REPRODUCTION
RESEARCH

Bovine germinal vesicle oocyte and cumulus cell proteomics

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Abstract

Germinal vesicle (GV) breakdown is fundamental for maturation of fully grown, developmentally competent, mammalian oocytes. Bidirectional communication between oocytes and surrounding cumulus cells (CC) is essential for maturation of a competent oocyte. However, neither the factors involved in this communication nor the mechanisms of their actions are well defined. Here, we define the proteomes of GV oocytes and their surrounding CC, including membrane proteins, using proteomics in a bovine model. We found that 4395 proteins were expressed in the CC and 1092 proteins were expressed in oocytes. Further, 858 proteins were common to both the CC and the oocytes. This first comprehensive proteome analysis of bovine oocytes and CC not only provides a foundation for signaling and cell physiology at the GV stage of oocyte development, but are also valuable for comparative studies of other stages of oocyte development at the molecular level. Furthermore, some of these proteins may represent molecular biomarkers for developmental potential of oocytes.

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Introduction

Mammalian oocytes are the female gametes, their molecular biology uniquely establishes the program of life after fertilization and they are crucial in reproductive biology. Through a series of developmentally regulated events oocytes develop from primordial, primary, secondary, and tertiary follicles in the ovary. The oocyte is ovulated at the metaphase II (MII) stage. In *in vitro* conditions, however, the germinal vesicle (GV) oocyte completes MI before arresting at the MII. At fertilization, the MII oocyte and male gamete spermatozoa fuse (Matzuk *et al.* 2002, Senbon *et al.* 2003, Gilchrist *et al.* 2004). In meiotic development, nuclear maturation is manifest by GV breakdown (GVBD), condensation of chromosomes, realization of first meiosis (MI), and another arrest of development at the metaphase of the second meiosis (MII). In addition, these events related to nuclear maturation, significant changes occur in the cytoplasm including structural changes of organelles, major translational activity in which while many new proteins are synthesized, synthesis of others is terminated (Moor *et al.* 1990, Coenen *et al.* 2004). Developmentally competent MII oocytes require four periods of protein synthesis; namely, synthesis required for GVBD, MI, MII, and maintenance of MII (Khatir *et al.* 1998).

Oocytes do not develop in isolation; they are intimately involved with cumulus cells (CC). CC bind to the zona pellucida of the oocyte and connect to the oocyte cytoplasmic membrane to form a cumulus–oocyte complex (COC) through transzonal cytoplasmic process. Gap junctions allow transfer of small molecules between the oocyte and the CC (Albertini *et al.* 2001). Although this bidirectional communication and paracrine signaling between cumulus cell and oocyte are critical for oocyte growth and regulation of meiotic maturation of the oocyte (Eppig *et al.* 1993, De La Fuenta & Eppig 2001, Gilchrist *et al.* 2003, Sugiura & Eppig 2005), their nature and effects on the transcriptomes and proteomes of both are poorly defined.

Functional genomics methods now enable the analysis of transcriptomes and proteomes. From these, we can derive the molecular networks that define oocyte maturation, fertilization, and embryonic development (Pan *et al.* 2005, Sagirkaya *et al.* 2006). Here, we identify proteomes from GV stage oocytes and their surrounding CC using differential detergent fractionation (DDF) two-dimensional

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liquid chromatography followed by electrospray ionization tandem mass spectrometry (DDF 2-LC MS^2; McCarthy *et al.* 2005). We obtained proteomes of GV oocytes and their surrounding CC, including membrane proteins, using proteomics in a bovine model. We identified 4395 and 1092 cumulus cell- and oocyte-specific proteins. Further, 858 proteins were common to both the CC and the oocytes. Our work has provided the first experimental confirmation of 5360 of these 'predicted/hypothetical' proteins and is the first proteogenomic mapping of the recently sequenced bovine genome. Next, we used gene ontology (GO) to functionally annotate our data and this provided the largest single entry of GO annotations for the cow. We then interrogated our GO annotations to model oocyte and cumulus cell function. Specifically, because they underlie oocyte–cumulus interactions, we focus here on membrane, nuclear, and signaling proteins; receptor and ligand pairs; and transcription factors.

Materials and Methods

GV oocytes and CC

Ovaries were obtained from a local abattoir. Immature oocytes were aspirated from follicles (2–8 mm diameter) using an 18-gauge needle attached to a vacuum system (Sagirkaya *et al.* 2006). COCs (Fig. 1) were selected, washed three times in TL-HEPES supplemented with polyvinylpyrrolidone (3 mg/ml polyvinylpyrroline-40; Sigma), Na-pyruvate (0.2 mM), and gentamycin (25 μg/ml). To obtain oocytes free of CC, cumulus cell and oocyte complexes were vortexed in TL-HEPES (3 min), oocytes were collected under a stereomicroscope, further vortexed with hyaluronidase to remove adhering CC completely (3 min), washed three times in saline and stored in a cell lyses buffer at 4 °C until use. The lysis buffer consisted of digitonin (0.15 mM), EDTA (100 mM), Phenylmethylsulphonyl fluoride (100 mM), sucrose (103 mg/ml), NaCl (5.8 mg/ml), and PIPES (3 mg/ml) at pH 6.8. Oocytes were examined under a sterio microscope to ensure the complete removal of the CC. The CC removed from the oocytes after the first vortex were centrifuged, washed twice with saline, and the pellets resuspended in the lyses buffer and stored (4 °C) until use. Our method provided pure populations of CC and oocytes.

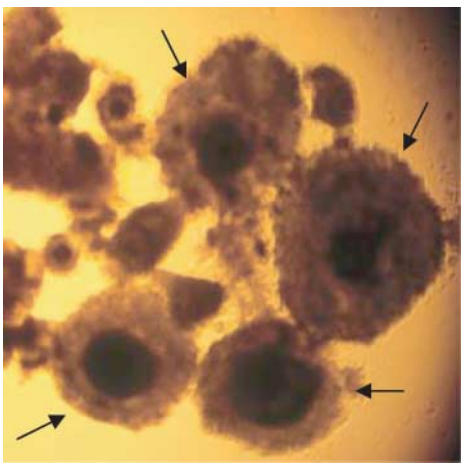

Figure 1 Morphological characteristics of bovine oocyte and their cumulus cells. Oocytes surrounded with several layers of cumulus cells (arrows) were used for this study. The is one of the most activity rich stages during oogenesis. Relatively compact cumulus cells undergo significant expansion during MI and MII stages of oocyte maturation.

