



Spontaneous in vitro hatching of the human blastocyst: the proteomics of initially hatching cells

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Abstract

Spontaneous in vitro hatching of human blastocysts starts with the formation of a tunnel through the zona pellucida (ZP) by cellular projections of trophoblast cells. Our aim was to identify the proteins that are upregulated in these initially hatching cells as compared to trophoblast (TE) cells from blastocysts that had not yet hatched. Forty seven women that underwent assisted reproduction treatment donated their ICSI-derived polyploid blastocysts for the study. In polyploid blastocysts that started spontaneous hatching, hatched clusters of cells were collected from the outer side of the ZP. Liquid chromatography mass spectrometry was applied to determine the proteins that were upregulated in these cells as compared to TE cells obtained from inside the ZP. Whole non-hatched polyploid blastocysts were used as controls. Overall 1245 proteins were identified in all samples. Forty nine proteins were significantly upregulated in hatching cells and 17 in the TE cells. There was minimal overlap between hatching and TE samples; only serine protease inhibitors (SERPINS) and lipocalin were detected in both samples. Myosin and actin were highly upregulated in the hatching cells as well as paraoxonase, N-acetylmuramoyl alanine amidase, and SERPINS clade A and galectin. In the TE cells, gamma butyrobetaine dioxygenase, lupus La protein, sialidase, lysosomal Pro-X carboxypeptidase, phospholipase b, and SERPINS clade B and A were among the most highly upregulated proteins. These findings may contribute to the basic knowledge of the molecular behavior of the specific cells that actively perforate the glycoprotein matrix of the ZP.

Keywords Human blastocysts · ICSI · Spontaneous hatching · Proteomics

Introduction

Successful hatching of the human blastocyst is mandatory for implantation and establishment of pregnancy. In assisted reproduction laboratories, hatching of blastocysts on days 5–6 of culture is often observed (Hardarson *et al.* 2012). The activation of the hatching process is not dependent on endocrine

effectors derived from the uterine milieu because it happens spontaneously in vitro.

Usually spontaneous in vitro hatching is preceded by expansion of the blastocyst (Hardarson *et al.* 2012). This induces stretching and thinning of the ZP. Then, at one point, a single trophoblast cell protrudes through the ZP forming a tiny slit and emerging on the outside. Several additional cells follow, creating a tunnel that gradually widens, enabling full hatching of the blastocyst.

The hatching patterns of conventional IVF-derived embryos and ICSI-fertilized embryos were compared using time-lapse monitoring (Kirkegaard *et al.* 2013; Araki *et al.* 2016). Initial penetration by small TE projections with gradual widening of the zona was observed in almost all (98%) of the ICSI embryos and in approximately half of the IVF embryos.

A previous study on the mechanics of blastocyst hatching in vitro defined the hatching cells as “zona breaker cells” (Sathanathan *et al.* 2003). They were shown to have surface microvilli and large bundles of contractile filaments and apparently they have lysosomes and secrete vesicles that interact

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with the ZP (Sathanathan *et al.* 2003). However, the molecular content of these vesicles was not identified. On the basis of mammalian models, it is assumed that proteases are involved in the initiation of the hatching process (Sathanathan *et al.* 2003; Seshagiri *et al.* 2016). Yet, the precise molecular mechanism that initiates and regulates hatching in the human blastocyst is still obscure.

In the present study, we isolated the cellular clusters which are the first to escape out of the ZP at the beginning of hatching and subjected them to mass spectrometry-based proteomic analysis. Their protein composition was compared to TE cells and whole blastocysts. Our hypothesis was that the contents of these specialized cells would reveal the molecular mechanism responsible for their ability to protrude through the matrix of the ZP and initiate the hatching process.

Materials and methods

Sample collection In our IVF laboratory, polyploid embryos (≥ 3 pronuclei) are discarded and are not transferred. The study was performed only with embryos that showed polyploidy. All women who agreed to donate their polyploid embryos for research signed an informed consent form. The study protocol was approved by the institutional review board (kmc-18-0004).

