Proteomics of bovine endometrium, oocytes and early embryos

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Summary

Embryo-maternal communication, as well as recognition and establishment of pregnancy, is predominantly controlled by secretion of hormones and proteins. Furthermore, uterine fluid serves as a histotroph and contains factors essential for embryo development and elongation, making it a meaningful source for proteomic approaches of early embryonic development. The studies included in this review address the analysis of uterine fluid and endometrium during the oestrous cycle, early pregnancy, maternal effects on embryo development and embryo-induced alterations to the maternal environment. Clear differences between the proteome of uterine fluid and blood plasma unravelled the active contribution of the endometrium to the composition of uterine luminal fluid. Proteins were identified which may contribute either to growth enhancement of blastocysts or influence secretion of "Interferon tau". Several studies suggested important roles of intrauterine factors including "Tissue inhibitor of metalloproteinases 2" for pregnancy establishment and "Acyl-CoA-binding protein" for the embryo prior to implantation. A meta-analysis of quantitative proteome studies revealed functional clusters including the terms "degradation of extracellular matrix", "platelet degranulation" and "MHC class II antigen presentation". Proteomic studies targeting oocytes and early embryos provided evidence for the function of "Cyclin B" in mitotic resumption, for "14-3-3 epsilon" to activate "maturation promotion factor" for promotion of mitosis during in vitro maturation, or for the inhibition of proteasomes during the late phase of maturation. Analysis of blastocyst derived trophectoderm cell lines suggested effects of annexin down-regulation on the failure rate of nuclear transfer pregnancies. Finally, absolute quantification of "Y-box-binding protein 2" and "Insulin-like growth factor 2 mRNA-binding protein 3" during early embryogenesis by "selected reaction monitoring" demonstrated the dynamism of proteome alterations during the first embryonic cell divisions.

Introduction

The term "proteome" was introduced in 1994 by Marc Wilkins at a symposium in Sienna, Italy, as "the entire set of proteins expressed by a genome", and is now used to describe the quantitative protein pattern present under exactly defined conditions in an organism, a tissue, a cell, a cellular compartment or a body fluid. The most popular analytical method for early proteomic experiments for a long period has been two-dimensional gel electrophoresis (2D-PAGE), which was described for the first time independently in 1975 by Klose (Klose 1975) and O'Farrell (O'Farrell 1975). Since 1997, the "2D difference gel electrophoresis" (2D-DIGE) approach introduced by Unlu et al. (Unlu et al. 1997) has significantly improved reproducibility of quantification between individual

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gels and has extended the dynamic range of spot quantification by an order of magnitude. An extremely sensitive modification of the DIGE concept is based on coupling fluorophores to sulfhydryl residues in proteins to completion and referred to as 'saturation labelling' (Kondo et al. 2003). This technique facilitates analysis of complex protein mixtures using just a few micrograms of total protein (for a detailed user guide see Arnold & Frohlich 2012).

For the last ten years, a powerful alternative to 2D gel-based approaches has been developed and is referred to as nano liquid chromatography–tandem mass spectrometry or nano-LC-MS/MS (for review see Frohlich & Arnold 2006). In this approach, protein lysates of the original samples are digested with protease, separated by nano-chromatography (for review see Frohlich & Arnold 2009) and subsequently analysed by a tandem mass spectrometer. For relative quantification of individual proteins in LC-MS/MS approaches, several methods are available, based on either a direct quantification of MS or MS/MS signals or on a pre-labelling of proteins or peptides with chemical tags containing different compositions of stable isotopes prior to mass spectrometry (for a detailed review of proteomic techniques see Arnold & Frohlich 2011).

Up to now, proteomic approaches require an expensive set of instruments and sophisticated know-how with respect to protein biochemistry, chromatography, mass spectrometry and bioinformatics. Due to the variety of chemical and physicochemical properties of proteins (e.g., solubility) and the high dynamic range of 10⁸ in biological sources, proteins have so far not been identified and quantified on the basis of microarrays, as easily as is possible for RNAs. Probably as a consequence of the complex technology and experience required for proteomic analyses, many researchers have focused on the analysis of the transcriptome, which is much faster, cheaper and more comprehensive. However, it does not and cannot give unambiguous evidence as to whether, and to what extent, the corresponding protein is either present in the sample or is going to be synthesized. This is a consequence of the numerous regulation steps in eukaryotic organisms between the appearance of the transcript or polyA + RNA and translation at the ribosome. Making matters even more complicated, protein processing by proteolytic events, protein activation and signalling cascade activation by (de)phosphorylation is not accessible from either a genome or transcriptome. Therefore, analysis of the transcriptome is not a suitable surrogate for proteome analysis, and information about protein expression levels, abundance changes, modification stages, or activation status can in general only be gained at the protein level.

This review provides a short overview of proteomic approaches relevant for ruminant reproduction and addresses typical examples of holistic gel based and LC-MS/MS based experiments. The examples describe applications of proteomic techniques to bovine endometrium, uterine laminar fluid, oocytes and early embryos and illustrate the power of current methods to decipher complex biological processes at a molecular level. As a forward look, we include an unpublished example from our laboratory illustrating the unprecedented power of this developing technology "Selected reaction monitoring" (SRM) (for review see Frohlich & Arnold 2011), a universally applicable, highly sensitive and antibody free quantification technique for targeted proteomic approaches.