Proteomics

Five hundred GV oocytes and their surrounding CC were each subjected to DDF exactly as described (McCarthy *et al.* 2005). The DDF fractions predominantly contain: DDF1, cytosolic; DDF2, membrane proteins; DDF3, cytoskeletal and nuclear proteins; and DDF4, remaining most insoluble proteins. The proteins in these DDF fractions were identified by two-dimensional liquid chromatography tandem mass spectrometry (2-$DLCMS^2$) exactly as described (McCarthy *et al.* 2006*a*,*b*). The resulting mass spectra were used to search subsets of the downloaded from the National Center for Biotechnology Institute (NCBI; 7/20/05) using TurboSEQUEST (Bioworks Browser 3.2; ThermoElectron, Waltham, MA, USA). We used a bovine subset of the nonredundant protein database (NRPD; 39 963 entries). Peptide matches were included only if they were ≥ 6 amino acids long and had $\Delta Cn > 0.1$ and Sequest cross-correlation (Xcorr) scores for charge states of 1.9, 2.2, and 3.75 for $+1, +2$, and $+3$ respectively (Washburn *et al.* 2001). All protein identifications and their associated MS data have been submitted to the PRoteomics IDEntifications database (PRIDE; Martens *et al.* 2005).

Modeling the proteomics data

We used GO and AgBase (McCarthy *et al.* 2006*a*,*b*) to identify the molecular functions, biological processes, and cellular components of the proteins in our dataset. Proteins without existing GO annotation, but between 70 and 90% sequence identities to presumptive orthologs with GO annotation, were GO-annotated using GOanna tool (McCarthy *et al.* 2006*a*). We next identified membrane, nuclear, and signaling proteins from our GO annotations and DDF profiles as described (McCarthy *et al.* 2006*a*). To identify receptor–ligand pairs, we used GO annotations and 'Bioinformatic Harvester' (Liebel *et al.* 2004) for proteins with human, mouse, or rat orthologs.

Since we did not find the ligands for all receptors in our data, we examined the amino acid sequences of these unidentified proteins to confirm whether they would be able to be identified by the DDF 2-$DLCMS^2$ method at all. To be reliably identified using our

proteomics method, a molecule must be a protein with tryptic peptides whose sequences are unique in the genome and these peptides must be within the detectable mass limits of the mass spectrometer. Also, post-translational modifications (such as glycosylation) can sterically hinder trypsin cleavage (Bark *et al.* 2001). We identified whether 'missing' proteins had peptide sequences that could be digested with trypsin (Gasteiger *et al.* 2005) whether the resulting peptides could be unique identifiers for the protein (using BLAST) and then whether or not these unique tryptic peptides would be detectable by mass spectrometry. Since 95% of our entire identified peptides were between 6 and 29 aa long (defined using our in-house 'peptide distribution analysis' program), we then removed all peptides that were <6 or >29 aa. The remaining 6–29mers were then analyzed for possible *N*- or *O*-linked glycosylation (Gupta & Brunak 2002, Julenius *et al.* 2005) that may cause steric hindrance during trypsin digestion.

To identify transcription factors we used GO annotations. We also manually inspected the entire dataset for terms that could identify transcription factors in the protein name: transcription factor, leucine zipper, DNA-binding protein, steroid hormone receptor, and corticoid receptor (http://www.gene-regulation.com/pub/databases/transfac/cl.html). Finally, we cataloged whether or not the transcription factors that we identified had previously been identified in oocytes or CC, by doing literature searches using PubMed.

Results

Proteomes

We identified 5253 and 1950 proteins in CC and GV stage oocytes respectively. Among these 858 (11.9%) were common to both cell types. Thus, this technique allowed us to identify 4395 and 1092 unique proteins in CC and oocytes respectively (Fig. 2). The lower number of proteins detected in the GV oocytes might be due to low concentration of proteins in the oocytes since fewer oocytes were used when compared with the CC. Among the 4395 proteins unique to CC, only 615 (14%) have been previously described; 3751 (85%) were annotated as 'predicted' (i.e. proteins are predicted based on sequence similarity to known proteins in other species and are frequently found in NRPD for species that have had their genomes sequenced (McCarthy *et al.* 2006*a*)); and 29 (0.65%) were annotated as 'hypothetical' (i.e. proteins predicted from nucleic acid sequences and that have not been shown to exist by experimental protein chemical evidence (Lubec *et al.* 2005)). Out of the 1092 proteins unique to oocytes, 141 (12.9%) were known, 947 (86.7%) were predicted, and only 4 (0.4%) were hypothetical. Among the 858 proteins common to both cell types, 191 (22.3%) were known, 662 (77.1%) were predicted, and only 5 (0.6%) were hypothetical (Fig. 2). This work, on only two cell types from a single organ, has contributed to the annotation of the newly sequenced bovine genome by experimentally confirming the *in vivo* expression of 5360 electronically predicted proteins (Supplementary Table 5, which can be viewed online at www.reproduction-online.org/supplemental/). The proteins in DDF fractions were identified by (2-DLCMS^2). The applied method of peptide detection does not exclude the presence of a protein absolutely. Thus, the protein might be present although there was no peptide discovered.

A schematic of the experimental design and results indicating specific findings exhibited in specific tables and figures is shown in Fig. 3.

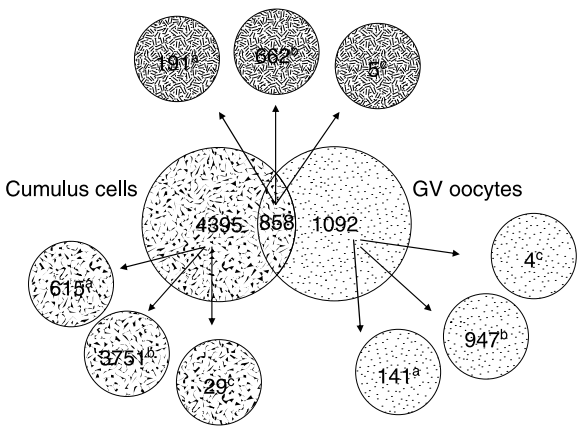

Figure 2 Distribution of predicted proteins, known and hypothetical proteins in oocytes, cumulus cells, and both cell types. [a]Known proteins; [b]predicted proteins; [c]hypothetical proteins.

