is unknown. Most structural models are proposed for mouse embryos, whose zona pellucida is composed of three proteins (ZP4 was lost in mouse during evolution). In general, all models agree that ZP proteins are organized into fibrils crosslinkable with each other in the extracellular environment. In mouse, the fibrils are formed by proteins ZP2 and ZP3, associated alternately with each other (these proteins account for approximately 90% of the zone mass of mouse oocyte), and ZP1 proteins are incorporated into the fibrils and responsible for crosslinking of different fibrils (ZP1s of different fibrils are associated with each other) (Han et al., 2010; Monné and Jovine, 2011; Louros et al., 2013). The role of the ZP4 protein in the zona pellucida structure is unclear. Considering the fact that ZP1 is absent in a number of mammals (cow, dog), one can suggest that ZP4 plays a similar role of ZP2/ZP3 fibril crosslinking.

The zona pellucida fibrils are too small to be examined even in an electron microscope (Familiari et al., 2008). Assumptions about the fibrils structure are based on the analysis of the three-dimensional structure of the zone proteins (Monné and Jovine, 2011). Based on the birefringent effect in polarized light peculiar exclusively for the inner layer of the zona pellucida, the assumption on the different orientation of the fibrils in the inner and outer layers of the zona pellucida was made. It is believed that the fibrils have predominantly radial arrangement in the inner layer, while they have laminal arrangement in the outer layer (Keefe et al., 1997).

Electron microscopy images indicate spongy and porous structure of the zona pellucida (Novo et al., 2012), but the origin of these pores and spongy structure is unknown. Probably, the porous structure of the zone is the result of exposure to follicular cells, which penetrate the zona pellucida with their outgrowths and, thus, contact with oolemma. Immediately before ovulation, follicular cells break contacts with the oocyte and take out the outgrowths from the zona pellucida pores (Huang and Wells, 2010), and the zone surface is quickly smoothed after ovulation and the pores are tightened (Familiari et al., 2008). However, there are no sufficient data to state that the zone porosity occurs as a result of the interaction of follicle cells with the oocyte. Embryonic zona pellucida is permeable to all substances dissolved in water. Furthermore, antibodies and certain viruses can pass through it (Sellens and Jenkinson, 1975; Van Soom et al., 2010).

FUNCTIONS OF ZONA PELLUCIDA

A number of papers indicate that the presence of the zona pellucida is required for the normal formation of the oocyte during folliculogenesis. Thus, in mice with artificially damaged gene ZP2 or ZP3 (gene knockout), the zona pellucida is not formed at all (Liu et al., 1996; Rankin et al., 1996, 2001; Wassarman and Litscher 2012). In the ovaries of these mice, most follicles undergo degeneration, and the oocytes are often absent in the follicles or only a small amount of the oocytes that have no zona pellucida is formed. The role of the zona pellucida in folliculogenesis and oogenesis remains unclear. In this context, the study of Turkish researchers, who showed accumulation and localization of vascular endothelial growth factor (VEGF) in the zona pellucida during folliculogenesis (beginning with the stage of antral follicle), is interesting (Celik-Ozenci et al., 2003). It can be assumed that the zona pellucida matrix serves as a reference for the establishment of follicular cell contacts with the oocyte.

The zona pellucida plays a special role in interaction of the spermatozoon with the oocyte during fertilization (Gupta, 2015). It is one of the barriers that implements the so-called polyspermy block—impediment for the penetration of two or more spermatozoa into the oocyte (Sun, 2003). The binding of the spermatozoon with the zona pellucida is species-specific: for example, rat spermatozoon does not bind to mouse zona pellucida and, vice versa, human spermatozoon does not bind to rat zona pellucida (Hoodbhoy et al., 2005). There are hypothetical recognition receptors of all four zona pellucida proteins on the surface of the spermatozoon. Although the receptors were not reliably investigated, the binding of spermatozoa with sin-

