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RESEARCH COMMUNICATION

## Proteomic analysis of bovine blastocoel fluid and blastocyst cells

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### Abstract

The understanding of the early mammalian development is a prerequisite for the advancement of *in vitro* fertilization and improvement of derivation and culturing of embryonic stem cells. While, whole genome transcriptomic analysis on bovine blastocysts has identified genes active in early development, little information is available about the protein complement of early embryos. Modern, sensitive proteomic technology (nano HPLC tandem mass spectrometry) allowed us to describe the proteome of the scarce blastocoel fluid and cell material of expanded bovine blastocysts isolated by micromanipulation. From two independent replicates, 23 proteins were identified in the blastocoel fluid while 803 proteins were identified in the remaining cell material. The proteins were grouped into categories according to their gene ontology (GO) terms by which proteins involved in cell differentiation, cell proliferation, development, and reproduction could be derived. Proteins classified in these categories could be candidates for further functional studies to understand pluripotency and early mammalian development.

**Abbreviations:** ESC: embryonic stem cells; GO: gene ontology; HILIC: hydrophobic interaction liquid interaction chromatography; HPLC: high-performance liquid chromatography; HSA: human serum albumin; ICM: inner cell mass; ICSI: intra cytoplasmic sperm injection; iPSC: induced pluripotent stem cells; IVF: *in vitro* fertilization; IVM: *in vitro* maturation; TE: trophoctoderm

### Introduction

The mammalian blastocyst is the early unimplanted embryo consisting of a spherical and fluid filled aggregation of 50–140 cells [Hardy et al. 1989]. It consists of an outer layer of differentiated trophoctoderm (TE) cells that gives rise to placental tissue as well as an undifferentiated inner cell mass (ICM), which gives rise to the developing embryo [Pedersen et al. 1986]. The function and composition of the blastocoel fluid, bathing both the TE and ICM, remains largely unknown. Originally, it was suggested that the cavity solely provides a compartment for migration of cells in the process of gastrulation [Stableford 1949]. However, later studies demonstrated that the blastocoel fluid may directly support the development of the blastocyst. In an elegant study, Pedersen and Spindle injected donor 8-cells and morulas into the blastocoel cavity of giant chimeric blastocysts formed by the aggregation of 8–10 embryos at the 4–8-cell stage, to

### Keywords

Blastocoels, blastocyst, bovine, IVP, proteome

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show that the injected embryos develop into normal blastocysts with their own blastocoel cavity [Pedersen and Spindle 1980]. This finding strongly supports the suggestion that the blastocoel fluid plays a critical role in supporting cellular processes during pre-implantation development other than merely providing a compartment for cell migration.

The ICM from a series of mammalian species can be isolated to form an undifferentiated cell line of embryonic stem cells (ESC) with the potential to develop *in vitro* into any cell type present in the adult body. Due to the far-reaching potential of stem cells in regenerative medicine [Mountford 2008], human ESC have gained growing interest ever since the first derivation of a human ESC cell line in 1998 [Thomson et al. 1998] and later the first derivation of induced pluripotent stem cells (iPSC) from a human adult skin biopsy [Takahashi et al. 2007]. Embryonic stem cells and iPSC technologies are also explored for utilization in livestock research, breeding, and transgenesis with a special focus on bovine and porcine cells. Pluripotent cells may be used to improve traits of agricultural importance, genetically manipulate species for the production of biomedical models, and potentially provide a model to test novel iPSC therapies

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[Lu et al. 2012]. However, it remains a challenging task to derive and culture stable pluripotent cell lines from large domestic animals [Ezashi et al. 2012; Telugu et al. 2010].

Evaluation of the protein profile of the blastocoel fluid would potentially advance our understanding of whether the fluid influences blastocyst development and more specifically on the regulation of proliferation of cells in the ICM. Furthermore, specific protein components in the blastocoel fluid would be candidates to use in culture media for both pre-implantation embryos and ESC/iPSC. Although several studies were performed in the 1970s on the physiochemical properties of especially rabbit blastocoel fluid [Borland et al. 1977; Cross 1973, 1974; Varma et al. 1979], the protein composition of the fluid is scarcely described. Nevertheless, the blastocoel fluid is expected to constitute a combination of proteins originating from the maternal environment through trans-cellular transport and/or through the release of components from ICM and TE cells of the blastocyst [Dardik and Schultz 1991]. Other prospects for examinations of blastocoel fluid are to select the best embryo for transfer after *in vitro* fertilization (IVF) procedures in humans [D'Alessandro et al. 2011], and also to identify genetic diseases in individuals before transfer, known as pre implantation genetic diagnosis or PGD [Palini et al. 2013].

Despite recent advances in various proteomic technologies, knowledge of the general mammalian blastocyst proteome remains very limited. The latest review in the field by Katz-Jaffe and McReynolds [2013] lists the main hurdles to be the combined effects of limited template, low protein concentration, deficient platform sensitivity, and limited protein database information. Recently, our group published the first proteomic study on the human blastocoel fluid and

blastocyst cells [Jensen et al. 2012]. In the present study we present a proteomic analysis of the blastocoel fluid and remaining cells of bovine blastocysts.