Role of the uterine environment during early pregnancy

In beef and milk production, early embryonic mortality is an important factor leading to enormous economic losses. In cattle, the majority of embryo losses happens before Day 16 after conception (Diskin & Morris 2008). Failure to maintain pregnancy during early stages of gestation can be caused by incorrect embryonic development, insufficient receptivity of the uterus, or be a consequence of an impaired biochemical communication between the conceptus and the maternal environment (Wolf et al. 2003). In cattle, the early embryo enters the uterus around Day

5 after fertilization (Hackett et al. 1993). From then on, in addition to embryo quality, the uterine environment plays a major role in the successful establishment and maintenance of pregnancy. Until embryo attachment around Day 19, the embryo develops to a blastocyst, hatching on Day 9 to 10 and continuing to grow and elongate to a conceptus with a tubular shape of 10-20 cm in length (Betteridge & Fléchon 1988). During this phase, the embryo/conceptus is surrounded by and dependent on the uterine fluid (Morris & Diskin 2008), which contains minerals, nutrients, vitamins and proteins. At this stage of pregnancy, progesterone released by the corpus luteum (CL) is a key hormone, modulating the endometrium and enabling growth as well as elongation of the conceptus. In ruminants, the conceptus secretes interferon tau (IFNT) (Mann et al. 1999, Spencer et al. 2008) in order to maintain CL progesterone secretion and to prevent luteolysis. IFNT interacts with endometrial receptors, leading to a decrease of estrogen receptors and, as a consequence, to decreased estrogen-induced oxytocin receptor expression. These IFNT induced effects subsequently hinder prostaglandin F2-alpha release, luteolysis and a resumption of the oestrous cycle (Thatcher et al. 2001). In cattle, IFNT production in the trophectoderm rises around Day 11 and reaches a maximum around Day 16, leading to maternal recognition of pregnancy (Mann et al. 1999). Although these crucial processes are well known, further biochemical mechanisms controlling receptivity and embryo implantation are still poorly understood. The analysis of the embryo-maternal interface with modern "-omics" technologies (Ulbrich et al. 2013) generate sophisticated datasets increasing the understanding of the biochemical processes taking place during early pregnancy. A variety of transcriptomic studies have been published, addressing alterations in gene expression of endometrium during the oestrous cycle (Bauersachs et al. 2008, Bauersachs et al. 2005), early pregnancy (Bauersachs et al. 2006, Bauersachs & Wolf 2012, Klein et al. 2006), as well as clarifying further effects of key molecules like progesterone (Forde et al. 2011, Forde et al. 2012). However, since the establishment of pregnancy is predominantly controlled by secretory events, proteome analysis of the uterine milieu to which the early embryo/ conceptus is exposed is particularly interesting. The broad majority of proteomic studies performed so far have targeted human uterine proteins in the context of diseases like endometrial/cervical cancer, endometriosis, and the diagnosis of infertility and will not be a focus of this review (for comprehensive reviews please refer to Meehan et al. 2010, Salamonsen et al. 2013). The use of proteomic techniques to study the uterine environment of ruminants during the oestrous cycle and early pregnancy has a long history. During the 1980s and 90s, endometrium, uterine luminal fluids (ULF), as well as uterine/endometrial secreted proteins of goat (Gnatek et al. 1989, Weise et al. 1993), sheep (Lee et al. 1998, Moffatt et al. 1993) and cow (Rueda et al. 1993) were analysed. However, the majority of these early studies were impaired by the fact that the sensitive and effective identification of proteins by mass spectrometry was not routinely available at that time. For the bovine system, the majority of more recent studies focused on ULF. Until implantation the embryo is surrounded by this fluid which serves as a supply of nutrients, minerals and vitamins. It is clear that this uterine histotroph represents a promising target for the detection and identification of proteins important for the survival and growth of the early embryo. Furthermore, ULF is the matrix facilitating the transport of signal molecules crucial for the molecular communication between the maternal environment and the conceptus (Ulbrich et al. 2012).

Modulation of the ULF proteome during oestrous cycle

It is known from various species that ULF contains blood proteins, as well as proteins secreted by the uterine epithelium (Fischer & Beier 1986, Kayser et al. 2006, Lee et al. 1998, Roberts & Parker 1974). Therefore, a quantitative proteome study was performed in order to detect proteins differing in abundance between both biological fluids (Faulkner et al. 2012). Using an 8-plex iTRAQ approach, the authors compared proteomes of uterine flushes from ipsilateral (n = 6) and contralateral (n = 6) uterine horns with blood plasma (n = 4) of cyclic Day 7 beef heifers. To increase analytical depth of the proteome analysis, the samples were albumin depleted, leading to a quantification of 112 proteins. In total, 35 of these proteins had higher and 18 had lower abundance in the uterine flush samples compared with plasma. In the dataset of higher abundant proteins in plasma, several with protease inhibitor functionality could be identified. Among the higher abundant proteins in ULF, various proteins involved in carbohydrate metabolism (e.g. aldose reductase (AKR1B1), a variety of proteins associated with response to stress, proteins with roles in the immune system and transport proteins like acyl-CoA-binding protein (DBI) were identified. Since the proteome of uterine fluid differs significantly from the plasma proteome, the authors concluded that the endometrium contributes actively to the ULF composition and may be regulated and influenced by other factors like local steroid stimuli.

The modulation of ULF composition during the oestrous cycle and the strong impact of ULF on the growth of the bovine embryo has been further demonstrated by an experiment performed by Ledgard and coworkers (Ledgard et al. 2012). In their study, they transferred Day 7 IVP blastocysts either to synchronous Day 7 or to asynchronous Day 5 and Day 9 uteri. The embryos were recovered on either Day 14 or Day 22. Comparing the developmental parameters of conceptuses after Day 14, they found that their average length was halved in case of transfer on Day 5, or increased fourfold in the case of transfer at Day 9, compared to conceptuses generated by artificial insemination (Al). On Day 22, the development of blastocysts transferred at Day 5 remained retarded, whereas differences of conceptuses derived from blastocysts transferred at Day 9 were less pronounced. To detect differences between Day 5, Day 7 and Day 9 in ULF at the proteome level, they performed 2D-PAGE experiments and identified 10 proteins where their abundance was altered between Day 5 and 9 of oestrous cycle, three of which were subsequently verified by Western blot experiments. All three proteins i) aldose reductase (AK1R1B1), ii) phosphoserine aminotransferase 1 (PSAT1) and iii) purine nucleoside phosphorylase (PNP) were enriched in uterine flushes obtained from Day 9 animals. Furthermore, legumain (LGMN) and tissue inhibitor of metalloproteinases 2 (TIMP2) were detected by Western blots exclusively in Day 14 ULF. This finding is in agreement with the distinct increase of TIMP2 protein in uterine fluid at Day 15 of oestrous cycle observed in a previous study (Ulbrich et al. 2011). In summary, the authors concluded that the growth enhancement of blastocysts transferred to uteri at Day 9 was correlated with the expression of several proteins. These proteins may contribute to the growth of the conceptus trophectoderm and positively influence the secretion of IFNT crucial for pregnancy establishment.

