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Proteomic analysis of the early bovine yolk sac fluid and cells from the day 13 ovoid and elongated preimplantation embryos

Pernille L. Jensen [a,b,*], Hans C. Beck [c], Tonny S. Petersen [a], Lotte Stroebech [b], Mette Schmidt [d], Lars M. Rasmussen [c], Poul Hyttel [e]

[a] Laboratory of Reproductive Biology, University Hospital of Copenhagen, Copenhagen OE, Denmark
[b] ORIGIO A/S, Maaloev, Denmark
[c] Centre for Clinical Proteomics, Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Odense, Denmark
[d] Department of Veterinary Reproduction and Obstetrics, University of Copenhagen, Frederiksberg C, Denmark
[e] Department of Clinical Veterinary and Animal Sciences, University of Copenhagen, Frederiksberg C, Denmark

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ABSTRACT

The bovine blastocyst hatches 8 to 9 days after fertilization, and this is followed by several days of preimplantation development during which the embryo transforms from a spherical over an ovoid to an elongated shape. As the spherical embryo enlarges, the cells of the inner cell mass differentiate into the hypoblast and epiblast, which remain surrounded by the trophectoderm. The formation of the hypoblast epithelium is also accompanied by a change in the fluid within the embryo, i.e., the blastocoel fluid gradually alters to become the primitive yolk sac (YS) fluid. Our previous research describes the protein composition of human and bovine blastocoel fluid, which is surrounded by the trophectoderm and undifferentiated cells of the inner cell mass. In this study, we further examine the changes in the protein composition in both the primitive YS fluid and the embryonic cells during early and slightly later stage cell differentiation in the developing bovine embryo. *In vitro*–produced Day 6 embryos were transferred into a recipient heifer and after 7 days of further *in vivo* culture, ovoid and elongated Day 13 embryos were recovered by flushing both uterine horns after slaughter. The primitive YS fluid and cellular components were isolated from 12 ovoid and three elongated embryos and using nano-high-performance liquid chromatography, tandem mass spectrometry, and isobaric tag for relative and absolute quantitation proteomic analysis, a total of 9652 unique proteins were identified. We performed GO term and keyword analyses of differentially expressed proteins in the fluid and the cells of the two embryonic stages, along with a discussion of the biological perspectives of our data with relation to morphogenesis and embryo-maternal communication. Our study thereby provides a considerable contribution to the current knowledge of bovine preimplantation development.

1. Introduction

Bovine *in vitro* production of preimplantation embryos offers a range of possibilities to study early mammalian development. The technology allows for research to be performed during the very early development stages, from

* Corresponding author. Tel.: +45 35455822; fax: +45 35455824.
E-mail address: pernille_linnert@hotmail.com (P.L. Jensen).

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the newly fertilized zygote to the first cell cleavages, as well as in the later stage of morula and blastocyst. When bovine blastocysts hatch 8 to 9 days after fertilization, development can no longer be sustained *in vitro* [1–3]. At the time of hatching, the first hypoblast cells (the primitive endoderm) have differentiated from the inner cell mass (ICM) of the blastocyst and by Day 10 have established a confluent hypoblast epithelium lining the inside of the embryo. The epiblast also forms following differentiation of the remaining portion of the ICM. The hypoblast encloses what may be referred to as the primitive yolk sac (YS). Around Day 12, the epiblast displaces the thin overlying trophectoderm lining (the Rauber layer) and establishes the embryonic disc. The embryonic disc then gives rise to the three germ layers of the embryo, whereas the hypoblast gives rise to the extraembryonic endoderm lining the YS of the developing embryo [4]. Along with the formation of the embryonic disc, the embryo is transformed from a spherical to an ovoid form, and from Day 12 the embryo elongates further to establish the extraembryonic membranes, which will extend throughout both uterine horns from about 3 weeks of development [5].