## Results and Discussion

In this study, we isolated and analyzed the blastocoel fluid and remaining cells from 20+11 bovine blastocysts using micromanipulation as shown in Figure 1. The expanded blastocyst with a clear blastocoel cavity was fixed on a holding pipette. The micropipette was inserted and blastocoel contents aspirated until collapse. The contents were then subjected to nano-HPLC tandem mass spectrometry.

An overview of the number of proteins identified is presented in Venn-diagrams in Figure 2. A total of 99 proteins could be identified in the blastocoel fluid; 23 of these were present in both samples and are described in Table 1. Fourteen of these 23 proteins could also be found in human blastocoel fluid [Jensen et al. 2012] and these common proteins are marked with an asterisk in the table. Of the 99 blastocoel proteins, 58 could also be identified in the bovine cell material which further supports the identity of these proteins.

In the blastocyst cell material, 2,341 unique proteins could be identified. The large pool of cells from 20 blastocysts contributed 2,201 protein identifications while the smaller pool of 11 blastocysts contributed 944 protein identifications. There was an overlap of 802 proteins between the two biological replicates and these proteins were analyzed further using the ProteinCenter software. Here, the blastocyst proteins were grouped according to their cellular function using gene ontology (GO) terms, and the distribution is presented in a pie chart in Figure 3. Proteins from the

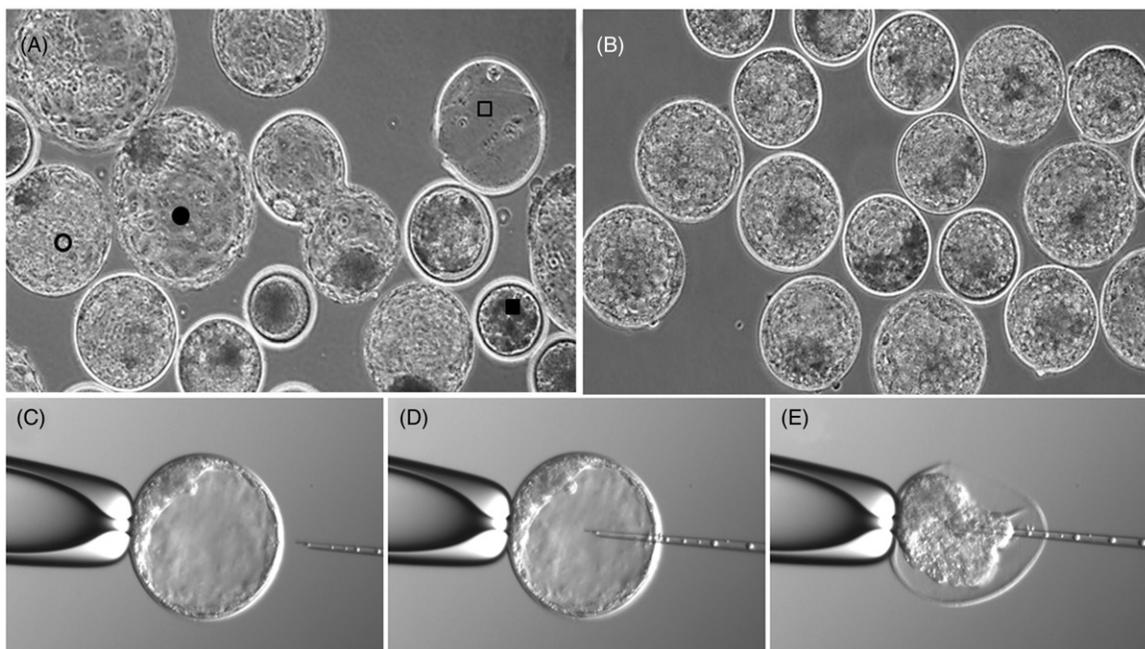


Figure 1. Isolation of bovine blastocoel fluid. (A) A mixed population of blastocysts with arrested embryos (■), expanded (○), and hatched blastocysts (●). Notice the empty zona pellucida (□). (B) Collection of expanded blastocysts with a clear blastocoel cavity chosen for micromanipulation. (C) A single blastocyst is fixed on a holding pipette with the inner cell mass (ICM) pointing towards the top of the holding pipette. (D) The micropipette is inserted in the blastocoel cavity. (E) Suction applied and the blastocoel is aspirated until the blastocyst collapses.

categories *cell differentiation*, *cell proliferation*, *development* and *reproduction* were retrieved and these are presented in Supplemental Table 1. Proteins from these categories could be of potential interest in the support of stem cell- and embryo culture and selected proteins are described in Table 2.

Comparing the present bovine data set to the human blastocyst proteomic data set [Jensen et al. 2012] nine proteins were found to be unique to the bovine blastocoel fluid: Alpha-S1-casein, Annexin, Beta-casein, Beta-lactoglobulin, Casocidin-I, Histone H1.1, METTL3 protein, Proline-rich protein P-B, and Ubiquitin C. An overlap of 309 proteins was found comparing the protein identifications from the cell samples of the two species. These proteins are listed in Supplementary Table 1 and a selection of the proteins is presented in Table 3. Seventeen proteins were identified in the native culture medium (BlastAssist<sup>®</sup>) and since the only

protein source in this medium is human serum albumin (HSA), the identified medium proteins must be co-purified proteins from the HSA production.