The dynamic character of the ULF proteome during the oestrous cycle was further investigated by a study performed by Mullen and coworkers (Mullen *et al.* 2012). They quantitatively analysed uterine flushes from Day 7 and Day 13 cyclic heifers (n = 6 per group), with the goal to identify proteins contributing to early embryo survival. In total, 300 proteins were identified from Day 7 samples and 510 proteins from Day 13 samples, of which 281 were present in both groups. To quantitatively compare the results, a spectral counting approach was applied and led to the detection of 5 proteins of higher abundance at Day 7 and 29 proteins which were more abundant on Day 13. Bioinformatics and literature studies revealed that among the proteins enriched at Day 13 several are related to metabolism, tissue remodelling, embryo growth/development, uterine health/immune function and free radical management. LGMN, TIMP2, chromogranin A (CHGA) and superoxide dismutase (SOD) were further validated by Western blotting. The authors concluded that the temporal proteome signatures detected in Day 7 and Day 13 uterine fluids may be used as markers associated with uterine health and receptivity as well as with embryo development. Importantly more abundant proteins on Day

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13 may have a role for embryo growth. Furthermore, the study is in-line with results of previous publications (Ledgard et al. 2012, Ulbrich et al. 2011), all of which support an important role of intrauterine TIMP2 for pregnancy establishment.

A further LC-MS based proteomic approach compared proteomes of Day 7 ULF with Day 15 ULF (Faulkner *et al.* 2013). In contrast to Mullen *et al.* (2012), they divided the samples into two further groups with respect to the plasma progesterone concentration of the donors at Day 5. For proteome analysis, the 8-plex iTRAQ strategy, including internal pooled standards, was used, leading to identification of 167 proteins in four or more biological replicates. No significant differences in abundance between the ULF proteomes between the high and low progesterone groups were detected. Nevertheless, the abundance of several proteins correlated with plasma progesterone concentrations. In addition, 20 proteins showed higher abundance at Day 15 compared to Day 7, and another 20 proteins showed lower abundance at Day 15. Among the proteins enriched at Day 15, LGMN, TIMP2, retinol binding protein 4 (RBP4) and isocitrate dehydrogenase 1 (IDH1) could be identified. The authors concluded that progesterone - as well as the cycle stage during early embryonic development - modulates the abundance of proteins related to similar functions. Furthermore, the study represents an excellent complement to other proteomic approaches described in this review investigating the role of ULF during the oestrous cycle.

Alterations of ULF proteome prior to pregnancy recognition at Day 16

Since the uterine environment prior to pregnancy recognition at Day 16 may not exclusively be modulated by maternal factors, some proteome studies studied the ULF proteome in the presence of early embryos. A 2D-gel based study analysed uterine fluid of beef heifers in either the presence or absence of embryos (Munoz et al. 2012). For sample generation, 29 to 56 morulae (Day 5) were transferred to the animals and flushed at Day 8. To eliminate the effects of genetic variability, the same animals were used for Day 5 sham transfers and flushed at Day 8. Furthermore, the animals were used for AI experiments and embryo transfers of single embryos to obtain further samples and to check if these procedures impaired the ability of the animals to become pregnant. The proteomes of uterine flushes (n = 8 per group) were analysed using a 2D-DIGE approach, leading to the identification of 38 proteins where abundance was altered. Although the changes in abundance of many of these proteins were fairly low, (the majority of the proteins showed differences in abundance lower than 1.5), bioinformatic analysis suggested an involvement of NF κ B pathway. In addition, the authors performed further validation and more targeted experiments, e.g. Western blots, embryo culture in either the presence or absence of uterine fluid dialysates, and immunocytochemical analysis of embryos. These data led to the hypotheses that the downregulated NFkB pathway in the uterus may contribute to the immune privilege of the early embryo, and that TNF α and IL-1B could be involved as embryonic signalling molecules.

A recently published proteomic study addressed ULF during early pregnancy (Beltman et *al.* 2014) and analysed the uterine histotroph of heifers at Day 7 after insemination. Uterine flushes were grouped into those derived from heifers with viable embryos (either morula or early blastocyst stage) and those from heifers with degenerate embryos arrested at the 2- to 16-cell stage (n = 3 per group). A 2D-LC-MS/MS approach, combined with spectral counting, was used for quantitative proteome analysis, and 40 proteins were identified from which 6 significantly differed in abundance. Five of these proteins, namely tubulins beta-4A (TUBB4A) and alpha-1D (TUBA1D), cytochrome c1 (CYC1), dihydropyrimidinase-related protein 2

(DPYSL2) and platelet-activating factor acetylhydrolase IB subunit gamma (PAFAH1B3) were more abundant in flushes from the group having viable embryos, whereas S100-A4 was less abundant. S100-A4 is involved in the regulation of a variety of cellular processes, amongst them cell differentiation and cell cycle progression. The authors suggested that the identified proteins may serve as markers for the successful development of early embryos. It was suggested that PAFAH1B3, belonging to the group of platelet-activating factors - known to play an important role for embryo development in other species, may represent a focus for further studies.