Membrane, intercellular signaling, and nuclear proteins

From the GO, we identified 378 membrane proteins (39% of the total known proteins): 266 unique to CC, 52 unique to oocytes, and 60 in both cell types. Our results agree with estimates that approximately one-third of all currently described genes code for membrane proteins (Wallin & von Heijne 1998, Stevens & Arkin 2000). Using GO associations, we identified 186 nuclear proteins: 73 unique to CC, 11 unique to oocytes, and 112 in both cell types. We also identified 36 proteins GO-annotated as involved in signaling: 25 unique to CC, 7 unique to CC oocyte, and 4 in both cell types. Only 154 (16.2%) proteins previously annotated as membrane proteins were present in DDF2. This difference between GO annotation and DFF fraction may be due to the presence of membrane proteins in fractions other than DDF2 (because proteins with greater numbers of transmembrane domains tend to be present in the later DDF fractions); because some proteins may have membrane-bound isoforms that are not currently annotated as such (McCarthy *et al.* 2005) or due to errors in GO annotation.

Membrane and nuclear proteins are fundamental for inter- and intracellular signaling and are thus fundamental for modeling cell–cell interactions. We identified

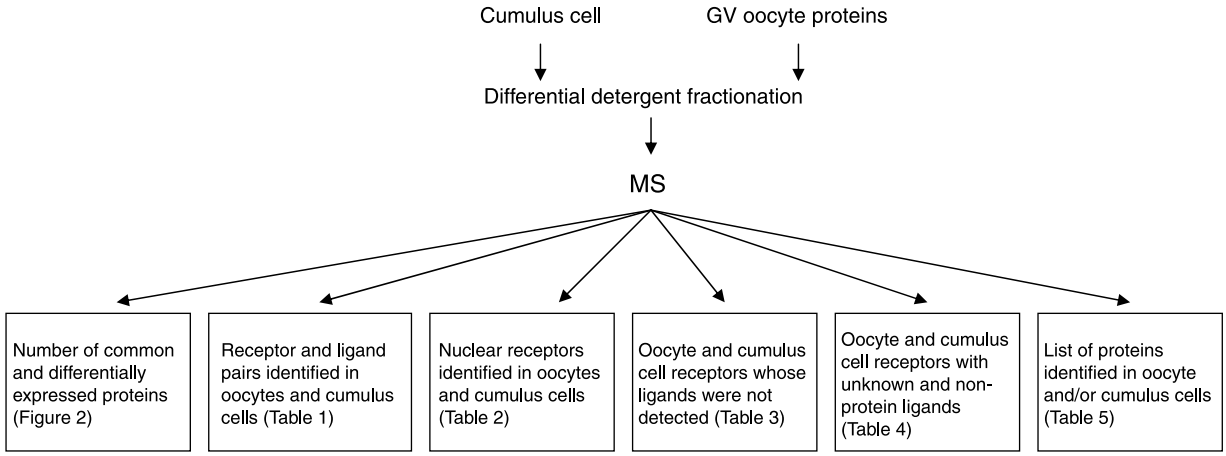

Figure 3 Schematic diagram of the experimental design and results indicating specific findings exhibited in specific tables or figures.

241 receptor–ligand pathways expressed in the CC and oocytes (Table 1). Among these were 18 growth factors (along with their binding proteins), which are likely involved in cell proliferation and cell differentiation. This is important in gametogenesis because oocyte-secreted growth factors play crucial roles in oocyte development and ovulation (Coskun *et al.* 1995). The cumulus cell dataset had numerically more growth factors (McCarthy *et al.* 2006*a*) when compared with oocytes (Matzuk *et al.* 2002) but, as a proportion of the total proteins identified from each cell type, the difference was much less striking: 0.29% (CC) versus 0.15% (oocytes). Endothelial growth factor-D, fibroblast (FGF), and epidermal growth factor (EGF) were present in both CC and oocytes, insulin-like growth factor (Igf) and transforming growth factor (TGF) were expressed only in CC (Table 1).

We also identified laminin receptors (cell adhesion molecules) in both oocytes and CC. These receptors interact with laminin, which is a major component of the basement membrane. Laminin receptors are thought to mediate the attachment, migration, and organization of cells into tissues by interacting with other extracellular matrix components (ECMs). Laminin-rich ECMs have contrasting regulatory effects on gap junction expression and thereby can alter specific cell–matrix interactions and gap junction-mediated cell-to-cell communication (Guo *et al.* 2001). This is directly relevant to the physiology of the COC, because the gap junctions between the CC and the oocyte allow transfer of molecules between CC and oocytes, as well as among the CC (Simon *et al.* 1997). We also observed 15 protein tyrosine phosphatase receptors (PTP); among these, 10 were in CC and 5 were in oocytes. PTPs are known signaling molecules regulating many cellular processes, including cell growth, differentiation, and mitotic cycle.

Nuclear hormone receptors were also present in oocytes and CC. Notably, estrogen receptor was expressed by oocytes and the estrogen receptor-binding protein was expressed by CC. Likewise, thyroid hormone receptor was expressed by the oocytes and its interacting proteins were expressed by CC. Differential expression of estrogen and thyroid hormone receptors may be a key signaling in oocyte development. Other nuclear receptors, such as peroxisome proliferators-activated receptors (PPARs), retinoic acid receptors (RXRs), and aryl hydrocarbon receptor nuclear translocators were also identified (Table 1). PPARs were identified only in CC, whereas RXRs and aryl hydrocarbon receptor were identified in both cell types. PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various target genes, such as retinoic acid (RA)-responsive genes (BTBD11, calmin, cyclin M2, ephrin B2, HOXD10, NEDD9, RAINB6, and tenascin R; James *et al.* 2003). RAs are absolutely essential for ovarian steroid production, oocyte maturation, and early embryogenesis (Mohan *et al.* 2003).