### Blastocoel proteins and pluripotency

In the search of proteins to support the undifferentiated growth of pluripotent stem cells (ESC and iPSC), the proteins in the blastocoel fluid are of obvious interest as they bathe the pluripotent ICM of the blastocyst while the undifferentiated cells proliferate. The study of human blastocoel fluid provided potential protein candidates such as various vitamin-binding proteins and specific heat shock proteins [Jensen et al. 2012]. It is well-known that bovine pluripotency in part is supported by factors different from those in humans [Maruotti et al. 2012], and it was therefore expected that previously unknown bovine blastocyst proteins of interest would be identified. However, not one of the nine proteins found to be unique to the bovine blastocoel fluid have any known relation to pluripotency or early developmental biology. Further functional studies are required to identify whether they function in early development.

Proteins found in the blastocoel fluid could potentially improve the production of stable bovine ESC or iPSC lines if added either alone or in combination to the culture medium during isolation and/or maintenance of the cells. The production of stable porcine ESC/iPSC lines as well as large numbers of porcine blastocysts has similarly proven to be a challenging task. While the developmental processes up until the hatching blastocyst are very alike between humans, cattle, and pigs, the post-hatching progression and placentation are substantially different between humans and the livestock species. Candidate proteins for improvement of culture media for porcine ESC/iPSC lines therefore may be

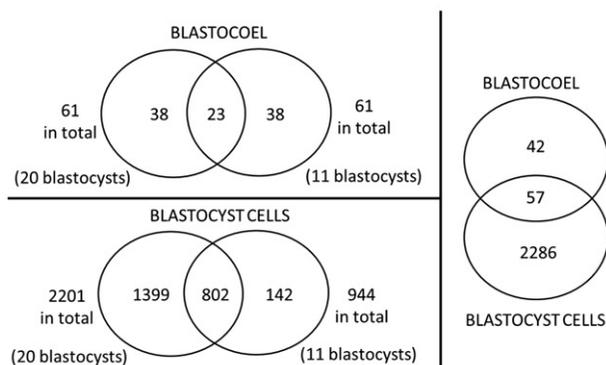


Figure 2. Venn diagrams over protein identifications in bovine blastocoel fluid and blastocyst cells with an overview of the proteins in each biological replicate. Figure not to scale.

Table 1. Proteins identified in the bovine blastocoel fluid.

PROTEINS IN THE BLASTOCOEL FLUID			
Protein description	Size (kDa)	Accession number	Functional keywords (Uniprot)
14-3-3 protein zeta/delta*	27.7	P63103	Adapter protein
Actin, cytoplasmic 1*	41.7	P60712	Cell motility
Alpha-S1-casein	23.0	P02662	Antioxidant
Annexin	39.0	F1N650	Binding of calcium and phospholipid
Annexin A2*	38.6	P04272	Calcium-regulated membrane-binding
Arginase-1*	35.0	Q2KJ64	Arginine metabolism and urea cycle
Beta-casein	23.6	P02666	Antioxidant
Beta-lactoglobulin	18.3	P02754	Retinol binding
Casocidin-I	24.3	P02663	Calcium phosphate transport
Desmoplakin*	332.2	E1BKT9	Major protein of desmosomes
Heat shock protein beta-1*	17.4	G3X7S2	Stress resistance and actin organization
Histone H1.1	10.4	P02253	Nucleosome assembly
Histone H2B*	13.9	A5D7N2	Nucleosome core
Junction plakoglobin*	81.8	Q8SPJ1	Cytoskeleton
Lysozyme*	3.7	Q27997	Lysozyme activity
Major vault protein*	98.9	F1MNN6	Required for normal vault structure
METTL3 protein	64.4	A6QQV4	mRNA methyltransferase activity
Proline-rich protein P-B	5.7	Q60FB3	Modulating effect on immune system
Thioredoxin*	10.9	G8JKZ8	Redox signaling
Ubiquitin C	34.3	E1B9K1	Activation of protein kinases and signaling
Zona pellucida glycoprotein 2*	75.4	Q9BH10	Secondary sperm receptor
Zona pellucida glycoprotein 3*	41.8	Q58DI4	Sperm binding and zona matrix formation
Zona pellucida glycoprotein 4*	57.1	Q9BH11	Sperm receptor

The functional keywords are found by searching the proteins at [www.uniprot.org](http://www.uniprot.org). \*marked proteins which were also identified in human blastocoel fluid [Jensen et al. 2012].