As a general practice in our laboratory, all fresh autologous oocytes that undergo either conventional IVF or ICSI are grown in EmbryoScopes (Unisense Fertilitech, Aarhus, Denmark) until days 5–6. We used only ICSI-derived polyploid blastocysts in order to avoid the morphological as well as molecular interference of spermatozoa attached to the outer surface of the ZP.

Polyloid ICSI embryos that were identified as such during routine daily annotations were grown until days 5–6 of culture. Some developed into blastocysts that started to hatch spontaneously. When hatching initiation was observed, the blastocysts were transferred to PBS (phosphate-buffered saline, Gibco, Paisley, U.K.). The blastocysts were held on the opposite side of the hatching point by a holding pipette and the first few hatching cells that emerged out of the ZP were collected using a large biopsy pipette. The cells that were obtained were transferred to 5% SDS (sodium dodecyl sulfate, Sigma, Rehovot, Israel) and immediately plunged in liquid nitrogen. A total of approximately 150 cells were collected from blastocysts donated by 47 women, pooled together and then divided into two separate samples.

If the polyploid blastocysts did not hatch, a laser beam was applied to breach the zona adjacent to the trophectoderm layer on the side opposite the inner cell mass and trophectoderm cells were isolated with a biopsy pipette, washed in PBS, lysed in 5% SDS and kept in liquid nitrogen until further evaluation. Approximately 250 trophectoderm cells were collected and divided into three samples.

Nine whole non-hatching blastocysts were also collected in 5% SDS and frozen in liquid nitrogen singly or in groups of 2 or 3.

According to a recent study, a non-expanded blastocyst has on average 70 cells (Iwasawa *et al.* 2019). In order to generate a calibration curve for mass spectrometry analysis, whole non-expanded blastocysts were collected. We then subjected them to mass spectrometry analysis in groups of 1, 2, 3, and 4 blastocysts in order to calculate the minimum number of cells necessary to receive a valid result. A single blastocyst sufficed to obtain full protein profile analysis.

Sample preparation for liquid chromatography mass spectrometry Samples were prepared as reported previously (Elinger *et al.* 2019). Briefly, lysates in 5% SDS in 50 mM Tris-HCl were incubated at 96°C for 5 min, followed by six cycles of 30 s of sonication (Bioruptor Pico, Diagenode, Denville, NJ). Proteins were reduced with 5 mM dithiothreitol and alkylated with 10 mM iodoacetamide in the dark. Each sample was loaded onto S-Trap microcolumns (Protifi, Farmingdale, NY) according to the manufacturer's instructions. After loading, samples were washed with 90:10% methanol/50 mM ammonium bicarbonate and then digested with trypsin for 1.5 h at 47°C. The digested peptides were eluted using 50 mM ammonium bicarbonate; trypsin was added to this fraction and incubated overnight at 37°C. Two more elutions were made using 0.2% formic acid and 0.2% formic acid in 50% acetonitrile. The three elutions were pooled together and vacuum-centrifuged to dry. Samples were kept at -80°C until analysis.

Liquid chromatography mass spectrometry ULC/MS grade solvents were used for all chromatographic steps. Each sample was loaded using splitless nano-ultra performance liquid chromatography (nanoUPLC, 10 kpsi nanoAcquity; Waters, Milford, MA). The mobile phase was (A) H_2O + 0.1% formic acid and (B) acetonitrile + 0.1% formic acid. Desalting of the samples was performed online using a reversed-phase Symmetry C18 trapping column (180 μm internal diameter, 20 mm length, 5 μm particle size; Waters). The peptides were then separated using a T3 HSS nano-column (75 μm internal diameter, 250 mm length, 1.8 μm particle size; Waters) at 0.2 $\mu\text{L}/\text{min}$. Peptides were eluted from the column into the mass spectrometer using the following gradient: 4% to 27%B in 90 min, 27% to 90%B in 5 min, maintained at 90% for 10 min, and then back to initial conditions.