Embryo-induced proteomic alterations in the uterine milieu after maternal recognition of pregnancy but prior to implantation

Just before implantation, molecular signalling between the embryo and endometrium appears to be crucial for the maintenance of pregnancy. For instance, as well as the suppression of prostaglandin F2-alpha release and CL luteolysis, the maternal immune system must be influenced to become tolerant to the immunologically 'foreign' conceptus (Bauersachs & Wolf 2013). To investigate the embryo maternal communication just before embryo implantation, Ledgard and co-workers analysed Day 16 and Day 18 ULF of pregnant (Day 16, n=3; Day 18, n=2) and non-pregnant (Day 16, n = 2; Day 18, n = 3) dairy cows using 2D-PAGE (Ledgard et al. 2009). In a gel based comparison of ULF from non-pregnant donors with ULF originating from the uterine horn ipsilateral and contralateral to the embryo, nine higher abundant (>2-fold) proteins in pregnant versus non-pregnant donors were detected. Mass spectrometry led to the identification of peroxiredoxin 1 (PRDX1), carbonic anhydrase, thioredoxin (TXN), ezrin (EZR), heat shock protein 70 (HSP70), IDH dehvdrogenase (IDH), nucleoside diphosphate kinase (NDPK), purine nucleoside phosphorylase (PNP), and triosephosphate isomerase (TPI1). Furthermore, four proteins, i.e. LGMN, TIMP2, retinol-binding protein (RBP) and cystatin E/M (CST6), were more abundant in ULF from non-gravid donors and from horns contralateral to the conceptus. Two of these proteins, LGMN and TIMP2, were analysed in more detail by Western blots of caruncular tissue and ULF samples. Furthermore, endometrial LGMN and TIMP2 mRNA levels during the oestrous cycle were monitored and localized by *in situ* hybridisation, and the corresponding proteins were localized by immunocytochemistry. Interestingly, it was demonstrated that LGMN and TIMP-2 mRNA expression was quite similar between uterine horns during early pregnancy, but that the corresponding protein abundance was significantly decreased in horns containing a conceptus. The authors speculated that the amount of these proteins may be modulated by IFNT or other factors secreted by the conceptus. This is a clear example demonstrating that protein abundance cannot be extrapolated from mRNA levels. Furthermore, the study identified a number of specific proteins which may be involved in conceptus-maternal interactions prior to placentation.

One of the few studies investigating the proteome of endometrial tissue during the periimplantation stage was performed by our group (Berendt *et al.* 2005) with the focus on Day 18 of pregnancy. To reduce genetic variability as a cause of proteome differences, we used monozygotic cattle twins (generated by embryo splitting). One twin (n = 3 per group) received two *in vitro*-produced blastocysts at Day 7, whereas the other twin received a sham-transfer as a non-pregnant control. Using 2D-DIGE technology, intercaruncular endometrium samples from the pregnant and corresponding non-pregnant twin were analysed. Image analysis of the 2D-gels and subsequent mass spectrometry led to the identification of four proteins of higher abundance (ratio \geq 2, p-values < 10⁻⁵) in the endometrium of pregnant animals: i) Rho GDP dissociation inhibitor 2 (ARHGDIB) ii) isocitrate dehydrogenase 1 (IDH1) iii) acyl-CoA-binding protein (DBI) and iv) aldose reductase (AKR1B1), referred to as 20 Alpha-hydroxysteroid dehydrogenase. The abundance of AK1RB1 was further validated in all replicates by a mass spectrometry based approach using the cleavable isotope-coded affinity tag (ICAT). Noticeably, three of these proteins were also detected in other studies discussed and cited in this review. For example IDH1, an essential enzyme of the Krebs cycle, was found to be enriched in Day 15 ULF compared to Day 7 ULF (Faulkner et al. 2013) and was shown to be more abundant in ULF of animals at Day 18 of pregnancy (Ledgard et al. 2009). Furthermore, acyl-CoA-binding protein (DBI), which binds medium- and long-chain acyl-CoA esters with very high affinity, was found to be enriched by a factor >7 in Day 7 ULF compared to plasma (Faulkner et al. 2012), strongly suggesting an important role of this protein in the embryo prior to implantation. Another finding supporting the importance of DBI was reported in mice, where DBI-null embryos are not able to achieve the blastocyst stage (Landrock et al. 2010). A further protein identified in several proteomic studies investigating the bovine uterine environment is AKR1B1. AK1RB1 is known to be a key enzyme for the conversion of glucose to sorbitol, the first step in the polyol pathway. In addition to our finding, AKR1B1 was i) found to be enriched in Day 7 ULF compared to plasma (Faulkner et al. 2012), was ii) detected in higher abundance in ULF at Day 9 and Day 14 compared to Day 5 (Ledgard et al. 2012), was iii) identified in Day 7 ULFs in groups with viable and degenerate embryos (Beltman et al. 2014) and iv) identified in Day 7 and Day 13 ULF samples (see supporting information in Mullen et al. 2012). The importance of this enzyme is further supported by additional findings in other species (Lee et al. 1998, Ross et al. 2007).