gle zona pellucida proteins was described. The spermatozoon associates with both polypeptide regions of ZP glycoproteins directly and specific oligosaccharide residues of ZP glycoproteins (Gupta, 2015). The binding sites (the head or midpiece of the spermatozoon) were characterized, and it was described whether the binding is carried out before or after the triggering of the acrosome reaction (Gupta, 2015). The interaction of the spermatozoon with the zona pellucida occurs in two stages: recognition/binding and triggering of the acrosome reaction (Gupta and Bhandari, 2011). It is known that there are many binding sites for spermatozoa on ZP glycoproteins, but it is still not completely established which of them cause the triggering of the acrosome reaction; obtained experimental data are contradictory even for mouse, the most well-studied object. But even these contradictory data cannot be automatically transferred to humans, since the zona pellucida structure and polyspermy block mechanism in mice and humans are different.

After the fusion of spermatozoon and oocyte membranes, the triggering of the cortical reaction occurs: up to ten different types of lytic enzymes that modify the zona pellucida are released from the oocyte (Sun, 2003; Liu, 2011). The enzymes of cortical granules cleave oligosaccharide residues from ZP proteins and also a polypeptide chain region from the ZP2 protein. As a result of this modification, the zona pellucida is no longer capable of initiating the acrosome reaction and does not allow spermatozoa to come close to the oolemma for repeated fertilization. Thus, the polyspermy block is implemented at the level of the zona pellucida. A number of experimental data indicates that there is a parallel mechanism for the polyspermy block implementation at the level of oolemma that prevents the repeated fusion of the spermatozoon with the oocyte (Mio et al., 2012; Bianchi and Wright, 2014).

The modification of the zona pellucida after the cortical reaction is often described as zona hardening. Real increase in physical hardness (2.6–2.7 times) and viscosity (4.44 times) was shown for the zona pellucida of mouse oocytes soon after fertilization (Drobnis et al., 1988; Murayama et al., 2006; Kim, J. and Kim, J., 2013). A similar study with similar results was also carried out for cow oocytes (Boccaccio et al., 2012). However, it should be noted that, in all similar studies, not hardness of isolated zona pellucida but deformation of the oocyte or embryo, surrounded by the zona pellucida, was measured in response to the pressing, assuming that the viscosity of the cytoplasm of the oocyte/zygote remains constant, which is not quite correct.

In addition to the participation in oogenesis and fertilization, the zona pellucida is obviously required to hold blastomeres together at the period of embryo division (Bronson and McLaren, 1970; Suzuki et al., 1995). Embryo cells at the stage of division have a poor adhesion to each other; human embryo without the zona pellucida easily disintegrates into individual cells for the first 4 days of development.