We have identified 338 transcription factors in oocytes and CC. More transcription factors were identified in the CC (249 factors) when compared with oocytes (89 factors). However, when the total numbers of proteins are taken into account, the proportion of transcription factors was higher in oocytes (8.1%) than that of cumulus cell (5.6%). Thus, our results agree with previous data that GV oocytes are transcriptionally highly active (Memili & First 1999, Dalbies-Tran & Mermillod 2003). Furthermore, most of the transcription factors we found in both CC and oocytes belonged to the zinc finger class of transcription factors. This is reassuring as this class of transcription factors is the most common in vertebrate genomes, accounting for an estimated 3% of all gene transcription (Klug 1999). PubMed searches showed that 9 out of 19 known transcription factors were previously identified in oocytes and CC: 3 retinoid receptors and PPARs (Mohan *et al.* 2003), 4 signal transducer and activator of transcription (STAT) proteins (Boelhauve *et al.* 2005), 1 C-fos (Davis & Chen 2003), and 1 transcription activator sox 9 (Lonergan *et al.* 2003). We have identified ten transcription factors that were not identified

Table 1 Receptors and ligand pairs identified in cumulus and oocyte. This shows membrane receptors and their ligands and associated signaling molecules in cumulus and oocyte.

Oocytes			Cumulus cells	
Intracellular	Membrane	**Extracellular**	Membrane	Intracellular
			Predicted: similar to epidermal growth factor receptor	Predicted: similar to growth factor receptor-bound protein 2
	Predicted: similar to epidermal growth factor receptor pathway substrate		Predicted: similar to epidermal growth factor receptor pathway substrate	
			Insulin-like growth factor 2 receptor	Predicted: similar to mannose 6 phosphate receptor-binding protein 1
		Predicted: similar to IGF-II mRNA-binding protein 1		
	Predicted: similar to muscle, skeletal, receptor tyrosine kinase, part[a]	FGF2 BOVIN heparin-binding growth factor 2 precursor (HBGF-2)	Predicted: similar to platelet-derived growth factor, A chain precursor	
	Vascular endothelial growth factor-D		Vascular endothelial growth factor-D	
Predicted: similar to C1q and tumor necrosis factor-related protein 2	Predicted: similar to tumor necrosis factor receptor superfamily member		Predicted: similar to tumor necrosis factor receptor superfamily member [a]	Predicted: similar to C1q and tumor necrosis factor-related protein 2
		Predicted: similar to insulin-like growth factor IB precursor (IGF-IB)	IGF-I receptor	
		Predicted: similar to insulin-like growth factor-binding protein-like		
		Predicted: similar to interferon-α	Interferon, α; receptor	
			Predicted: similar to interleukin 1 receptor-like 2, partial[a]	
	Predicted: similar to interleukin-1 receptor-associated kinase 1	ICBO1B interleukin-1 β precursor	Predicted: similar to interleukin-1 receptor-associated kinase 1	
	Predicted: similar to interleukin-1 receptor-like 1 precursor[a]	ICBO1B interleukin-1 β precursor	Predicted: similar to interleukin-1 receptor-associated kinase 1	
	Predicted: similar to interleukin-1 receptor-associated kinase 1			
			MPRD_BOVIN cation-dependent mannose-6-phosphate receptor precursor[a]	Predicted: similar to mannose 6 phosphate receptor-binding protein 1
	Predicted: similar to glutamate receptor, ionotropic, *N*-methyl-D-aspar[a]		Predicted: similar to glutamate receptor, ionotropic, *N*-methyl-D-aspar[a]	
	EAA1 _BOVIN excitatory amino acid transporter 1 (sodium-dependent glutamate/asp)		Excitatory amino acid transporter 1 (sodium-dependent glutamate/asp)	
			Predicted: similar to glutamate receptor δ-2 subunit precursor[a]	Predicted: similar to glutamate receptor-interacting protein 2
			Predicted: similar to mannose receptor, C type 2, partial[a]	Predicted: similar to mannose-6-phosphate isomerase
Predicted: similar to GDP-mannose pyrophosphorylase B isoform 2			Predicted: similar to GDP-mannose pyrophosphorylase B isoform 2	
		Latent TGF-β-binding protein-2	Predicted: similar to transforming growth factor β type II receptor[a]	
		Predicted: similar to bone morphogenetic protein 10 precursor (BMP-10)		

Table 1 *(Continued).*

Oocytes		Extracellular	Cumulus cells	
Intracellular	Membrane		Membrane	Intracellular
			Predicted: similar to transforming growth factor β 3 precursor	
			Predicted: similar to glutamate receptor 1 precursor (GluR-1)[a]	Predicted: similar to glutamate receptor-interacting protein 1
	Predicted: similar to activin A type IB receptor isoform b precursor	Predicted: similar to bone morphogenetic protein 10 precursor		
			Predicted: similar to glutamate receptor KA2 precursor[a]	Predicted: similar to glutamate receptor-interacting protein 1
	Predicted: similar to ALK tyrosine kinase receptor precursor		Predicted: similar to ALK tyrosine kinase receptor precursor[a]	Predicted: similar to protein tyrosine kinase
	Predicted: similar to laminin receptor 1 (ribosomal protein SA)[a]	Laminin Bl	Predicted: similar to laminin receptor 1 (ribosomal protein SA)[a]	
		Predicted: similar to laminin α-2 chain precursor		
		Predicted: similar to laminin α 5		
		Predicted: similar to laminin β 2-like chain		
		Predicted: similar to laminin α 3 subunit isoform 1		
		Predicted: similar to laminin α 3 subunit isoform 1		
			Predicted: similar to phospholipase A2 receptor[a]	
Predicted: similar to phospholipase A2, activating protein				Predicted: similar to phospholipaseA2-like
				Predicted: similar to cytosolic phospholipase A2
	Predicted: similar to glutamate receptor KA1 precursor, partial[a]		Predicted: similar to glutamate receptor KA1 precursor [a]	
	Excitatory amino acid transporter 1 (sodium-dependent glutamate/asp)		Excitatory amino acid transporter 1 (sodium-dependent glutamate/asp)	
			Predicted: similar to glutamate receptor, ionotropic, δ 1, D14[a]	Predicted: similar to glutamate receptor interacting protein 2
	Predicted: similar to glutamate receptor, ionotropic, kainate 1 isoform		Predicted: similar to glutamate receptor, ionotropic, kainate 1 isoform[a]	Predicted: similar to glutamate receptor-interacting protein 1 (GRIP1)
			Excitatory amino acid transporter 1 (sodium-dependent glutamate/asp)	
			Predicted: similar to glutamate receptor, metabotropic 8, partial[a]	Predicted: similar to glutamate receptor-interacting protein 2
			Predicted: similar to metabotropic glutamate receptor 5 precursor	Predicted: similar to glutamate receptor-interacting protein 2
			Predicted: similar to metabotropic glutamate receptor 4 precursor	
	Predicted: similar to kinase insert domain receptor (a type III receptor)[a]			