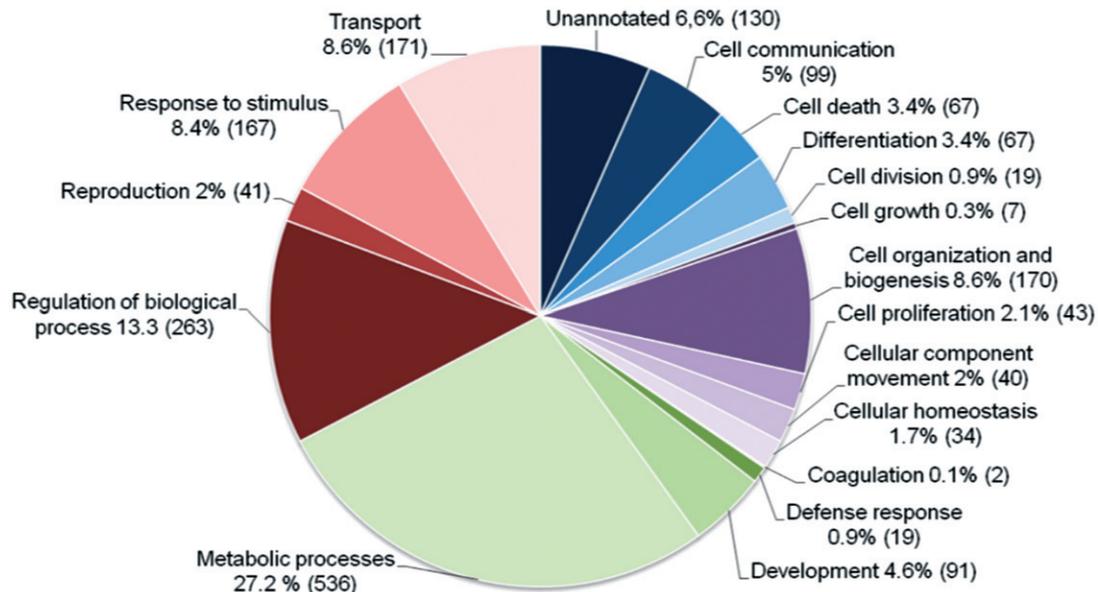


Figure 3. The distribution of gene ontology (GO) terms of the proteins from the bovine blastocyst cells. A protein can appear in more than one category.

found in both the human and bovine blastocoel proteomes, although the bovine results may be more relevant to the pig developmental biology.

It is clear that we have only identified a few of the most abundant proteins present in the blastocoel fluid in this bovine study. While the concentration of a specific protein is important for the biological effect, it is likely that a low-abundance protein could have an essential effect on cellular pluripotency or differentiation. However, such low abundance proteins could not be detected in this study. Isolation and analysis of blastocoel fluid is technically challenging and a larger study is needed to reveal more proteins in the important fluid of the mammalian blastocyst.

### Development and signaling

In Table 2 and Supplementary Table 1, proteins involved in *cell differentiation*, *cell proliferation*, *development* and *reproduction* are listed. For many proteins, this study is the first to link their expression to the mammalian blastocyst. Although our study does not provide understanding about the effects and kinetics of each blastocyst protein identified, the knowledge of which proteins are present in the blastocyst provide an important platform to qualify further functional studies. The recent progress in the mapping of the bovine embryo transcriptome [Chitwood et al. 2013; Driver et al. 2012; Filliers et al. 2011; Mamo et al. 2012] are invaluable to support this work.

Specifically in the GO category *development*, 89 proteins were identified in the bovine blastocyst cell material. Several of these development-associated proteins are known to have functions in cytoskeletal organization and cell adhesion and thus may exert functions in modulation of cell shape and migration in the developing embryo. Examples of such proteins from the present data are Drebrin, Desmoplakin, Fibronectin, Filamin-A, Integrin beta-1, Junction plakoglobin, Lactadherin, Transgelin-2, and Tubulin-folding cofactor B.

Few proteins were identified with known roles in cellular signaling, examples are STAT3 protein and Catenin delta-1.

Surprisingly, none of the major proteins known to be involved in embryo-maternal signaling were detected in the blastocyst cells in our study (e.g. Interferon Tau, Interleukin 1B, TNF alpha, or IGF binding proteins [Munoz et al. 2012; Staggs et al. 1998; Wolf et al. 2003]). These proteins may be present in undetectable concentrations or may appear at later stages of development. Interferon Tau has been detected in other studies at the blastocyst stage [Johnson et al. 2006; Kimura et al. 2004; Kubisch et al. 2003] and this indicates that we probably have been unable to detect the known embryo-maternal signaling components in our study with the available blastocyst material and proteomic technology.

### Proteins in the human and bovine blastocyst cells

A total of 309 of the 802 internally validated bovine blastocyst proteins were also identified in the human blastocyst cells [Jensen et al. 2012]. The development of the embryo up until the blastocyst stage is rather similar between human and cattle; however, there are differences in the developmental kinetics and in the subsequent early developmental steps. The blastocyst is formed on day 5–6 in humans and day 7–8 in cattle, and further the implantation of human blastocysts takes place shortly after the blastocyst is formed while the bovine blastocyst continues to grow and later elongate extensively for about three weeks of detached development [Blomberg et al. 2008]. The bovine conceptus size increases more than 1000-fold during the elongation [Maddox-Hyttel et al. 2003] and this is accomplished by an increase in cell number and accompanying protein synthesis [Degrelle et al. 2005; Thompson et al. 1998]. Furthermore, bovine placentation is synepitheliochorial, which means that the uterine epithelium is modified by invasion and fusion of trophoblast cells; however, it shows very little invasiveness [Wooding 1992]. It is therefore not surprising that the protein expression is not identical

Table 2. Selected proteins from the bovine blastocyst cells with the gene ontology (GO) terms *cell differentiation*, *cell proliferation*, *development*, and *reproduction*.