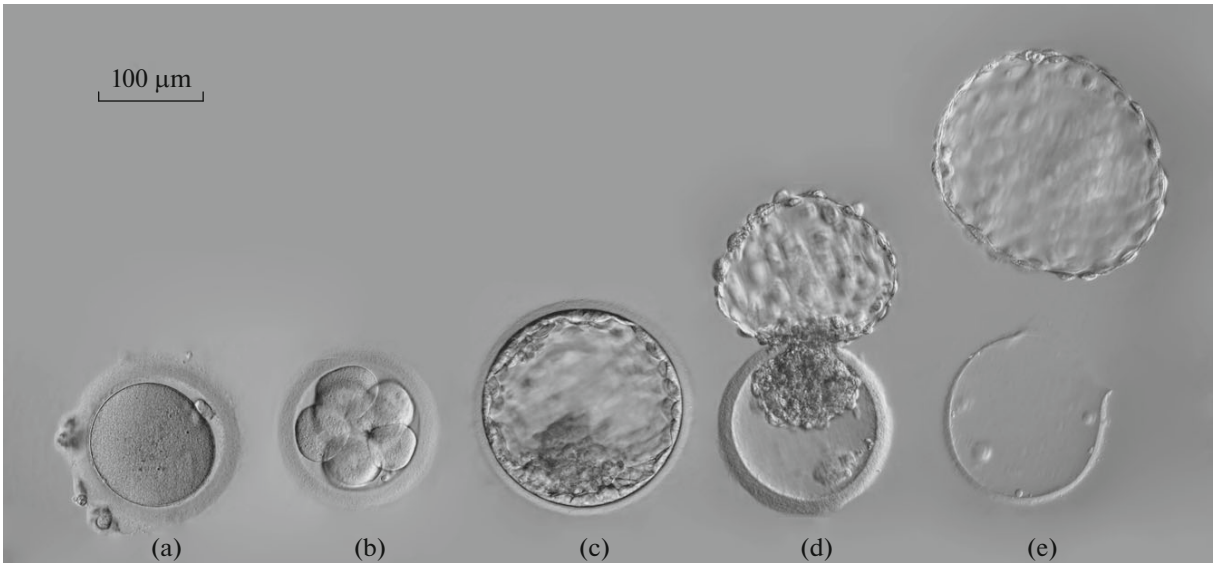
Because of the permeability of the zona pellucida for all water-soluble substances, its barrier function does not seem to be obvious. Large molecules (for example, serum albumin), antibodies, and even some viruses penetrate the zona pellucida (Sellens and Jenkinson, 1975; Van Soom et al., 2010). There are studies indicating the possibility of regulatory protein accumulation in the zona pellucida or perivitelline space. For example, vascular endothelial growth factor (VEGF) is detected in the zona pellucida during folliculogenesis (Celik-Ozenci et al., 2003), while the GP215 glycoprotein from tubular fluid accumulates in the perivitelline space (Kapur and Johnson, 1986).

Also, it can be assumed that the zona pellucida protects embryonic cells from contact with the mother's immune cells and prevents the initiation of the immune response (Clark, 2010). However, if such a barrier function did exist, it would function for only 2–3 days at the last stages of preimplantation development: with the appearance of specific embryonic antigens on the cell surface to the embryo coming out of the zona pellucida. The importance of the embryo protection from the mother's immune system in such a short period appears doubtful.

DYNAMIC CHARACTERISTICS OF ZONA HATCHING

Modern conceptions of zona hatching are based on numerous observations of blastocysts in human and mammals in vitro (figure). The general scheme of human embryo release from the zona pellucida is as follows: 4–5 days after fertilization, the embryo at the blastocyst stage actively transports water into the cavity of the blastocyst, thereby increasing its diameter approximately 1.5 times. According to Kirkegaard et al., the average diameter of the blastocyst increases from 120 to 160–180 μm, the blastocyst mechanically affects the zona pellucida—it is stretched and becomes thin—its thickness reduces from 18 to 5 μm (Kirkegaard et al., 2012). Then, the break of the zona pellucida with the formation of a slot-like opening occurs, and the blastocyst, while continuing to increase in the size, bulges the trophoblast into the formed opening. During this period, the blastocyst begins to make so-called contractions—a series of successive compressions and bloatings—due to which the embryo completely leaves the zona pellucida (Sathananthan et al., 2003).

There are significant contradictions in the data on timing of hatching for human blastocysts in vitro. In early studies, it was indicated that the blastocysts begin the process of hatching on the sixth day after fertilization (Lopata and Hey, 1989), while most of the studies at the turn of the 20th and 21st centuries showed the fifth day (Sathananthan et al., 2003) or the sixth day (Veeck and Zaninovic, 2003) after fertilization as the



Zona pellucida at different stages of human preimplantation development in vitro (micrograph, magnification of 200×, contrasting according to Hoffman). (a) Oocyte, surrounded by the zona pellucida (0 h of development). (b) Embryo at stage of division, surrounded by the zona pellucida (44 h of development). (c) Embryo at the blastocyst stage, the zona pellucida is thinned due to blastocyst expansion (118 h of development). (d) Blastocyst performs hatching, release from the zona pellucida (126 h of development). (e) Blastocyst is completely out of the zona pellucida (140 h of development).