The ICM can be isolated from murine and human embryos and can be cultured as embryonic stem cell (ESC) lines [6,7]. However, *bona fide* ESC lines have not yet been established in cattle, potentially because the relevant signaling pathways required for retaining pluripotency *in vitro* in these species remain to be identified [8]. In the blastocyst, the ICM is partially surrounded by blastocoel fluid. We have recently described the protein composition of the human and bovine blastocoel fluid [9,10] for the purpose of identifying factors involved in the regulation of mammalian pluripotency, and several protein candidates from the blastocoel fluid were suggested for further testing in ESC cultures. However, in the present study, we wanted to further investigate the protein composition of the fluid in the later stage of posthatching embryo. The blastocoel fluid is encapsulated by the epithelial trophectoderm and the ICM, and these cellular compartments define the composition of this fluid. After formation of the hypoblast, the internal fluid-filled cavity, i.e., the primitive YS, is enclosed by two epithelia, including internal hypoblast and the external trophectoderm and epiblast. Hence, the composition of the fluid in the primitive YS may be more complexly regulated. To investigate the fluids of the bovine posthatching embryo, we first transferred *in vitro*–produced Day 6 embryos into a recipient heifer and recovered Day 13 ovoid and elongated embryos after slaughter by flushing of the uterine horns. From these embryos we have isolated the primitive YS fluid and using nano-high-performance liquid chromatography (HPLC), tandem mass spectrometry, and isobaric tag for relative and absolute quantitation (iTRAQ) proteomic analysis, we have compared the proteins present in both the YS fluid and the remaining cell material of the ovoid vs. the elongated embryonic stages.

2. Materials and methods

Reagents and media were purchased from Sigma-Aldrich (Brøndby, Denmark) unless otherwise stated. All media were prewarmed and pre-equilibrated in a CO_2 incubator before use. All plastic ware were purchased from NUNC (Thermo Scientific, Waltham, MA, USA).

An overview of the experimental design and methods is presented in Figure 1.

2.1. In vitro production of bovine embryos

2.1.1. Follicle aspiration and in vitro maturation (Day 1)

Sixty bovine ovaries were obtained from a local slaughterhouse (Herlufmagle Slagteri, Herlufmagle, Denmark) and transported to the laboratory within 3 hours of retrieval at 29 °C to 32 °C in a thermocontainer. The temperature of the ovaries and oocytes during handling was kept within this range. The ovaries were rinsed twice in 0.9% saline, and antral follicles with surface diameters of between 3 and 15 mm were aspired by use of a vacuum pump using an 18-ga short needle and collected into a

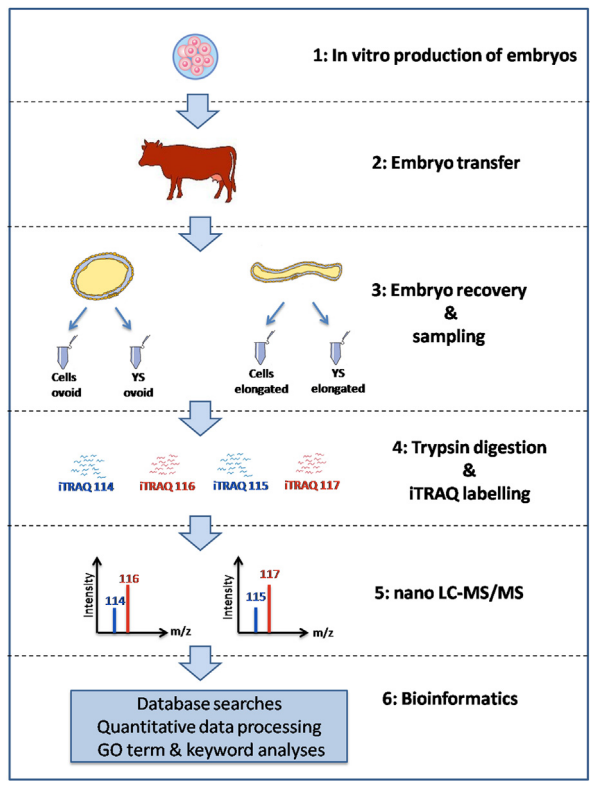

Fig. 1. Workflow. 1: Day six bovine morula embryos were generated by *in vitro* production. 2: The embryos were transferred into the uterine horns of a recipient heifer. 3: At day 13, ovoid (left) and elongated (right) embryos were recovered from the uterus of the slaughtered heifer and yolk sac (YS) fluid and cells were sampled separately from the two embryonic stages resulting in four samples. 4: Proteins were extracted from the sample material and enzymatically digested. The peptides were labeled with isobaric tags and combined (cell samples combined and YS fluids amples combined). 5: The proteomic analysis was performed using isobaric tag for relative and absolute quantitation (iTRAQ): The combined peptides were then fractionated by Hydrophilic Interaction Chromatography HILIC) and analyzed by nano High-Performance Liquid Chromatography (HPLC) tandem mass spectrometry (MS/MS). 6: The identified proteins were analyzed using the bioinformatics software ProteinCenter.