All the endometrium related proteome studies presented above have provided new insights into the complex biochemical processes of pregnancy establishment. The uterine proteins, of which alterations in abundance were detected so far, represent valuable targets for functional studies. To create a functional overview of these proteins, we performed a combined Cytoscape/ ClueGO/CluePedia analysis (Bindea et al. 2013, Bindea et al. 2009, Smoot et al. 2011) for all the proteins in which an alteration in abundance in either the ULF or the endometrium during the oestrous cycle or early pregnancy was detected in at least one of the studies. The two most prominent clusters are shown in Fig. 1. Interestingly, the ClueGO analysis revealed an enrichment of proteins related to the terms "Degradation of the cellular matrix", "Platelet degranulation", "Activation of Chaperones by IRE1alpha", "Glycosphingolipid metabolism" and "MHC class II antigen presentation". Notably, the formation of these clusters is based on different studies addressing different time points of the oestrous cycle and early pregnancy. This demonstrates that studies investigating multiple time points during early pregnancy are necessary to comprehensively describe the uterine milieu at the proteome level. The knowledge and understanding about the uterine proteome is still far from being complete. However, future studies, making use of the newest developments in mass spectrometry, will allow a deeper understanding of the uterine proteome, and contribute substantially to the unravelling of the biochemical processes crucial for bovine pregnancy establishment.

Quantitative proteome analysis of oocyte maturation and early embryogenesis

Though mammalian oocytes and embryos have been extensively investigated, only a few have so far taken a proteomic approach. Moreover, most of those studies were performed in mice, and results can only be extrapolated to both non-rodent mammals and humans to a very limited extent because of the fundamental differences in their reproductive physiology and morphology. Furthermore, both cattle and humans are monovulatory and the duration of oestrous and menstrual cycle are significantly longer (21 and 28 days, respectively). The physiology, morphology and size of the ovaries and oocytes are also very similar between humans and cows (Gordon 2003,



6 Increased in ULF at Day 8 after embryo transfer (Munoz et al. 2012)
7 Decreased in ULF at Day 8 after embryo transfer (Munoz et al. 2012)
8 Increased in the endometrium of Day 18 pregnant animals (Berendt et al. 2005)
9 Decreased in ULF of horns containing Day 18 conceptus (Ledgard et al. 2009)

Fig. 1. ClueGO / CluePedia analysis of proteins shown to be altered in abundance in either ULF or endometrium during the oestrous cycle or early pregnancy. Enriched terms from the Reactome database are displayed as nodes. The node size corresponds to the statistical significance (adjusted p-value) for each term. Gene symbols of the corresponding proteins are shown as small circles and are connected to related terms from the Reactome database.

Guzman et *al.* 2012). Therefore, besides addressing problems in ruminant reproduction and development, bovine oocytes and embryos can in a number of aspects also serve as animal models for human reproduction and corresponding disorders and/or therapies (Hall et *al.* 2013), making research in this area especially valuable. The following discussion highlights some recent research covering this field. As a convenient overview, key results of each study, together with IDs of the altered proteins are shown in Table 1.

Study subject / approach	Major findings	Conclusions	Reference
Oocyte maturation/ radiolabelling + 2D-PAGE	Identification of cyclin B	Cyclin B may have an initiator role for meiotic resumption	Levesque & Sirard 1996
Oocyte maturation/ radiolabelling + 2D-PAGE	Characteristic patterns of protein synthesis detected	Protein synthesis affects different proteins during the transition of GV to MII stage; 3 patterns of protein synthesis detected	Coenen <i>et al.</i> 2004
Oocyte maturation/ 2D-PAGE	40 proteins identified; 4 were differentially expressed: tubulin-chain (TBB), cyclin E2 (CCNE2) and truncated CCNE2, protein disulfide isomerase (PDIA3), and peroxiredoxin 2 (PDX2)	CCNE2 and PDX2 as molecular markers for meiotic maturation suggested	Bhojwani et al. 2006
Oocyte maturation/ 2D-DIGE saturation labelling	38 significant differences in protein abundance between GV and MII oocytes; 10 identified: clusterin (CLU), translationally controlled tumour protein (TCTP), E2, 6-phosphogluconolactonase (6PGL), peroxiredoxin-3 (PRDX3), Eukaryotic translation (EF-1γ), and 14-3-3 protein epsilon (YWHAE) and 3 different isoforms of glutathione-s-transferase Mu 5 (GSTM5)	Varying abundance changes between different isoforms of GSTM5 revealed; Decrease of YWHAE fits MPF activation to promote mitosis; Protein candidates for in vitro maturation suggested	Berendt et al. 2009
Oocyte maturation/ iTRAQ labelling + LC-MS/MS	669 proteins identified and 653 quantified; relative abundance of 7 and 24 proteins increased and decreased in response to the proteasomal inhibitor MG132, respectively	Inhibition of proteasomes during the late phase of oocyte maturation can improve the developmental competence of oocytes	You et al. 2012b
Embryogenesis/ radiolabelling + 1D-PAGE	Protein synthesis decreased between the zygote and the 8-cell stage and then increased until the blastocyst staged	The maternal-to-embryonic transition occurs at the 8-16-cell stage	Frei <i>et al</i> . 1989
Oocyte maturation + embryogenesis/ radiolabelling + 2D-PAGE	46 proteins present during oocyte maturation as well as early embryogenesis; 10 identified: HSC71, HSP70, CypA, UCH-L1, GSTM5, Cct5, E-FABP, 2,3-BPGM, E2D3 and β-actin/γ-actin	Translation affects different proteins during oocyte maturation and embryogenesis; Housekeeping proteins suggested	Massicotte et al. 2006
Trophectoderm cell lines / 2D-PAGE + MALDI-TOF-MS or LC-MS/MS	118 common protein spots detected from trophectoderm cell lines established from differently produced blastocysts	Down-regulation of annexin genes in cultured NT-derived trophectoderm cell lines may play a role in the high failure rate of nuclear transfer pregnancies	Talbot et al. 2010

Table 1. Summary of proteomic studies concerning bovine oocyte maturation and early embryogenesis.