time when hatching starts. In current studies carried out on a large sample of embryos (more than 100) using the method of time-lapse shooting, it is indicated that the time when hatching starts is less than 5 days from the moment of fertilization: 107.8–116.1 h (Kirkegaard et al., 2012) and 106.2–118.2 h (Serdarogullari et al., 2014). These differences in the data are evidently due to the significant progress in the technology of preimplantation human embryo culturing in vitro, which occurred over the past 20 years. Faster development of embryos and increase in pregnancy frequency indicate the approximation of cultivation conditions to the natural ones. In this regard, one can assume that human blastocyst in vivo proceeds to zona hatching between 4 and 5 days after fertilization. The question how hatching processes observed in vitro correspond to hatching processes occurring in vivo remains open. Some authors indicate that the proteolytic enzymes, secreted by the endometrium, may accelerate the processes of hatching in vivo (Vajta et al., 2010).

The break of the pellucid zone occurs in the region of mural trophectoderm (a part of the trophectoderm that surrounds the blastocyst cavity). The question whether the place where the zone break occurs is specific or the break occurs in a random place due to the total reduction in the zona pellucida strength remains open. The process of human blastocyst release from the zona pellucida was thoroughly analyzed by light and electron microscopy in the paper published 12 years ago by the international research group (Sathananthan et al., 2003). These researchers found morphological features of trophectoderm cells, which are located in the region of the zona pellucida break, and they called them as zona-breaker cells. During hatching, these cells acquire a spherical shape and form finger-like outgrowths. It was also shown in this paper that the zona-breaker cells are arranged in the zone of upcoming hatching, characterized by a large number of microvilli, lysosomes, and, apparently, actively secrete vesicles. This is consistent with the concept that the hatching process depends on both mechanical and chemical factors. In addition, large bundles of contractile tonofilaments passing along the break of the zona pellucida like a sphincter or funnel were visualized in these cells. However, as discussed above, such cells are located in the region of already formed break of the zona, and it is not very clear whether morphologically distinguishable zona-breaker cells appear as part of the trophectoderm or the shape of their cells changes during the trophectoderm movement through the break in the zona pellucida. The trophectoderm of late blastocysts strongly responds to mechanical stress; for example, a single touch of a thin glass needle leads to acute collapse of the blastocyst cavity, while the trophectoderm integrity is not affected (Kimura et al., 2012). This reaction to mechanical stress is associated with the activity of desmosomes, which integrate the trophoblast in a common mechano-dependent system. Under the local impact on trophectoderm cells of late blastocyst, a sharp reorganization of tonofilaments and cell compression occur, while desmosomes, which provide the mechanical link between the cells, initiate the process of the same reorganization of the cytoskeleton in neighboring cells of the layer. Thus, the appearance of

"compressed" trophectoderm cells at the site of the zona break may be associated precisely with the local influence of the edges of the zona break on the mechano-sensitive cell system but not with the specific function of "zona breaking" of the certain trophectoderm cells.