SELECTED PROTEINS FROM THE BLASTOCYST CELLS				
GO term	Protein description	Size (kDa)	Accession number	Functional keywords (Uniprot)
Cell differentiation	Calpain-2 catalytic subunit	78.0	Q27971	Cytoskeletal remodeling and signal transduction
	Cullin-3	88.9	E1BIN5	Required for embryonic stem cells division
	Disabled homolog 2	82.4	F1N371	Adapter molecule in neural development
	DnaJ homolog subfamily A member 3, mitochondrial	49.2	A1A4J9	Modulates apoptotic signal transduction
	ERO1-like protein alpha precursor	51.9	A5PJN2	Antioxidant
	Nuclease-sensitive element-binding protein 1	36.0	F1MZ92	Stimulates cell migration and proliferation
	Nucleoside diphosphate kinase B	17.3	Q3T0Q4	Neural patterning and cell fate determination.
	Platelet-activating factor acetylhydrolase IB subunit alpha	46.6	A5D7P3	Proliferation and migration of neuronal precursors
	Ras-related protein Rab-11A	24.4	F2Z4D5	Regulator of membrane delivery during cytokinesis
	Redox-regulatory protein FAM213A	24.3	Q3ZBK2	Antioxidant
Cell proliferation	14-3-3 protein sigma	27.8	Q0VC36	Regulates protein synthesis and epithelial cell growth
	Drebrin	72.1	Q1RMT6	Cell migration and plasticity
	E3 ubiquitin-protein ligase UHRF1	88.3	A7E320	Epigenetic regulator
	Eukaryotic translation initiation factor 5A-1	16.8	Q6EWQ7	Muscle stem cell differentiation a.o.
	High-mobility group AT-hook 1	10.4	Q2V9X0	Cell cycle regulation
	Inhibin alpha chain	36.9	P07994	Hormone secretion, embryonic axial development a.o.
	Peroxiredoxin-1	22.2	Q5E947	Antioxidant
	Tax1-binding protein 3	13.7	Q3ZCD3	Inhibitor of Wnt signaling
	Thioredoxin-dependent peroxide reductase	21.5	P35705	Antioxidant
	Ubiquitin-conjugating enzyme E2 L3	16.9	F1MC72	Regulates nuclear hormone receptor activity
Development	Anamorsin	33.2	Q5EAC7	Anti-apoptotic effects
	Catenin alpha-1	100.0	F1MM34	Cell differentiation
	LGALS3 protein	27.6	A6QLZ0	Differentiation of epithelial cells during early embryogenesis
	MAP2K1 protein	43.4	Q0VD16	MAP kinase signaling
	Nucleoplasmin-2	22.6	D2DSG3	Chromatin reprogramming in fertilization and early embryonic development
	Oocyte-expressed protein homolog	15.9	A0JNQ6	Essential for zygotes to progress beyond the first embryonic cell divisions
	Prohibitin-2	33.3	Q2HJ97	Estrogen regulation
	Prosaposin	58.1	A1L555	Sphingolipid activation
	STAT3 protein	83.1	Q32LP6	Mediates cellular responses to growth factors
	Transforming protein RhoA	21.8	P61585	Essential in cleavage furrow formation
Reproduction	Dihydrolipoyl dehydrogenase, mitochondrial	54.2	F1N206	Hyperactivation of spermatozoa
	Lactadherin	45.5	Q95114	Zona pellucida-binding protein
	NACHT, LRR and PYD domains-containing protein 5	120.9	F1MT67	Essential role for zygotes to progress beyond the first embryonic cell divisions
	Peptidyl-prolyl cis-trans isomerase FKBP4	51.5	F1MU79	Role in trafficking of steroid hormone receptors between cytoplasm and nucleus
	Protein DJ-1	20.0	Q5E946	Plays a role in fertilization
	UV excision repair protein RAD23 homolog B	43.1	Q29RK4	Modulation of proteasomal degradation
	Vesicle-associated membrane protein-associated protein B	27.1	A2VDZ9	Cellular calcium homeostasis regulation
	Zona pellucida glycoprotein 2	75.4	Q9BH10	Secondary sperm receptor
	Zona pellucida glycoprotein 3	46.5	Q58DI4	Sperm binding and zona matrix formation
	Zona pellucida glycoprotein 4	82.9	Q9BH11	Sperm receptor

The functional keywords are found by searching the proteins at [www.uniprot.org](http://www.uniprot.org).

between the two species. However, human and bovine embryos can be successfully cultured up until the blastocyst stage in the same culture medium (e.g. BlastAssist®) and the expression of many of the basic housekeeping and conserved proteins are expected to be similar.

Our data can be used to pinpoint molecular differences in the developmental pathways between the two species in support of comparative studies. Such studies, preferably including the mouse embryo, are important with perspectives

in both evolutionary biology and comparative physiology in a field where data regarding culture conditions and molecular functions often are extrapolated between the model-species cow and mouse to the human pre-implantation embryo.

### Technical considerations

A day seven expanded bovine blastocyst with a size of around 200–250 µm is expected to contain 2–8 nl of blastocoel fluid.

Table 3. Selected proteins identified in both human and bovine blastocyst cells.