The nanoUPLC was coupled online through a nanoESI emitter (10 μm tip; New Objective; Woburn, MA) to Q Exactive HF-X mass spectrometer (Thermo Scientific, Waltham, MA). Data was acquired in data-dependent acquisition (DDA) mode, using a Top10 method. MS1 resolution was set to 120,000 (at 200 m/z), mass range of 375–1650 m/z, AGC of 3e6, and maximum injection time was set to

100 msec. MS2 was performed by isolation with the quadrupole, width of 1.7 Th, 27 NCE, 15 k resolution, AGC target of 150 msec, and dynamic exclusion of 30 s.

Isolated TE cells were subjected to liquid chromatography mass spectrometry with samples of one, two, and three whole blastocysts as controls. Isolated hatching cells were also analyzed against samples of one and two blastocysts as controls.

Data processing Raw data were processed with MaxQuant v1.6.0.16. The data was searched with the Andromeda search engine against the human proteome database appended with common lab protein contaminants and the following modifications: carbamidomethyl of Cys, oxidation of Met, and acetylation of protein N terminal. Quantification was based on the LFQ method (Cox *et al.* 2014), based on unique/all peptides.

Results

Videos 1 and 2 of hatching blastocysts clearly show the hatching point at which the zona breaching cells interact with the ZP. They penetrate the zona by cellular projections that finally emerge through a tunnel created in the zona. Subsequently the tunnel widens to allow herniation and finally dynamic hatching of the entire blastocyst. The initial hatching and the breached zona are also shown in Fig. 1 (images a, b, c, d). Samples of clusters of hatched cells that squeezed out of the zona and were collected for proteomics analyses are demonstrated in Fig. 2. Intracellular vacuoles can be seen in the hatched clusters in both Figs. 1 and 2.

Proteomic analysis of hatching, TE, and whole blastocysts identified a total of 1245 proteins (with at least 2 peptides) in all samples.

Forty nine proteins in the hatching cells and 17 proteins in the TE cells that were significantly upregulated are shown in supplementary Tables S1 and S2, respectively. These proteins are listed according to their relative intensity as compared to whole blastocysts. The first 45 proteins in the list of hatching cells and the first 12 proteins in the list of TE cells showed $> \times 100$ -fold changes as compared to control blastocysts.

In this study, we chose to discuss from each list several proteins that showed the highest expression in hatching and TE cells (Tables 1 and 2, respectively).

A minimal overlap between hatching and TE proteins was observed. Only serine protease inhibitors and lipocalin were detected in both samples.

Myosin and actin were highly upregulated in the hatching cells as well as paraoxonase, N-acetylmuramoyl alanine amidase, and SERPINS clade A and galectin.

In the TE cells, gamma butyrobetaine dioxygenase, lupus La protein, sialidase, lysosomal Pro-X carboxypeptidase,

phospholipase b, and SERPINS clade B and A were among the most highly upregulated proteins.

Discussion

Blastocyst hatching is a critical prerequisite for implantation in a receptive endometrium. Blastocyst-stage embryos hatch from the ZP and expose TE cells which will establish the interface with the epithelial endometrium. The hatching process seems to depend on physical (blastocyst expansion) as well as molecular factors (initiation of zona breaching by hatching cells).

The purpose of our study was to investigate the contents of the first hatching cells in order to elucidate the molecular network underlying their activity.