Table 1 *(Continued).*

Oocytes			Cumulus cells	
Intracellular	Membrane	**Extracellular**	Membrane	Intracellular
		Vascular endothelial growth factor-D		
		Predicted: similar to apolipoprotein B-100 precursor	Predicted: similar to apolipoprotein E receptor 2, partial[a]	
		Predicted: similar to apolipoprotein B		
		Predicted: similar to neuregulin 2 isoform 3	Predicted: similar to receptor tyrosine-protein kinase erbB-3 precursor[a]	
Predicted: similar to pro-neuregulin-3 precursor (Pro-NRG3)			Predicted: similar to pro-neuregulin-3 precursor (Pro-NRG3)	
	Predicted: similar to protein tyrosine phosphatase, receptor type, N p		Predicted: similar to protein tyrosine phosphatase, receptor type, f p	
Predicted: similar to protein tyrosine phosphatase 4a1	Predicted: similar to protein tyrosine phosphatase, receptor type, U i		Predicted: similar to protein tyrosine phosphatase, receptor type, U i	Predicted: similar to protein tyrosine phosphatase 4a1
Predicted: similar to protein tyrosine phosphatase, nonreceptor type			Predicted: similar to protein tyrosine phosphatase, receptor type, Q i	Predicted: similar to protein tyrosine phosphatase, nonreceptor type
Protein tyrosine phosphatase, nonreceptor type 13 (APO-1/CD95 (Fas)-a)			Predicted: similar to protein tyrosine phosphatase, receptor type, K p	
			Predicted: similar to glycoprotein receptor gp330/ megalin precursor[a]	Predicted: similar to myomegalin
			Predicted: similar to Eph receptor A2, partial[a]	
	Predicted: similar to EPH receptor A8 isoform 1 precursor		Predicted: similar to EPH receptor A8 isoform 1 precursor, partial[a]	
	Predicted: similar to Ephrin type-A receptor 1 precursor		Predicted: similar to Ephrin type-A receptor 1 precursor[a]	
			Predicted: similar to Epha5 protein, partial	
	Predicted: similar to interleukin-1 receptor-like 1 precursor[a]			
	Predicted: similar to interleukin-1 receptor-associated kinase 1		Predicted: similar to interleukin-1 receptor-associated kinase 1	
			Predicted: similar to transforming growth factor β 3 precursor	
			Bovine T-cell receptor γ chain	
	T-cell receptor β J8		T-cell receptor β chain variable segment	
	T-cell receptor δ chain			
	Predicted: similar to T-cell receptor α chain			
	MHC class I heavy chain		MHC class I heavy chain	
	MHC class H β-chain		MHC class n β-chain	
		Predicted: similar to putative CCL21 chemokine	Chemokine receptor 7[a]	

Table 1 *(Continued).*

Oocytes			Cumulus cells		
Intracellular	Membrane	Extracellular	Membrane	Intracellular	
			Predicted: similar to chemokine receptor		
	Predicted: similar to fibroblast growth factor receptor 1 isoform 1 precursor[a]		Predicted: similar to fibroblast growth factor receptor 1 isoform 1 precursor[a]		
		Heparin-binding growth factor 2 precursor (HBGF-2)			
		Predicted: similar to netrin 4	Predicted: similar to netrin receptor Unc5h4, partial[a]		
	Predicted: similar to netrin receptor UNC5C precursor	Predicted: similar to netrin 4	Predicted: similar to netrin receptor UNC5C precursor[a]		
	Predicted: similar to polycystic kidney disease and receptor for egg jelly[a]		Predicted: similar to polycystic kidney disease and receptor for egg jelly		
	Predicted: similar to polycystin 2, partial				
		Predicted: similar to transferrin	Predicted: similar to transferrin receptor[a]		
		TRFE_BOVIN serotransferrin precursor (transferrin)			

[a]Indicates receptor and ligand present in the same cell type.

previously in bovine oocytes and CC, and these include a forkhead transcription factor, nuclear transcription factor-Yα, Pax6, basic transcription factor 3a, zinc finger DHHC, DNA polymerase δ subunit zinc finger protein 313, zinc finger protein 470, and zinc finger protein ZFY. We have also identified 83 predicted proteins as transcription factors in oocytes and 236 predicted proteins as transcription factors in cumulus cells (Supplementary Table 5, which can be viewed online at www.reproduction-online.org/supplemental/).

'Missing' ligands

Ligands for 121 receptors were not identified, of which only 27 are proteins (Table 3). For the remaining 94, either the ligand is unknown (30 ligands) or known, but it is not a protein; axiomatically in either event the ligand cannot be identified by DDF 2-LCMS[2] (64 ligands; Table 4). Out of the 27 known protein ligands, 7 have no entries in the NCBI, which rendered them undetectable by the Sequest search. Eight of the remaining 20 have no unique peptides; 38 (of 60 peptides in total) are probably *O*-glycosylated and 2 are probably *N*-glycosylated. Therefore, only 20 unique peptides, representing 7 proteins, could theoretically be detected (Table 3).

Discussion

Although most basic reproductive biology work is done in the mouse (Eppig *et al.* 1993), significant species differences in oocyte biology exist (Sutton *et al.* 2003). Here, we used the bovine system because it is important for both agricultural and biomedical studies. Coenen *et al.* (2004) pioneered proteomics of bovine female gametogenesis. Using radio labeling and two-dimensional gel electrophoresis, they demonstrated three major patterns of translational activity during bovine oogenesis (one at the initiation of maturation, 0–4 h; one in the middle, 4–16 h; and one after completion of MI, 6–28 h) suggesting a developmentally regulated series stage-specific protein synthesis. However, the identities, functions, and expression patterns of these proteins are largely unknown. Here, we studied GV stage oocytes because these are highly active both transcriptionally and translationally (Memili & First 1999). Furthermore, interactions between the oocyte and its surrounding CC at this stage are crucial for development of a matured oocyte (MII) – the only cell type that can be fertilized to initiate a new organism. The GV stage is also one of the most active stages in the regulation of cumulus cell functions (Gilchrist *et al.* 2004). Although our methods used tenfold fewer cells to identify a ten time larger proteome, our work complements that of Coenen *et al.* (2004). Our comprehensive approach using DDF to model bovine oocytes also has significant impact on annotation of the bovine genome by demonstrating the

Table 2 Nuclear receptors identified in cumulus and oocyte. This shows nuclear receptors other than receptors related to membrane and their associated signaling molecules of cumulus and oocyte.