SELECTED PROTEINS IN COMMON BETWEEN BOVINE AND HUMAN BLASTOCYST CELLS			
Protein description	Size (kDa)	Accession number	Functional keywords (Uniprot)
Anamorsin	33.2	Q5EAC7	Anti-apoptotic effect
Aurora kinase A	45.4	Q2TA06	Critical role in various mitotic events
Basigin	29.8	Q3ZBX0	Spermatogenesis, embryo implantation a.o.
Catenin alpha-1	100	F1MM34	Cell differentiation
Ezrin	68.7	P31976	Cytoskeletal structure
Fascin	54.8	Q3MHK9	Cell motility and migration
Heat shock 70 kDa protein 4	94.4	E1BBY7	Stress response
Heat shock protein 75 kDa, mitochondrial	73.2	Q2TB14	Chaperone
Heat shock protein HSP 90-alpha	84.7	Q76LV2	Chaperone
Hypoxia up-regulated protein 1	111.6	E1B748	Cytoprotective role after oxygen deprivation
Inositol polyphosphate 1-phosphatase	43.9	P21327	Regulation of phosphatidylinositol signaling pathways
Insulin-like growth factor 2 mRNA-binding protein 1	63.4	E1BKJ7	IGF2 regulation
Lamin-B1	66.1	E1B8N6	Component of the nuclear lamina
Nucleophosmin	32.2	E3SAZ8	Protein chaperoning, cell proliferation a. o.
Oocyte-expressed protein homolog	15.9	A0JNQ6	Essential for zygotes to progress beyond the first embryonic cell divisions
Peroxiredoxin-1	22.2	Q5E947	Antioxidant
Prostaglandin E synthase 3	18.7	Q3ZBF7	Chaperone
Protein DJ-1	20	Q5E946	Plays a role in fertilization
Protein S100-A14	11.4	Q3MHP3	Regulation of cell survival and apoptosis
Thioredoxin	10.9	G8JKZ8	Antioxidant
Ubiquitin carboxyl-terminal hydrolase isozyme L1	25.2	F1MQ16	Processing of ubiquitin precursors and of ubiquitinated proteins
Vitronectin	53.5	Q3ZBS7	Cell adhesion and spreading factor
Zona pellucida glycoprotein 2	75.4	Q9BH10	Secondary sperm receptor
Zona pellucida glycoprotein 3	46.5	Q58DI4	Sperm binding and zona matrix formation
Zona pellucida glycoprotein 4	82.9	Q9BH11	Sperm receptor

A total of 309 proteins were identified in both human and bovine blastocysts [Jensen et al. 2012]. The functional keywords are found by searching the proteins at [www.uniprot.org](http://www.uniprot.org).

The collection of this fluid by micromanipulation is technically challenging and it is difficult to obtain sufficient clean sample material for a comprehensive proteomic mapping of the proteins in the fluid. In our study we examined two samples of pooled blastocoel fluid and even though the first sample contained double the amount of blastocoel fluid compared to the second (from 20 and 11 blastocysts, respectively), the resulting number of proteins identified are the same (61 proteins in each sample). Reasons for these results can be due to handling difficulties during the micromanipulation procedure leading to a smaller fluid yield per blastocyst or loss of sample material during their preparation for proteomic analysis. The yield from the proteomic analysis can also vary with such small samples because we are working at the limit of detection for the proteomic equipment. Blastocoel fluid from a larger number of blastocysts will be needed to obtain a full protein profile for the blastocoel fluid.

Regarding the cell material, it seems that the number of protein identifications increases proportionately when the number of blastocysts are increased (944 proteins in 11 blastocysts compared to 2,201 proteins in 20 blastocysts). That 802 of the 944 proteins from the small sample were also detected in the larger sample means that 85% of the cellular protein identifications could be reproduced. Analyzing a larger number of blastocysts, possibly without initial isolation of blastocoel fluid, would potentially provide a more detailed proteomic description of the bovine blastocyst.

To summarize, we report to have identified 23 proteins in two replicates of bovine blastocoel fluid and 803 proteins in

the corresponding replicate blastocyst cell material. To our knowledge, this study is the first proteomic study of bovine blastocoel fluid and blastocyst cells and the data will supplement the recent advances in bovine embryo transcriptomics in improving the understanding of early mammalian development. This will potentially support progress in the field of *in vitro* fertilization and stem cell derivation and culture in both livestock animals and humans.

## Materials and Methods

### Production and handling of bovine blastocysts

Production of bovine embryos from slaughterhouse material with the sole purpose of research, does not require ethical approval by Danish law. Bovine blastocysts were produced at ORIGIO, Denmark and micromanipulation and sample collection was performed at the fertility clinic at Herlev Hospital, Denmark. Reagents and media were purchased from Sigma Aldrich (Brøndby, Denmark) unless otherwise stated. All media were pre-warmed and pre-equilibrated in a CO<sub>2</sub> incubator before use.

### Follicle aspiration and *in vitro* maturation (IVM)

Bovine ovaries, originating mainly from Danish dairy cows and heifers, were obtained from a local slaughterhouse (Herlufmagle Slakteri, Herlufmagle, Denmark), and transported to the laboratory within 3 h of retrieval at 29–32 °C in a thermo container. The temperature from ovary collection throughout all manipulations outside the incubator was kept within this temperature range.