Quantitative proteome analysis of oocyte maturation

The earliest molecular approaches investigating qualitative and quantitative changes in the proteome of bovine oocytes addressed alterations of protein profiles during *in vitro* oocyte maturation. Radiolabelling approaches, combined with one-dimensional electrophoresis, were used to assess *de novo* protein synthesis as well as protein phosphorylation patterns. They demonstrated that maturation is associated with the synthesis of distinct and temporally expressed proteins (Kastrop *et al.* 1990, Khatir *et al.* 1998, Wu *et al.* 1996). Due to the lack of sensitive mass spectrometry based protein identification methods at that time, quantitative changes in protein synthesis rates could be assessed, but not assigned to individual protein IDs. One of the first proteins identified using a proteomic approach was cyclin B. This protein was suggested to have an initiator role in meiotic resumption and was identified by comparison of two-dimensional (2D) gel patterns of *de novo* synthesized proteins involved in meiotic resumption (Levesque & Sirard 1996).

In a more recent study, the process of oocyte maturation was investigated with respect to *de novo* protein synthesis during a 28 h period using a radiolabelling approach (³⁵S-methionine) and 2D gel separation (Coenen *et al.* 2004). Cumulus-oocyte complexes were incubated in conventional maturation medium and metabolically labelled at seven different time points over 24h. The proteins of the denuded oocytes were separated using 2D-PAGE, autoradiograms of 2D gels were scanned and optical density of protein spots used as a quantitative measure of *de novo* protein synthesis. A comparison between the seven 4 h time intervals revealed three major patterns of protein synthesis: one at the beginning of maturation (0-4 h), one in the middle (4-16 h), and a further one after the completion of MI stage (16-28 h), demonstrating that protein synthesis affects different proteins during the transition of GV to MII stage. Interestingly, the number of synthesized proteins was highest at the beginning of maturation until the MI stage, and then decreased after 12 h and then stayed fairly constant.

In a further 2D gel based approach, differentially expressed proteins involved in meiotic maturation of oocytes were identified and their phosphorylation pattern assessed (Bhojwani et al. 2006). Bovine oocyte extracts from 400 oocytes, each in either the GV (0 h), MI (10 h), or MII (24 h) stages were treated either with or without the cyclin dependent kinases (CDK) inhibitor butyrolactone I (BL I), subjected to 2D-PAGE and stained with Sypro Ruby. For staining of phosphoproteins, the fluorescent Pro-Q diamond phosphoprotein gel stain was applied, which makes it possible to distinguish between phosphorylated and non-phosphorylated proteins (Peluso & Pappalardo 2004, Steinberg et al. 2003). In total, 4800 bovine oocytes were analysed in three independent series of experiments from which 550 protein spots could be detected using Sypro Ruby-stained gels at any given maturation stage. On ProQ diamond-stained gels, 190 spots in GV stage, 270 spots in MII stage, and 250 in MII and after treatment with BL I were observed. There is increasing evidence that meiotic resumption is under the control of CDK1 kinase, which forms the maturation promoting factor MPF together with cyclin B, and mitogen-activated protein kinases MAPK (for review see Fan & Sun 2004, Kishimoto 2003). BLI is supposed to impair resumption of meiosis due to its CDK-inhibiting quality. In a preceding study (Tomek et al. 2002), the group had already demonstrated that BL I inhibits phosphorylation of essential components of the translation initiation complex and therefore modulates translation. Nevertheless, BL I treatment seemed to have no apparent effect on the overall expression of proteins during IVM in the follow-up study (Bhojwani et al. 2006) and the authors conclude, that alterations in the proteome are mainly caused by protein degradation and not by changes in the turnover of proteins. From ProQ diamond staining, a phosphorylation ratio of 30% at the GV stage, 50% at the MI stage and 45% at the MII stage was estimated. Seven proteins were claimed to be differentially phosphorylated between the stages and the phosphorylation of

peroxiredoxin-2 responded to BL I treatment, suggesting control via MPF or MAPK. To identify the proteins separated on the gels, Sypro Ruby stained spots were picked, digested with trypsin and analysed with MALDI-TOF-MS. Spots were only considered significant when differences in spot intensities were greater than a factor of two. Forty spots could be identified and four of them were differentially abundant, from which cyclin E2 and peroxiredoxin-2 were suggested to serve as molecular markers for meiotic maturation (Bhojwani *et al.* 2006).

A very sensitive non-radioactive protein labelling method, 2D-DIGE saturation labelling, was applied to study bovine oocyte maturation in vitro (Berendt et al. 2009). In this 2D gel-based approach, two overlapping pH gradients (pH 4–7 and pH 6–9) were used to detect and quantify more than 3000 protein spots by fluorescence scanning from a total protein amount of only $0.5 \,\mu g$ per gel, which equals about six oocytes. Six biological replicates were analysed which led to the detection of 38 significant differences in protein abundance (p < 0.01 in Student's t-test, abundance ratio >2) between GV and MII oocytes. Of these 38 spots, the intensities of 21 increased during maturation, while the intensities of 17 decreased. A preparative gel of saturation-labelled protein extracts from 2200 oocytes rendered it possible to identify differentially abundant proteins of matched spots by nano-LC-MS/MS analysis. Among the proteins decreasing in abundance during maturation were clusterin (CLU), translationally controlled tumour protein (TCTP), peroxiredoxin-3 (PRDX3), and 14-3-3 protein epsilon (YWHAE). The latter has been suggested to mediate Cdc25 phosphatase inhibition (Yang et al. 1999), which is a phosphatase required for MPF activation (Yurttas et al. 2010). A particular advantage of 2D gel-based proteomics is the possibility to detect changes between different isoforms of proteins. The 2D-DIGE saturation labelling approach by Berendt et al. revealed changes in abundance of different isoforms of glutathione-s-transferase Mu 5 (GSTM5). While two isoforms (pl values 5.3 and 5.5) increased by a factor of 10.3 and 2.7, respectively, a third isoform (pl value 6.3) was decreased by a factor of 4.1. 2D-DIGE-based protein guantification was verified by Western blotting and validated by confocal laser scanning microscopy.