Oocyte			**Cumulus cells**	
Nucleus	Intracellular	**Extracellular**	Intracellular	Nucleus
Predicted: similar to estrogen-related receptor γ			Predicted: similar to estrogen receptor-binding protein	
			Sulfotransferase, estrogen-preferring	
Predicted: similar to thyroid hormone receptor β			Predicted: similar to thyroid hormone receptor interactor 12	
			Predicted: similar to thyroid hormone receptor interactor 3	
			Predicted: similar to thyroid hormone receptor interactor 6	
	Predicted: similar to thyroid hormone receptor interactor 11		Predicted: similar to thyroid hormone receptor interactor 11	
			Predicted: similar to thyroid hormone receptor-associated protein	
				Predicted: similar to glucocorticoid receptor (GR)
				Predicted: similar to glucocorticoid receptor DNA-binding factor 1
	Heat shock 90 kDa protein 1, α		Heat shock 90 kDa protein 1, α	Predicted: similar to aryl hydrocarbon receptor nuclear translocator-1
			Cellular retinoic acid-binding protein 1	
			Cellular retinoic acid-binding protein 2	Predicted: similar to nuclear receptor ROR-β
Predicted: similar to retinoic acid receptor, α			Cellular retinoic acid-binding protein 1	
			Cellular retinoic acid-binding protein 2	
				Peroxisome proliferators-activated receptor α[a]
				Predicted: similar to peroxisome proliferator-activated receptor-binding protein
Predicted: similar to aryl hydrocarbon receptor nuclear translocator 2[a]	90 kDa heat shock protein β		90 kDa heat shock protein β	
			Cellular retinoic acid-binding protein 1	
			Cellular retinoic acid-binding protein 2	Predicted: similar to retinoic acid receptor β (RAR-β)
			Cellular retinoic acid-binding protein 1	Predicted: similar to retinoic acid receptor γ
Predicted: similar to retinoic acid receptor, α			Cellular retinoic acid-binding protein 2	Predicted: similar to retinoic acid receptor RXR-β
Predicted: similar to nuclear receptor coactivator 7				
Predicted: similar to nuclear receptor-binding SET domain protein 1 isoform				

[a]Indicates receptor and ligand present in the same cell type.

existence of 5360 'predicted' and 38 'hypothetical' proteins for the first time (Supplementary Table 5, which can be viewed online at www.reproduction-online.org/supplemental/).

Not only are oocyte proteomes virtually undescribed, but there is also a general lack of knowledge of how interactions between the oocytes and surrounding CC lead to oocyte maturation. Interactions between oocytes and CC are considered essential for proper maturation or 'programming' of oocytes, which is crucial for normal fertilization and embryonic development (Buccione *et al*. 1990). CC are unique in that they are

Table 3 Cumulus and oocyte receptors whose ligands were not detected. This lists known cumulus and oocyte ligands that were not detected by mass spectrometry. Although the ligands were not detected the expression of its receptor indicates possible signaling mechanisms.

Cell type[a]	Protein name	Ligand
C	5-Hydroxytryptamine receptor 2A	5-Hydroxytryptamine
	Bradykinin receptor B2	Bradykinin
	Chemokine receptor 7	Mip-3-β chemokine|chemokine ligand 21
	Chemokine (C-X-C motif) ligand 13-like	CCR2b and CCR3 receptors
	Prolactin receptor long form	Prolactin
	Thyrotropin (TSH) receptor variant	Thyrotropin
	Predicted: similar to AXL receptor tyrosine kinase	Growth arrest-specific gene 6
	Predicted: similar to chemokine (C–C motif) receptor 9 isoform A, part	Chemokine scya25
	Predicted: similar to ephrin receptor EphB3 precursor	EphB1
	Predicted: similar to glucagon-like peptide 1 receptor precursor	Glucagon-like peptide 1
	Predicted: similar to glucagon-like peptide 2 receptor precursor	Glucagon-like peptide 2
	Predicted: similar to interleukin 20 receptor, α	Interleukin 20
	Predicted: similar to interleukin 4 receptor α	Interleukin 4 and IL13
	Predicted: similar to melatonin-related receptor	TOMM20 and TOMM40
	Predicted: similar to mineralocorticoid receptor, partial	Mineralocorticoids (mc) such as aldosterone
	Predicted: similar to opioid-binding protein/cell adhesion molecule–ligand	Opioid
	Predicted: similar to opioid growth factor receptor-like 1	Opioid growth factor
	Predicted: similar to prostaglandin D2 receptor	Prostaglandin D2
	Predicted: similar to ryanodine receptor 2	Ryanodine
	Predicted: similar to vasoactive intestinal polypeptide receptor 2	Vasoactive intestinal polypeptide
O	Predicted: similar to interleukin 3 receptor, α precursor	Interleukin-3
	Predicted: similar to NT-3 growth factor receptor precursor	Neutotropin 3
	Predicted: similar to proteinase-activated receptor 2 precursor	Trypsin and trypsin-like enzymes
	Predicted: similar to ryanodine receptor 2	Ryanodine
CO	ANPRB_BOVIN atrial natriuretic peptide receptor B precursor (ANP-B)	Atrial natriuretic peptide (anp)|brain natriuretic peptide (bnp)
	Predicted: similar to ephrin receptor EphB2 isoform 2 precursor	EphB2
	Predicted: similar to interleukin-17B receptor precursor	IL17B and IL17E

[a]C, cumulus cell; O, oocyte; CO, both cell types.

differentiated somatic cells essential for development of a competent oocyte. A comparative functional analysis of oocyte–cumulus cell biology between mouse and livestock oocytes is important to fully understand early mammalian development. For example, differences have been demonstrated in oocyte regulation of cumulus cell metabolism, and in cumulus cell expansion between mouse and bovine (Zuelke & Brackett 1992, Eppig *et al.* 1993, Sutton *et al.* 2003). Our work provides the first detailed definition of both CC and oocytes at the same time in development.