The ovaries were rinsed twice in 0.9% saline and antral follicles with surface diameters between 3 and 15 mm were evacuated by use of a vacuum pump connected to an 18-G needle into a 50 ml tube containing 140 µl heparin (5,000 IE/ml, Leo Pharma, Ballerup, Denmark) to prevent clotting. The cumulus–oocyte complexes (COCs) were washed three times in TCM-199 wash medium supplemented with 50 µg/ml gentamycin and 0.5 mg/ml BSA. After retrieval and wash, the COCs were washed once in IVM medium consisting of DMEM supplemented with 5% FBS, 15 IE/ml PMSG-hCG (Suigonan, VetPharm, New York, USA) 1 mM Pyruvate, 50 ng/ml EGF and 50 µg/ml gentamycin before being transferred to the same medium for *in vitro* maturation in groups of 35–50 COCs in 4-well dishes (NUNC, Roskilde, Denmark) for 22.5–24.0 h at 35.8 °C in 5% CO<sub>2</sub> in air and in saturated humidity.

#### *In vitro fertilization (IVF)*

After IVM, the groups of mature oocytes were transferred to new 4-well plates with TALP-IVF medium consisting of sterile water, 5 µg/ml phenol red, 0.5 mM MgCl<sub>2</sub>, 6H<sub>2</sub>O, 2 mM CaCl<sub>2</sub>, 2H<sub>2</sub>O, 0.4 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM lactic acid, 25 mM NaHCO<sub>3</sub>, 3.2 mM KCl, 114 mM NaCl, 6 mg/ml BSA, 0.25 mM pyruvate, 30 µg/ml heparin, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine, 0.25 mM Na-pyruvate, 0.6% BSA fraction V, and 50 µg/ml gentamycin. Frozen straws of semen from two different SDM bulls – Elvis and Haslund (Vikinggenetics, Randers, Denmark) were thawed, pooled, and washed twice in modified non-capacitation (mNCM) medium consisting of sterile water, 113.1 mM NaCl, 4.78 mM KCl, 1.19 mM KH<sub>2</sub>PO<sub>4</sub>, 1.19 mM MgSO<sub>4</sub>, 21.6 mM Na-lactate syrup, 5 µg/ml phenol red, 50 µg/ml gentamycin, and 0.5 mM NaHCO<sub>3</sub>. Sperm cells were added to the 4-well dish with oocytes in a final concentration of 2 × 10<sup>6</sup> sperm cells/ml. The oocytes were incubated for fertilization overnight at 38.8 °C in an atmosphere of 5% CO<sub>2</sub> in air and in saturated humidity.

#### *In vitro culture (IVC)*

After IVF, cumulus cells and excess spermatozoa were removed by vortex agitation in wash medium for 2 min. The oocytes were washed twice in wash medium, counted, and washed once in IVC medium (BlastAssist, ORIGIO, Måløv, Denmark). Finally the oocytes were transferred to 4-well dishes with IVC medium covered with paraffin oil (ORIGIO). Twenty-five to 35 oocytes per well were incubated at 38.8 °C in an atmosphere of 6% CO<sub>2</sub>, 6% O<sub>2</sub>, and 90% N<sub>2</sub> in saturated humidity. The blastocysts were evaluated at day 8 after fertilization and expanded but not hatched blastocysts were chosen for further sample preparation using micromanipulation.

#### **Isolation of the blastocoel fluid and sample collection**

The blastocoel fluid was isolated from the blastocyst cells by micromanipulation using Nikon Eclipse 2000 microscope (200X) with Narishige manipulators (Narishige, Tokyo, Japan). The pipettes used were Cook Micro-Injection Pipette (K-MPIP-3135) and Cook Holding Pipette (K-HP-2135). Three droplets of 5–10 µl IVC medium and a droplet of 3 µl PBS were prepared in an ICSI dish and covered

with mineral oil. Five to ten blastocysts were transferred to each droplet with IVC medium and the dish was placed in the micromanipulator. The injection pipette was moved to the droplet of PBS and a small volume of PBS was taken into the pipette using the suction. This way the pipette was coated with PBS before sampling. In the media droplet, one blastocyst at the time was fixed on the holding pipette so that the ICM was placed in the holding-pipette side. The ICSI pipette was then gently lead in to the blastocyst cavity and suction applied to extract the fluid until the blastocyst collapsed (see Figure 1). The ICSI pipette was then moved to the PBS droplet where the blastocoel fluid was ejected. A small amount of PBS was left in the pipette and henceforth the blastocoel isolation was repeated on remaining blastocysts. When all blastocysts were emptied the dish was moved to a stereo microscope where the PBS/blastocoel droplet was transferred with a PBS-coated pipette tip to a low bind, protein free 500 µl tube (Eppendorf, Hamburg, Germany). Emptied blastocysts were collected in the smallest possible amount of medium and transferred to another low bind, protein free 500 µl tube with 5 µl PBS. Blastocoel and corresponding cell material from 21 + 11 bovine blastocysts were collected this way from two separate blastocyst *in vitro* productions. Both samples were immediately frozen at –80 °C. See the sampling procedure in Figure 1 and in the supplementary video (Supplementary Figure 1). The frozen samples were transported on dry ice for proteomic analysis at Odense University Hospital.