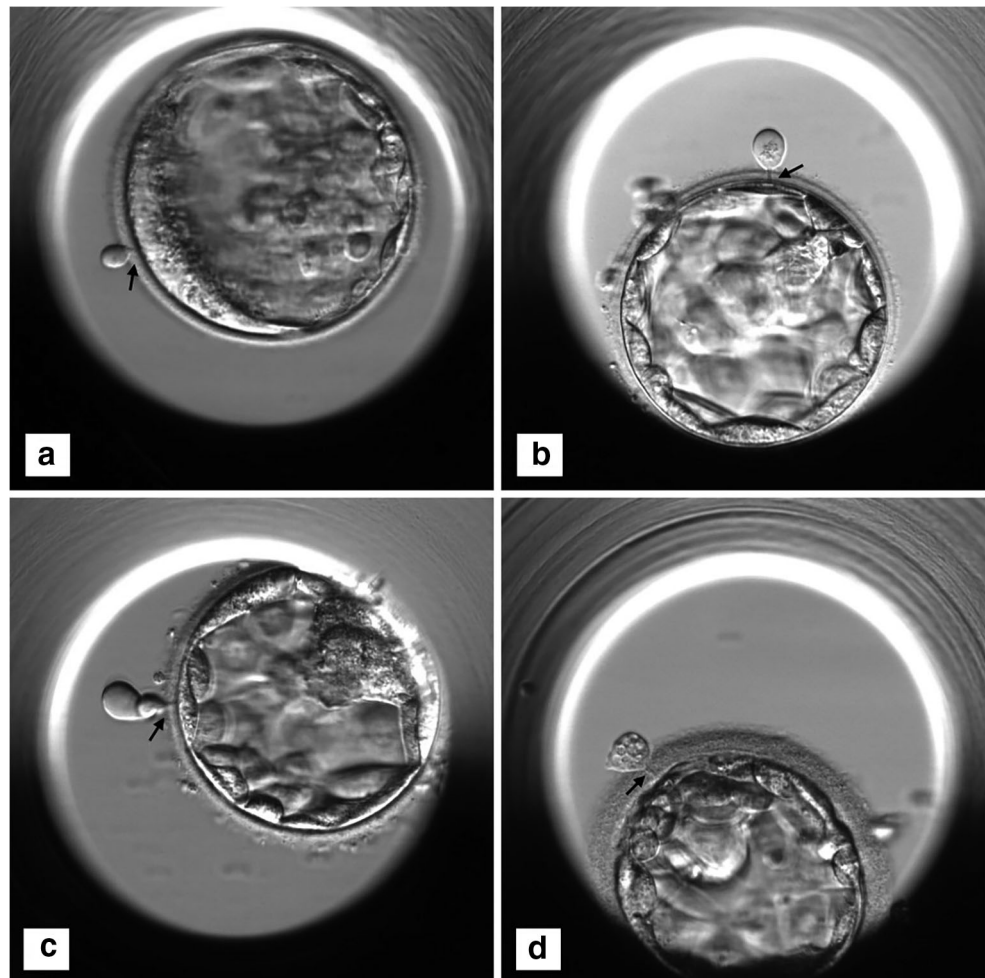
It is not surprising that actin and myosin were found to be among the most highly upregulated proteins in the hatching cells as they are actively involved in breaching and migration through the zona layer.

Paraoxonase 1 (PON1) which was highly expressed in the hatching cells (Table 1) is a high-density lipoprotein-associated enzyme that has a broad substrate specificity (Furlong *et al.* 2010). PON1 is important in metabolizing oxidized lipids. Previous studies have indicated that the inhibition of PON1 in human spermatozoa causes oxidative stress (Aitken *et al.* 2016) and its activity in follicular fluid was a significant positive predictor for day 3 embryo cell number (Browne *et al.* 2008). Furthermore, high expression of lipid metabolic genes and lipid metabolism was observed in preimplantation embryos and blastocysts, respectively (Dunning *et al.* 2010; Altmäe *et al.* 2012). It is therefore reasonable to assume that in actively hatching cells, paraoxonase 1 is important in maintaining oxidative balance.

Peptidoglycan recognition proteins (PGLYRP) are innate immunity proteins induced by interleukins. Their role in the hatching cells is obscure and needs further studies. However, it is noteworthy that pro-inflammatory and anti-inflammatory cytokines were identified in mammalian blastocysts (Seshagiri *et al.* 2016). It was assumed that cytokines play an important role during blastocyst differentiation and hatching. Additionally, it was suggested that cytokine-signaling systems activate zonal proteases associated with hatching (Seshagiri *et al.* 2016).