We used both physical and enzymatic separations to isolate pure cell populations (Memili & First 1999). We expected many proteins to be common to both CC and oocytes, particularly heat shock proteins, histones, ribosomal proteins, mitochondrial proteins, and proteins related to basic ubiquitous cellular and molecular functions (Supplementary Table 5, which can be viewed online at www.reproduction-online.org/supplemental/). We detected peroxiredoxin 4 in the oocytes (Table 5, supporting data). Also detected in pig oocytes, peroxiredoxin proteins have important roles in the maintenance of intracellular redox balance and protection of cells against oxidative stress due to reactive oxygen radicals (Ellederova *et al.* 2004). This suggests a conserved mammalian mechanism for cellular protection against oxidative stress. Our previous work and studies by others demonstrated that bovine oocytes have high transcriptional activity early on during GV leading to the MII stage in which mRNAs and proteins constitute a reservoir of molecular support for early embryogenesis following fertilization (Memili & First 1999, Dalbies-Tran & Mermillod 2003, Vallee *et al.* 2005). However, proteins are the primary functional units of the genome. Thus, we initiated the foundation for comprehensive proteome modeling of the dynamics of oocyte development through cell–cell interactions with the oocyte and the CC at the GV stage.

Mainly driven by the paracrine growth factors secreted by the oocyte, bidirectional interactions between the oocytes and the CC are essential for the development of competent MII oocytes, to support early embryogenesis, and for developmental potential of embryos for fetal development (Gilchrist *et al.* 2003). We detected expected proteins, including growth factors along with their binding proteins, such as Igfs and TGF in CC and oocytes respectively (Supplementary Table 5, which can be viewed online at www.reproduction-online.org/supplemental/). We detected other expected proteins in the oocyte included zona pellucida proteins, many zinc finger proteins consistent with a high level of transcriptional activity, and heat shock proteins (Supplementary Table 5, which can be viewed online at www.reproduction-online.org/supplemental/). The expected cumulus cell proteins included prohormone convertase, Igf2r, and binding proteins. Although oocytes have gamete and

Table 4 Cumulus and oocyte receptors with unknown and nonprotein ligands. Receptors whose ligands were not detected are listed.

Cell type[a]	Protein name	Ligand
C	MPRD_BOVIN cation-dependent mannose-6-phosphate receptor precursor	Mannose 6 phosphate
	Leptin receptor long form	Unknown
	Transient receptor potential cation channel TRPC4 middle region 1	Unknown
	Toll-like receptor 2	Lipopolysaccharide
	Predicted: similar to candidate taste receptor T1R2, partial	Unknown
	Predicted: similar to c-kit receptor	Unknown
	Predicted: similar to G protein-coupled receptor	Unknown
	Predicted: similar to G protein-coupled receptor 103	Unknown
	Predicted: similar to G protein-coupled receptor 149	Unknown
	Predicted: similar to G protein-coupled receptor 45	Unknown
	Predicted: similar to G protein-coupled receptor 82	Unknown
	Predicted: similar to G protein-coupled receptor 88	Unknown
	Predicted: similar to G protein-coupled receptor family C, group 5	Unknown
	Predicted: similar to γ-aminobutyric acid (GABA) B receptor 1	GABA
	Predicted: similar to γ-aminobutyric acid type B receptor, subunit	GABA
	Predicted: similar to γ-aminobutyric acid type B receptor, subunit	GABA
	Predicted: similar to γ-aminobutyric-acid receptor β-2 subunit	GABA
	Predicted: similar to G protein-coupled receptor SALPR	Unknown
	Predicted: similar to hypocretin receptor 2	Unknown
	Predicted: similar to killer cell immunoglobulin-like receptor KIR3DL1	Unknown
	Predicted: similar to leukemia inhibitory factor receptor precursor	Unknown
	Predicted: similar to muscarinic acetylcholine receptor M5	Acetyl choline
	Predicted: similar to neuronal acetylcholine receptor protein, α-6	Acetyl choline
	Predicted: similar to neuronal acetylcholine receptor protein, β-2	Acetyl choline
	Predicted: similar to neuronal acetylcholine receptor protein, β-3	Acetyl choline
	Predicted: similar to nuclear receptor subfamily 2, group E, member 1	Unknown
	Predicted: similar to nuclear receptor subfamily 4, group A, member 2	Unknown
	Predicted: similar to olfactory receptor	Oderants
	Predicted: similar to olfactory receptor 10A3 (HTPCRX12)	Oderants
	Predicted: similar to olfactory receptor 1257	Oderants
	Predicted: similar to olfactory receptor 12D2 (Hs6M1-20)	Oderants
	Predicted: similar to olfactory receptor 2C3	Oderants
	Predicted: similar to olfactory receptor 5H2	Oderants
	Predicted: similar to olfactory receptor 5U1 (Hs6M1-28)	Oderants
	Predicted: similar to olfactory receptor 6M1, partial	Oderants
	Predicted: similar to olfactory receptor MOR107-1, partial	Oderants
	Predicted: similar to olfactory receptor MOR14-2, partial	Oderants
	Predicted: similar to olfactory receptor MOR156-5, partial	Oderants
	Predicted: similar to olfactory receptor MOR157-1	Oderants
	Predicted: similar to olfactory receptor MOR235-2	Oderants
	Predicted: similar to olfactory receptor MOR241-1	Oderants
	Predicted: similar to olfactory receptor MOR256-13	Oderants
	Predicted: similar to olfactory receptor MOR258-6	Oderants
	Predicted: similar to olfactory receptor MOR264-5	Oderants
	Predicted: similar to olfactory receptor MOR267-8	Oderants
	Predicted: similar to olfactory receptor MOR34-1	Oderants
	Predicted: similar to olfactory receptor Olfr366	Oderants
	Predicted: similar to olfactory receptor Olr105	Oderants
	Predicted: similar to olfactory receptor Olr1466	Oderants
	Predicted: similar to olfactory receptor Olr1537	Oderants
	Predicted: similar to olfactory receptor Olr245	Oderants
	Predicted: similar to olfactory receptor Olr315	Oderants
	Predicted: similar to olfactory receptor Olr374	Oderants
	Predicted: similar to olfactory receptor Olr39	Oderants
	Predicted: similar to olfactory receptor Olr4	Oderants
	Predicted: similar to olfactory receptor Olr641	Oderants
	Predicted: similar to olfactory receptor Olr659	Oderants
	Predicted: similar to olfactory receptor Olr879	Oderants
	Predicted: similar to olfactory receptor, family 10, subfamily X	Oderants
	Predicted: similar to olfactory receptor, family 2, subfamily M	Oderants
	Predicted: similar to olfactory receptor, family 2, subfamily T	Oderants
	Predicted: similar to olfactory receptor, family 9, subfamily Q	Oderants
	Predicted: similar to orphan nuclear receptor NR4A1	Unknown
	Predicted: similar to short transient receptor potential channel 7	Unknown
	Predicted: similar to toll-like receptor 7 precursor (UNQ248/PRO285)	Lipopolysaccharide