#### **Proteomic analysis**

The proteins of the two replicates of the blastocoel fluid or the corresponding lysed cells collected in PBS were digested by the addition of 10 ng lysC (Wako Chemicals, Richmond, VA, USA) in 8 M urea followed by a reduction of the disulfide bonds using dithiothreitol (DTT) and blocking of free cysteine with iodoacetamide. The samples were diluted to a urea concentration of 0.75 M and further proteolytically cleaved by adding 25 ng sequencing grade trypsin (Promega, Madison, WI, USA). The resulting peptide mixtures were purified using reversed phase microcolumns and further fractionated using hydrophobic interaction liquid interaction chromatography (HILIC) using the fraction collection option of the Thermo/Dionex Ultimate 3000 nano/capillary HPLC system (Thermo Scientific, Sunnyvale, CA, USA). Briefly, the peptide samples were loaded onto a custom made HILIC column packed with TSKgel Amide-80 column material (3 µm bead size, 10 cm length, 300 µm ID, Tosoh Bioscience LLC, PA, USA). The resulting fractions were analyzed by nano-LC MSMS analysis using a Dionex Ultimate 3000 nano HPLC coupled to a Thermo Scientific Orbitrap Q-Exactive mass spectrometer (Thermo Scientific, Bremen, Germany); briefly, the samples (5 µl) were loaded onto a custom made fused capillary pre-column (2 cm length, 360 µm OD, 75 µm ID) with a flow of 5 µl per min for 7 min. Trapped peptides were separated on a custom made fused capillary column (20 cm length, 360 µm outer diameter, 75 µm inner diameter) packed with ReproSil Pur C18 3-µm resin (Dr. Maish, Ammerbuch-Entringen, Germany) with a flow of 300 nl per min using a linear gradient from 95% solution A (0.1% formic

acid) to 35% B (100% Acetonitrile in 0.1% formic acid) over 86 min, followed by 10 min at 90% B, and 14 min at 98% A at a flow rate at 300 nl per min. Mass spectra were acquired in positive ion mode applying automatic data-dependent switch between one Orbitrap survey MS scan in the mass range of 300 to 1,500 m/z followed by HCD fragmentation and Orbitrap detection of the ten most intense ions observed in the MS scan. Target value in the Orbitrap for MS scan was 1,000,000 ions at a resolution of 70,000 at m/z 200. Fragmentation in the HCD cell was performed at normalized collision energy of 30 eV. Ion selection threshold was set to 50,000–100,000 counts. Selected sequenced ions were dynamically excluded for 45 s.

### Data analysis

A combined MASCOT-SEQUEST search was performed where peak lists (mgf files) were processed using the Proteome Discoverer 1.4, version 1.4.0.288. The search parameters were set to: MS accuracy 10 ppm, MSMS accuracy 0.1 Da for HCD data, with two missed cleavages allowed, fixed modification of cysteine blocked with carbamidomethyl, and variable modifications; methionine oxidation and deamidated asparagines. Tandem mass spectra were searched against a matured and merged Swissprot-trEMBL bovine database downloaded on demand 16th of May 2012 from <http://www.ebi.ac.uk>. Proteins were inferred on a basis of at least one unique peptide identified with a high confidence (FDR < 1%). The proteins were grouped according to their associated functions in specific biological processes using GO terms provided by the bioinformatics tool ProteinCenter (<http://www.proxeon.com/home/index.html>). Human contaminants as keratins and trypsin were deleted from all data using the cRAP database as reference (<http://www.thegpm.org/crap/>). The background medium used for the blastocyst culture (BlastAssist<sup>®</sup>) was analysed as well and the identified proteins deleted from the blastocyst data. Only proteins identified in both analyses (biological replica) are presented in the results and discussion, however, the full lists of proteins can be found in Supplementary Table 1. The chosen bovine database search resulted in 790 uncharacterized proteins identified in the blastocyst material. The protein sequence of these proteins (identified by Uniprot search: [www.uniprot.org](http://www.uniprot.org)) were subjected to a BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) to provide a protein identification.

### Declaration of interest

Pernille Linnert Jensen and Lotte Stroebech were employed at ORIGIO A/S at the time of conducting the experimental work, however, no conflict of interest exists. This work was funded by the The Novo Nordic Foundation and The Lundbeck Foundation (STC), and the Danish Agency for Science, Technology and Innovation, Danish Ministry of Science, Innovation and Higher Education (PLJ).

### Author contributions

Conception and design, acquisition of data, data analysis and interpretation, manuscript writing, final approval of

manuscript: PLJ; Acquisition of data, manuscript writing, final approval of manuscript: MLG; Conception and design, acquisition of data, data analysis and interpretation, manuscript writing, final approval of manuscript: HCB; Acquisition of data, manuscript writing, final approval of manuscript: JP; Conception and design, acquisition of data, data analysis and interpretation, manuscript writing, final approval of manuscript: LS; Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript: STC; Conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript: CYA.

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Supplementary material available online

**Supplementary Table 1**

**Supplementary Figure 1 (Video and Video Still)**