The serine protease inhibitors (SERPIN) clades A and G and clades A, B, and G appear in hatching and TE cells, respectively. Clades A and G are extracellular, whereas SERPINB3 is intracellular. A serpin-enzyme complex is formed and the conformational change causes the inhibitory effect (Heit *et al.* 2013; Lucas *et al.* 2018). SERPINA3 and G1 genes have been previously identified in human frozen-thawed blastocysts and endometrial samples using genome expression analyses (Altmäe *et al.* 2012).

Fig. 1 Hatching blastocysts on day 5, *arrows* indicate slit in the ZP



We harvested the initial hatching cells after their passage through the zona when they were still attached to its outer surface (as shown in Fig. 2). It seems plausible that they had already secreted their proteolytic enzymes when they were in close proximity to the zona on the inside of the blastocysts, prior to hatching. Therefore these enzymes were not expressed in the cellular clusters that we collected. However, the impressive presence of serine protease inhibitors may imply that their function was to terminate the enzymatic activity of serine proteinases once the zona matrix was cleaved from the inside. We were not able to identify and isolate initial hatching cells while they were still inside the blastocyst. Furthermore, the collection process of hatching cells involved their washing in PBS in order to avoid the interference of culture media components. If proteases were released during zona penetration, they were washed out during the collection of the hatched cellular clusters.

Transmission electron microscopy of human blastocysts showed the presence of lysosomes and vesicles in zona breaker cells close to the hatching point on the inside of the embryos (Sathanathan *et al.* 2003). Similarly, intracellular vesicles in

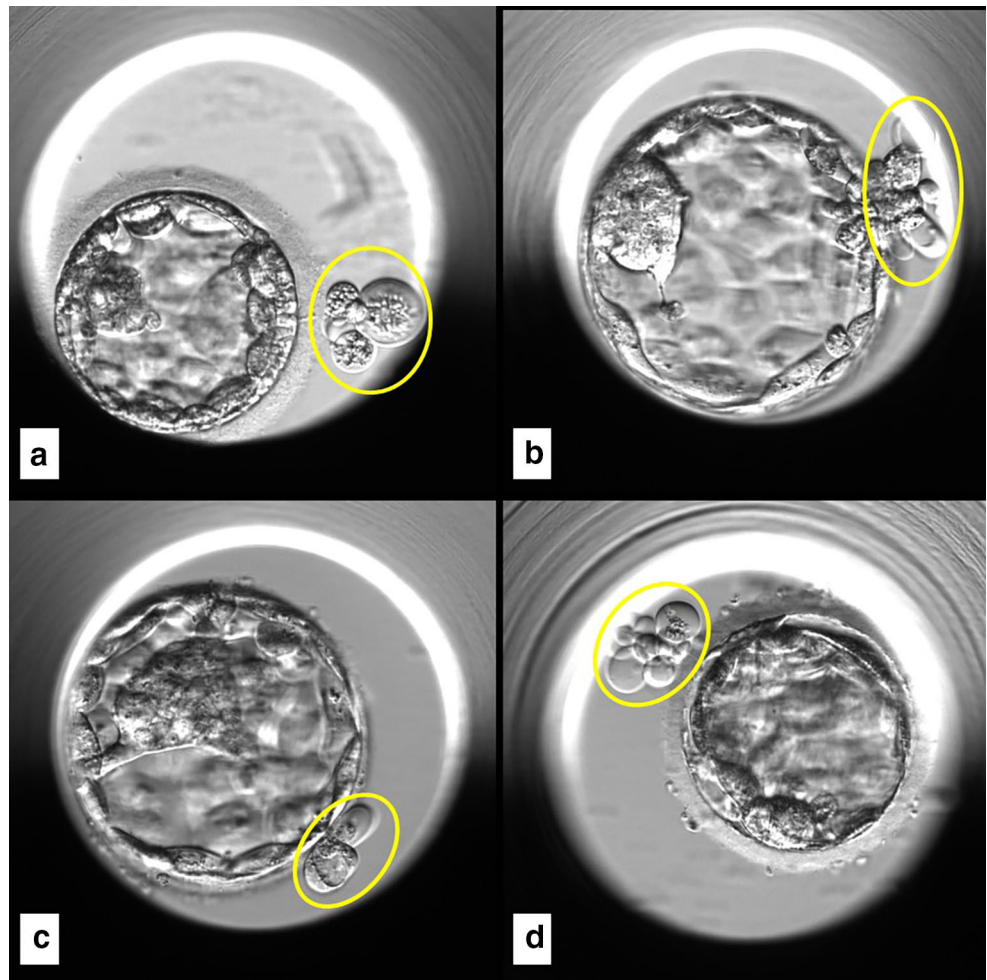
the hatched cells are shown in Figs. 1 and 2. These observations also point to a secretory activity of the hatching cells upon interaction with the ZP.