The formation of so-called trophoectodermal projections (TEPs), whose existence was shown for many species of mammals (rodents, sheep, cows, and primates), is of great interest to researchers in the field of studying of the hatching process. The formation of TEPs in human blastocysts was described using the method of time-lapse shooting in real time (Gonzales et al., 1996). In this paper, TEPs were described as a single, finger-like cytoplasmic outgrowths of the trophectoderm with the length up to 29 μm, perforating the zona pellucida, but their detailed morphology was not shown. When the blastocyst collapsing formed TEPs are detached from the trophectoderm, they remain outside the zone and rapidly degenerate. Such TEPs were not detected in early blastocysts or degenerating blastocysts; however, recently it was shown that long finger-like filopodia are formed at earlier stages of preimplantation development. Thus, it was shown that mouse embryo compaction occurs with the participation of long finger-like filopodia (Fierro-Gonzalez et al., 2013). At the very beginning of the compaction, some embryo cells form several long filopodia containing a large amount of E-cadherin, which are flattened along the surface of the neighboring cells, initiating the process of morula cell adhesion. Also, the authors of the paper believe that these filopodia control the process of the change in the cell shape; as a result, the cells are stretched along the surface of the neighboring cells and form the trophectoderm. The high content of actin in TEPs was shown in experiments using immunofluorescence staining and confocal microscopy (Seshagiri et al., 2009; Lu et al., 2012). The data of studies on human TEPs coincide with those obtained in other mammalian species, and they indicate the participation of TEPs in the hatching process of the blastocyst by passing through the zona pellucida and initiation of its break (Seshagiri et al., 2009). It is also possible to assume that, after zona hatching, such lamella-like TEPs are involved in the movement of the blastocyst across the surface of endometrial epithelium and initiation of the implantation process.

MOLECULAR MECHANISMS OF HATCHING

In addition to dynamic mechanisms of zona hatching, enzymatic degradation of the zona pellucida plays an important role in the attenuation process of its strength. The role of proteases in the hatching process is not in doubt: it was shown that the addition of protease inhibitors into the culture medium of the blastocysts leads to complete blocking of the process (Pan et al., 2015).

Depending on the species of mammal, different classes of proteases—serine proteases, cysteine proteases, metalloproteinases—participate in the hatching process. At the moment, the main issue remains the identification of hatching proteases that are specific to humans; however, there are little studies in this area due to ethical reasons, and they have the character of assumptions and extrapolations. Previously, there was a hypothesis that strypsin, a protease responsible for the hatching process in some other mammalian species, participates in hatching in humans. It is assumed that this protease is synthesized by both embryo cells and cells of uterine endometrium (Gogolevskii, 1998; O'Sullivan et al., 2002).

The involvement of cysteine proteases in hatching is the most detailed studied on the model of the Syrian hamster, whose structure of the zona pellucida is very similar to the zone structure of human oocytes (Sireesha et al., 2008). In the experiments using protease inhibitors, it was shown that only cathepsins out of three classes of cysteine proteases participate in zona hatching. These enzymes, predominantly localized in lysosomes, have also other important functions. For example, they are involved in the degradation of extracellular matrix in humans in the cases when they are secreted by the cells (Koblinski et al., 2000). It was also found that the addition of cathepsin L, P, or B, which have a high protease activity, to the culture medium results in complete dissolution of the zona pellucida and synthesis of cathepsin L and P in blastocyst cells during the period prior to hatching. It was shown by the immunofluorescence method that these cathepsins are clearly visualized in the TEP region of the blastocyst during zona hatching (Sireesha et al., 2008).

The human genome also contains the genes of cathepsin L (*CTSL*), B (*CTSB*), and other cathepsins. The presence of cathepsin L was found in the cells of the trophectoderm and inner cell mass (Adjaye, 2005); but the data that would directly confirm their role in zona hatching in humans are absent in literature. Determination of transcription, synthesis, and excretion of cathepsins by human blastocyst could be a promising topic for further studies.

Matrix metalloproteinases, which are capable of hydrolyzing any proteins of the extracellular matrix, may also participate in hatching of human blastocysts. The role of zinc metalloproteinases in early embryonic development was shown for many species of vertebrates (Sterchi et al., 2008). Special attention in this study is paid to astacin protease subfamily, which also includes hatching proteases of vertebrates—alveolin, ovastacin, nephrosin—and some other proteases of this group. In humans, there is only one gene, ovastacin, which is also present in house mouse (*Mus musculus*). Ovastacin refers to the group of phylogenetically related hatching proteases that are involved in the destruction of fertilization membranes in many invertebrates and vertebrates (Quesada et al., 2004). In