A recently published study (You et al. 2012b) used a nano-LC-MS/MS approach in combination with iTRAQ labelling to analyse the effect of the proteasomal inhibitor MG132 during bovine oocyte maturation. The proteasome is a multisubunit proteolytic complex (for review see Tanaka 2013) and early in maturatio, completion of meiosis I requires an inactivation of MPF through proteasomal cleavage of ubiquitinated cyclin B1 (for review see Karabinova et al. 2011). Treatment of oocytes with MG132 late in maturation had been shown to improve developmental competence of parthenogenetically activated oocytes in the pig (You et al. 2012a). To test the effect of MG132 in the cattle, You et al. applied the inhibitor between 16 h to 22 h after initiation of maturation. Proteomic analysis from three biological replicates was performed by labelling 100 ug of tryptic peptides of oocyte proteins per sample with iTRAQ and separating the peptides by an off-line 2D-LC method (SCX followed by RP chromatography) coupled to a O-TOF MS/MS system. A total of 669 proteins were identified in MII oocytes, and 653 proteins could be quantified. Exposure to MG132 increased the abundance of six proteins (minimum abundance ratio > 1.2 or < 0.8; p < 0.05 using Fisher's combined probability test). These comprised of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme involved in glycolysis and related to developmental competence (Krisher & Bavister 1999), tubulin alpha-1C chain (TUBA1C), important for organellar movement (Rivera et al. 2004), proteins involved in protein folding, and protein disulfide-isomerase (P4HB), which is an anti-apoptotic protein (Hashida et al. 2011). The abundance of 23 proteins was decreased, among them several proteins that exert anti-apoptotic actions and cyclin-dependent kinase 5 (CDK5), which can lead to apoptosis if aberrantly activated (Lopes et al. 2010). Finally, the study demonstrated that treatment with MGF132 late in maturation improves oocyte competence in cattle.

Quantitative proteome analysis of early embryogenesis

Several recent transcriptomic studies have studied bovine embryogenesis (Gad *et al.* 2012, Graf *et al.* 2014, Kues *et al.* 2008), but there are only few studies that have used proteomic approaches. One of the earliest studies using molecular approaches investigated quantitative changes in the proteome of bovine embryos, using radiolabelled ³⁵S-methionine and one-dimensional electrophoresis. The amount of *de-novo* protein synthesis during early embryogenesis was found to decrease between the zygote and the 8-cell stage and then to increase until the blastocyst stage (Frei *et al.* 1989). These results helped to determine the time of the maternal-to-embryonic transition (MET) (for review see Memili & First 2000).

Another study in bovine embryos (Massicotte *et al.* 2006) aimed to identify the maternal housekeeping proteins (MHKP) translated during bovine oocyte maturation and early embryo development. The group radiolabelled bovine oocytes and cultured embryos in presence of α-amanitine 15 h post-insemination (hpi), which limited protein synthesis to translation of maternal mRNAs (Plante *et al.* 1994). Embryos were then radiolabelled for 4 h between 36-40 hpi at the 2-cell stage, 48-52 hpi at the 4-cell stage and 60-64 hpi at the 8-cell stage. Pools of 10 embryos were prepared for 2D-gel analysis. For protein identification, a preparative gel containing 3000 GV oocytes was prepared. It was demonstrated, that 92 proteins were synthesized *de novo* throughout oocyte maturation and a total of 123 proteins during all three stages of embryogenesis. 46 proteins were synthesized during maturation as well as embryogenesis and were therefore considered as possible MHKPs. Using mass spectrometry, 10 of these potential MHKPs were identified, including the heat shock 70 kDa protein (HSP70), ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) and bisphosphoglycerate mutase (2,3-BPGM). Furthermore, pattern analysis of *de novo* translated proteins demonstrated a transition period until the embryo reaches the MET.

Proteomic analysis of trophectoderm cell lines established from bovine blastocysts

The mammalian blastocyst develops three distinct cell lineages before implantation, i.e., the epiblast, the endoderm and the trophectoderm. The latter forms the outer epithelial layer of the blastocyst which becomes the trophoblast of the placenta (for review see Rossant 2008). The regulation of the formation and maintenance of the trophectoderm has been widely studied in mammalian embryos (for review see Yamanaka et al. 2006), and trophectoderm has been used for the generation of cell lines. A recent study (Talbot et al. 2010) analysed different trophectoderm cell lines established from 8-11 day cattle embryos and examined differences in their proteome by 2D-gel electrophoresis combined with either MALDI-TOF-MS or LC-MS/MS. Trophectoderm cell lines were established from either in vivo-produced blastocysts (n = 5) or from in vitro-produced blastocysts derived by either in vitro maturation, fertilization and culture (IVF, n = 16), somatic cell nuclear transfer (NT, n = 16) or parthenogenetic (P) activation (n = 12) of oocytes. The success rate of cell line establishment was similar regardless of the source of blastocysts, except for in vivo-derived blastocysts, which had 100% efficiency. Comparison of gels led to the identification of 118 common protein spots of which 95% were identified. It was demonstrated that both of the calcium-dependent lipid-binding proteins, annexin I and II, were either reduced in abundance ($p \le 0.05$), or even absent, in NT-derived trophectoderm cell lines compared to IVF- and P-derived cell lines. Several proteomic studies of cow and pig placentas revealed that annexins and annexin interacting proteins, like \$100, are modulated when compared to normal control placentas (Chae et al. 2006, Kim et al. 2005, Lee et al. 2007). It was suggested by Talbot et al. (2010) that down-regulation of annexin genes in cultured NT-derived trophectoderm cell lines may play a role in causing the high failure rate of nuclear transfer pregnancies.