Galectin-1 is a beta-galactosidase binding lectin. Galectin-1 was detected in preimplantation human embryos on days 3 and 5 in the TE and inner cell mass (Tirado-González *et al.* 2013). The embryos also secrete galectin-1, suggesting that it may be important for the implantation process (Barriento *et al.* 2014). The presently observed upregulation of galectin-1 in hatching cells corresponds well with that notion.

Histidine-rich glycoprotein (HRG) was found in human preimplantation embryos and blastocysts (Nordqvist *et al.* 2010). The upregulated HRG in hatching cells observed in our study supports Nordqvist's research. Additionally, HRG has been recently found in the human embryo secretome (Kaiholo *et al.* 2019). It has been suggested that HRG can be used as a marker of embryo quality (Kaiholo *et al.* 2019). However, no significant differences in HRG levels were found between the secretomes from high-quality and low-quality blastocysts (Kaiholo *et al.* 2019).

The proteomics analysis of TE cells revealed upregulation of 17 proteins as compared to 49 proteins in the initial hatching cells

Fig. 2 Samples of hatched cellular clusters isolated for mass spectrometry analysis circles indicate the hatching cells collected for proteomics analysis



(supplementary Tables S1, S2). There was no substantial overlap in protein expression between TE and hatching samples except for the SERPIN protease inhibitors. It seems that many genes are

necessary for the initiation and successful completion of blastocyst hatching.

It is intriguing that in TE cells, gamma butyrobetaine hydroxylase is the most highly upregulated enzyme. This

Table 1 Eleven upregulated proteins in hatching cells

Protein names	Gene names	Fold change hatching cells/2 blastocysts
Myosin-4	MYH4	> × 100
Serum paraoxonase/arylesterase 1	PON1	> × 100
N-acetylmuramoyl-L-alanine amidase	PGLYRP2	> × 100
A0a024r617; A0A0G2JRN3; G3V2B9; G3V544; G3V5R8	SERPINAL1	> × 100
A0A0G2JMB2		> × 100
Actin, alpha skeletal muscle; actin, alpha cardiac muscle 1; actin, gamma-enteric smooth muscle; actin, aortic smooth muscle	ACTA1; C1; G2; A2	> × 100
Galectin-1	LGALS1	> × 100
Histidine-rich glycoprotein	HRG	> × 100
Kallistatin	SERPINA4	> × 100
Lipocalin-1; putative lipocalin-1-like protein 1	LCN1; LCN1P1	> × 100
Plasma protease C1 inhibitor	SERPING1	> × 100