addition, ovastacin participates in the formation of polyspermy block in mice. It is accumulated in cortical granules of the oocyte, and then the excretion of the cortical granules and ZP2 cleavage by ovastacin occur under the fusion of the spermatozoon membrane with oolemma (Burkart et al., 2012). Ovastacin synthesis in mouse is observed not only in the oocyte but also in the embryo at the division stage, starting from the second day of the development, indicating the possible presence of other functions for ovastacin. Considering the evolutionary relationship and structural similarity of ovastacin with hatching proteases of other species (Quesada et al., 2004), it is logical to assume the involvement of ovastacin in the hatching processes of human and mouse; however, temporal disconnection between registered ovastacin appearance in the cells of the early embryo and hatching remains unexplained. To study ovastacin participation in the hatching processes in humans, further studies on the ovastacin content in the embryo trophectoderm on the fifth day of culturing are needed, especially in the zona-breaker cells, whose active involvement in the local lysis of the zona pellucida is not proven yet. The question on the identification of other hatching proteinases at the moment is still open.

A great number of cytokines, growth factors, second messengers, transcription factors, and hormones are also involved in the hatching regulation (Kane et al., 1997; Sargent et al., 1998; Seshagiri et al., 2009). For example, the addition of heparin-binding epidermal growth factor (HB-EGF) to the culture medium of human developing embryos not only accelerated the formation of the blastocyst but also led to an increase in the proportion of blastocysts capable of independent hatching (Sargent et al., 1998; Kawamura et al., 2012). Similar data were obtained in animal models for epidermal growth factor (EGF), transforming growth factor beta (TGF-β), and leukemia inhibitory factor (LIF) (Mishra and Seshagiri, 2000; Seshagiri et al., 2002). Apparently, this involvement in the hatching process is mediated—the stimulation of intracellular processes in preimplantation embryos after the addition of growth factors to the culture medium that results in the formation of a greater number of blastocysts capable of hatching. Thus, it was shown in a number of studies that, normally, the cells of human preimplantation embryos release a set of growth factors and their receptors, which is necessary to maintain the normal rate of the development (Richter, 2008; Thouas et al., 2015).

The role of calcium in the regulation of embryogenesis has been studied for a long time and does not cause doubts; calcium-dependent processes related to hatching are actively investigated. For example, the expression of the *KCNN3* gene (SK3, Ca^{2+}-activated K^+ channel) in blastocysts, which performed normal hatching, and blastocysts incapable of hatching was studied in vitro (Lu et al., 2012). The *KCNN3* gene is present in the genome of a large number of animal species (over 100); its expression in the blastocyst was detected both in human and mouse. At the same time, the expression of related genes *KCNN1* and *KCNN2* in the blastocyst was not revealed, and the *KCNN4* gene expression was not investigated. It was shown that the expression level of the *KCNN3* gene in human blastocysts, which are not capable of hatching, is two times lower than that in normal blastocysts ($p < 0.05$, $n = 3$), indicating the important role of Ca^{2+}-dependent potassium channels in the hatching processes. In addition, in the mouse model, the level expression of the *KCNN3* gene was studied; it was higher in the trophectoderm and increased during preimplantation development of the blastocysts. It was shown that the *KCNN3* gene knockout results in reduced synthesis of F-actin, involved in the formation of TEPs, and blastocyst hatching disorders (Lu et al., 2012). When studying *KCNN3* in other human tissues, including tumor tissues, it was shown that *KCNN3*-mediated hyperpolarization leads to increased cell motility (Potier et al., 2006; Girault et al., 2011; Clarysse et al., 2014), and this process also may be important for normal hatching. It was also suggested that activation of Ca^{2+}-dependent K^+ channels may be a mechanism for the initiation of the hatching process, but there is no experimental evidence for this hypothesis yet.