Table 4 *(Continued).*

Cell type[a]	Protein name	Ligand
O	Toll-like receptor 9	Lipopolysaccharide
	Predicted: similar to feline leukemia virus subgroup C receptor-related	Unknown
	Predicted: similar to G protein-coupled receptor 128	Unknown
	Predicted: similar to G protein-coupled receptor 154	Unknown
	Predicted: similar to killer cell immunoglobulin-like receptor KIR3DL1	Unknown
	Predicted: similar to nuclear receptor subfamily 5, group A	Unknown
	Predicted: similar to olfactory receptor 1J4 (HTPCRX01)	Oderants
	Predicted: similar to olfactory receptor 5T2	Oderants
	Predicted: similar to olfactory receptor 5T2	Oderants
	Predicted: similar to olfactory receptor MOR173-1	Oderants
	Predicted: similar to olfactory receptor MOR195-1	Oderants
	Predicted: similar to olfactory receptor MOR234-3	Oderants
	Predicted: similar to olfactory receptor MOR256-15	Oderants
	Predicted: similar to olfactory receptor Olfr1357	Oderants
	Predicted: similar to olfactory receptor Olr245	Oderants
	Predicted: similar to olfactory receptor Olr701	Oderants
	Predicted: similar to olfactory receptor, family 51, subfamily E	Oderants
	Predicted: similar to scavenger receptor class A, member 3 isoform 2	Unknown
	Predicted: similar to sphingosine 1-phosphate receptor Edg-5	Lysosphingolipid sphingosine 1-phosphate
	Predicted: similar to transient receptor potential cation channel	Unknown
CO	Hyaluronic acid-mediated motility receptor	Hyaluronic acid
	Predicted: similar to G protein-coupled receptor 133, partial	Unknown
	Predicted: similar to G protein-coupled receptor 171	Unknown
	Predicted: similar to G protein-coupled receptor 64, partial	Unknown
	Predicted: similar to inositol 1,4,5-trisphosphate receptor type 1	Inositol 1,4,5-trisphosphate
	Predicted: similar to inositol 1,4,5-trisphosphate receptor type 2	Inositol 1,4,5-trisphosphate
	Predicted: similar to olfactory receptor 5B2 (OST073)	Oderants
	Predicted: similar to olfactory receptor 7A5	Oderants
	Predicted: similar to transient receptor potential cation channel	Unknown

[a]C, cumulus cells; O, oocytes; CO, both cell types.

totipotency-related proteins but CC are differentiated, we detected many more unique proteins in CC than oocytes (Supplementary Table 5, which can be viewed online at www.reproduction-online.org/supplemental/). Another reason for this discrepancy may be the relative lack of previous research on CC. A PubMed search shows that there are 36 times more papers describing research on oocytes than CC, which is probably because the oocyte is the unique progenitor for life. However, CC are essential to oocyte development, and for reproductive biology and are as important as oocytes (Sugiura & Eppig 2005). Our model is that oocytes orchestrate their environmental conditions by signaling cumulus cell development and physiology and that the soluble and membrane-bound signals from CC support oocyte development. This is because oocytes are dependent on CC in metabolic processes, such as glycolysis and amino acid uptake (Buccione *et al.* 1990). Here, we have been able to reconstruct signaling pathways from the intracellular space and cell membranes to the nucleus.

Paracrine growth factors secreted by oocytes are involved in a number of developmentally important events, including expansion of cumulus cell numbers and functions, regulation of follicular cell functions, and regulation of ovulatory and post-ovulatory events (Gilchrist *et al.* 2001). Among the expected growth factors, receptors, and ligands found in CC and oocytes (Table 1), there were remarkable numbers of nuclear receptors and binding proteins, for example, the RXRs in oocytes, and cellular RA-binding proteins in the CC (Table 1). Our evidence of retinoid signaling is consistent with the existing literature (30). RA, which is a metabolite of vitamin A, plays important roles in growth and differentiation by changing expression of certain genes (Mangelsdorf *et al.* 1994). RA improves development of bovine preimplantation embryos *in vitro* (Livingston *et al.* 2004) and supplementation of 9-*cis* RA in oocyte maturation medium influences trophectoderm differentiation and total cell number of the inner cell mass (Hidalgo *et al.* 2003).

Surrounding the oocyte and is made of three glycoproteins, zona pellucida has a role in fertilization and cleavage. We did not apply special treatment to the zona pellucida but we know that we could solubilize it because we identified proteins ZP2, ZP3, and ZP4 in DDF3 fraction (Supplementary Table 5, which can be viewed online at www.reproduction-online.org/supplemental/). However, the ZP has few known proteins (ZP1, 2, 3, and 4) and we may have identified previously unidentified ZP proteins but, because we did not specifically focus on the ZP, we cannot definitively identify these proteins' locations to the ZP. Notably we did not detect ZP1. This could be because ZP1 protein has no entry in the database we have used for sequest searchers which render them undetectable.

In conclusion, we have established a method that provides a basis for the proteomics of bovine oocyte

and surrounding cumulus cell biology, which will allow modeling the complex cell–cell interactions in oocyte development. This complements transcription analyses, and together the two methods may be used in the future for systems biology modeling of early mammalian development. We have also established the foundations necessary for further structural and functional annotation of the bovine genome aimed at identifying markers for developmental competency that are essential for selecting oocytes for mammalian reproduction.

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