Table 2 Eight upregulated proteins in TE cells

Protein names	Gene names	Fold change TE/3 blastocysts
Gamma butyrobetaine dioxygenase	BBOX1	> × 100
Lupus La protein	SSB	> × 100
Lysosomal Pro-X carboxypeptidase	PRCP	> × 100
Plasminogen activator inhibitor 2	SERPINB2	> × 100
Plasma protease C1 inhibitor	SERPING1	> × 100
Alpha-1-antichymotrypsin; alpha-1-antichymotrypsin His-Pro-less	SERPINA3	18.3
Lipocalin-1; putative lipocalin-1-like protein 1	LCN1; LCN1P1	14.6
A0A024R617; A0A0G2JRN3; G3V2B9; G3V544	SERPINA1	11.3

enzyme catalyzes the formation of L-carnitine which is essential in fatty acid metabolism. Carnitine allows the transport of activated fatty acids across the mitochondrial membrane during mitochondrial beta oxidation. Upregulation of beta oxidation improves ATP generation in the mitochondria which is beneficial for blastocyst hatching and implantation. Supplementation of L-carnitine in the culture media of human embryos has been shown to improve embryo quality and pregnancy rates (Kim *et al.* 2018). It has been suggested that carnitine plays an important role both as an antioxidant and in energy production (Kim *et al.* 2018).

Lupus La (second in line, Table 2) functions as an RNA-binding protein that promotes the maturation of tRNA precursors. It is also associated with ribosome biogenesis. It contributes to the establishment and survival of embryonic stem cells produced by mouse blastocysts (Park *et al.* 2006). The disruption of the La gene in mice leads to embryonic lethality (Park *et al.* 2006).

Polycarboxypeptidase (PRCP) is a member of a family of serine exopeptidases. The PRCP gene encodes preproprotein that is cleaved to generate the mature lysosomal PRCP. This enzyme has been shown to be an activator of the cell matrix-associated prekallikrein (Wang *et al.* 2014). Interestingly, in our study, kallikrein was upregulated in the hatching cells (Table 1), but the interrelationship between PRCP and kallikrein in the blastocyst needs future studies.

Previous mass spectrometry analysis of glycans in the human ZP demonstrated that Sialyl-Lewis^x is profusely expressed on the zona glycans (Clark 2013). Whether TE-derived sialidase 2 is involved in the processing of these glycans is not yet clear.

Our present analysis indicates that lipocalin-1 is upregulated in TE as well as in hatching cells. Lipocalin-1 was previously identified in the secretome of human blastocysts and was associated with chromosome aneuploidy (McReynolds *et al.* 2011). Our results support these findings. Lipocalin-1 may be a marker of aneuploidy as all of the blastocysts we

analyzed were polyploid and probably had unbalanced sets of chromosomes.

Kirkegaard *et al.* (2013) and Araki *et al.* (2016) investigated whether spontaneous hatching process was affected by the fertilization method. They reported that gradual hatching by small TE projections through the ZP was observed in almost all ICSI blastocysts and approximately half of IVF blastocysts. They surmised that their observations might reflect the impact of the ICSI needle which leaves a mechanical breach in the zona. However, in their studies, the ICSI needle penetration site was not labeled and therefore could not be identified as the hatching site. Since in the present report we focused on the characterization of initial hatching cells, which appear in both types of fertilization, the fertilization method is irrelevant for the interpretation of our results.

For ethical reasons, our experimental system included only polyploid human blastocysts. It seems reasonable that the proteins that regulate hatching in normal blastocysts are the same as those in hatching polyploid blastocysts.

Conclusions

We have shown that the initiation of hatching of the human blastocyst is accompanied by upregulation of specific proteins in the zona breaching cells and that the majority of these proteins are not upregulated in TE cells. Possibly, single-cell gene analysis of isolated hatching cells or RNAseq could precisely identify the specific genes involved in zona lysis.

We believe that our study may contribute to basic knowledge regarding the molecular behavior of the specific hatching cells that are able to invade the glycoprotein matrix of the ZP and help understand why some blastocysts are unable to hatch and fail in this process which is mandatory for successful implantation.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11626-020-00522-w>.

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