EFFECT OF in vitro MANIPULATIONS ON HATCHING PROCESS IN HUMAN

The development of embryos in vitro, presumably, is slower than in vivo, and, therefore, hatching during fertilization in vitro may potentially occur more slowly. We also assume that the embryo during in vitro culturing is under suboptimal conditions, because the composition of culture media is not identical to the content of the cavity of fallopian tubes and uterus. Because of this, a change in the configuration of the zona pellucida may occur. Some additional extracorporeal procedures may also lead to structural changes of the zona pellucida. Thus, according to some data, cryopreservation of oocytes may cause the zona pellucida induration and its greater resistance to proteolytic enzymes (Ko et al., 2008).

Recently, a group of scientists from Denmark conducted a study on the effect of in vitro fertilization method on the course of hatching and the clinical outcome of IVF (Kirkegaard et al., 2013). Zona hatching of embryos, produced by the method of insemination in vitro and intracytoplasmic spermatozoon injection into the oocyte (ICSI), was compared in the study. The researchers identified two types of hatching: with prior penetration of the zona pellucida with trophectoderm outgrowths and without it. In the first case, the blastocyst release occurs through gradually expanding hole of the zona pellucida, and there is a rapid and extensive break of the zona pellucida with rapid blastocyst extrusion in the second one. It was shown that

the use of ICSI method leads to the development of hatching of the first type (97.7% of all observations vs. 55.8% in the group without ICSI, $p < 0.001$). The authors also showed that, under fertilization using ICSI method, hatching begins earlier than without ICSI (95.9 vs. 99.7 hours after fertilization, $p < 0.001$). Thus, the local damage of the zona under ICSI has a significant impact on the dynamics of the hatching process. At the same time, it is important to note that the effect of the fertilization method and type of spontaneous hatching on the level of pregnancy was not revealed in this study.

In addition, when analyzing the hatching process in humans, one should consider that individual characteristics of the patient, both genetic and associated with abnormal course of previous processes such as oogenesis, may affect the structure of the zona pellucida. At the same time, no correlation with such factors as the patient's age or smoking was revealed in the study on zona pathologies (Pökkylä et al., 2011).

Most likely, the change in the zona pellucida structure, namely, its thickening, may lead not only to a lack of fertilization but also to hatching failure. At the same time, single studies on the hatching failure in humans do not contain any information on the morphology assessment of the zona pellucida of oocytes and embryos; thus, this hypothesis has no experimental data to be confirmed. Either change in the zona pellucida itself or slowed division of the embryo, during which physiological induration of the zona pellucida occurs, may be the causes of the zona pellucida thickening.

To date, abnormal secretion of proteases is the most common hypothesis of hatching failure, confirmed by experimental data (Pan et al., 2015). Genes responsible for hatching in mammals are known, and studies on pathogenetic mechanisms of abnormal protease secretion in humans are of great interest (Ozawa et al., 2012).

Also, one of the causes of hatching failures may be a lack of trophoblast differentiation and the emergence of zona-breaker cells. However, we did not find papers devoted to this phenomenon under thorough analysis of the literature data.

The disturbance of hatching due to the presence of extra cells between the trophectoderm and the zona pellucida, which are not involved in the formation of the blastocyst, was demonstrated by morphokinetic observation (Sathananthan et al., 2003).

CONCLUSIONS

Thus, the mechanisms of in vitro hatching of the human blastocyst are not fully understood; many conclusions have the nature of animal model extrapolations. The obtained data require confirmation for human embryos. However, it is known that zona hatching occurs due to dynamic mechanisms (increase in blastocyst leading to the zona pellucida thickening, its intense contraction, formation of trophectoderm cell outgrowths) and biochemical mechanisms (protease secretion). The data on the dynamic mechanisms of hatching is more complete due to ethical reasons, while the data on the molecular mechanisms of hatching in humans are mainly contradictory.

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