Selected Reaction Monitoring - a new and highly sensitive method for proteomics

A recently established method called Selected Reaction Monitoring (SRM) allows pre-selected proteins to be measured in a targeted approach in significantly smaller samples consisting of one to ten oocytes or embryo per analysis (Frohlich & Arnold 2011, Lange *et al.* 2008, Picotti *et al.* 2010). Our group is currently developing a multiplexed SRM assay for simultaneous quantification of developmentally relevant proteins (Deutsch *et al.*, unpublished data). As a proof of concept, we established SRM assays for Y-box-binding protein 2 (YBX2) and IGF2 mRNA-binding protein 3 (IGF2BP3), which we had previously detected in embryos at the 2-cell and 4-cell stages (Demant *et al.*, unpublished data). YBX2 has been reported to have an important role in storage and translational regulation of maternal mRNAs (Vigneault *et al.* 2009). The IGF2BP family influences the cytoplasmic fate of mRNAs (Fig. 2) and modulates cellular function and morphology, metabolism, proliferation and differentiation during development. IGF2BPs are highly expressed in both embryos and metastatic tissue (for review see Bell *et al.* 2013).



Fig. 2. Functions of the IGF2BP family. IGF2BPs regulate the cytoplasmic fate of mRNAs together with other RNA-binding proteins (RBPs). RBPs associate with specific target mRNAs (insulin-like growth factor II, ß-actin and proto-oncogene c-Myc), which are supposed to enable directed transport of transcripts throughout the cell along the microtubule and/or actin cytoskeleton. Adapted from Bell *et al.* 2013.

For absolute quantification, pools of GV oocytes, 2-cell embryos, 4-cell embryos and morulae were digested and measured on a QTrap mass spectrometer. Addition of stable isotope labelled synthetic peptide standards (so-called AQUA[™] peptides) corresponding to the peptides of interest enabled absolute protein quantitation. A typical SRM chromatogram illustrating the quantification of YBX2 and IGF2BP3 in a protein extract from 10 GV oocytes is shown in Fig. 3.

A total of 20 fmol AQUA peptide each was added and the three most intense transitions of each measured peptide, either endogenous or AQUA, are illustrated. The ratio between the most intense transitions, called quantifier, of endogenous and AQUA peptides was used



Fig. 3. SRM chromatogram of a simultaneous quantification of GAEAANVTGPGGVPVK of Y-box-binding protein 2 (YBX2) and IPVSGPFLVK of IGF2 mRNA-binding protein 3 (IGF2BP3) in a pool of 10 GV oocytes with 20 fmol AQUA peptide each. The quantifier transitions (fragment y3 for YBX2 and fragment y7 for IGF2BP3, both charged [2+]) are indicated by arrows. The remaining coloured lines represent two additional transitions serving as qualifiers for each endogenous peptide / AQUA peptide. Y-axis: Intensity of SRM signals in counts per second; x-Axis: Chromatographic retention time in minutes.



Fig. 4. Absolute SRM based quantification of Y-box-binding protein 2 (YBX2) and IGF2 mRNA-binding protein 3 (IGF2BP3). YBX2 and IGF2BP3 were quantified simultaneously in pools of 10 oocytes or embryos each. Absolute protein amounts are represented as fmol per oocyte or embryo (mean \pm SD, n = 3). A Mann-Whitney test was performed with GraphPad Prism 6 software and demonstrates statistically significant differences (p-value < 0.05):

Panel A: YBX2 content of GV oocytes versus 2-cell embryos, 4-cell embryos and morula (a, b, c), 2-cell embryos and 4-cell embryos versus morula (d, e);

Panel B: IGF2BP3 content of GV oocytes, 2-cell and 4-cell embryos versus morula (a, b, c).

to calculate absolute protein amounts per oocyte or embryo. The results are presented in Fig. 4. GV oocytes contained about 5 fmol YBX2 per oocyte, and the YBX2 content significantly decreased during development from GV oocytes to 2-cell embryos, remained constant at about 2 fmol per embryo during the next cell division to a 4-cell embryo, and decreased further to about 1 fmol per embryo during morula formation. The high amount of YBX2 in GV oocytes and the restricted window of YBX2 expression are consistent with the function of this protein in storage and translational regulation of maternal mRNAs (Vigneault *et al.* 2009). The amount of IGF2BP3 protein remained constant at a concentration of about 1 fmol per oocyte or embryo up to the 4-cell stage and decreased significantly during transition from the 4-cell embryo to the morula stage to about 600 amol per embryo. This suggests IGF2BP3 has a particularly important role in RNA trafficking and translational control during very early embryogenesis until at least the 4-cell stage. These results demonstrate the considerable dynamics in the abundance of proteins with even low abundance at stages of embryogenesis which, from a morphological point of view, seem to only redistribute the molecular environment of the inner cell mass to increasing numbers of blastomeres.

Conclusions / Outlook

In conclusion, the approaches described demonstrate impressively the power of modern proteomic techniques and approaches to effectively discover and quantify proteins crucial for ruminant reproduction. Furthermore, bioinformatic methods can facilitate in the functional assignment to either signal transduction and/or biochemical pathways. Proteomics continues to contribute substantially to the discovery and improved understanding of key physiological processes relevant in early development.

In the future, proteomics is expected to have an even greater impact on the understanding in life sciences as its rapidly evolving analytical power is increased even further. Ongoing technical developments in mass spectrometry and protein separation techniques will lead to considerably increased sensitivity and performance, facilitating more comprehensive proteome datasets from even smaller sample quantities. Moreover, the recently developed antibody free SRM technology enables highly sensitive protein quantification in the attomole range, and thus opens up the field of protein science to more quantitative approaches across all animal species. With respect to mammalian reproduction, the ultimate ambition of comprehensive proteomic analysis in single oocytes and embryos will soon be affordable, leading to significantly increased and rapid understanding of the processes in ruminant early development.

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