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JOSÉ RENATO DE SOUSA PASSOS

PERFIL PROTEÔMICO DE COMPLEXOS CUMULUS-OÓCITO E EMBRIÕES OVINOS PRODUZIDOS *IN VITRO*

FORTALEZA
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Tese apresentada ao Programa de Pós-Graduação em Zootecnia da Universidade Federal do Ceará, como parte dos requisitos para obtenção do título de Doutor em Zootecnia. Área de concentração: Produção Animal.

Orientador: Dr. Arlindo de Alencar Araripe Noronha Moura
Coorientador: Dr. José Ricardo de Figueiredo

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Ainda que a figueira não floresça, nem haja fruto na vide; o produto da oliveira minta, e os campos não produzam mantimento; as ovelhas sejam arrebatadas do aprisco, e nos currais não haja gado, todavia, eu me alegro no SENHOR, exulto no Deus da minha salvação. (Habacuque 3:17-18).

RESUMO

O estudo 1 teve como objetivo descrever o proteoma de complexos cumulus-oócito (COCs) ovino utilizando a espectrometria de massa (EM) com abordagem de proteômica *shotgun*. O estudo 2 teve como objetivo caracterizar o proteoma de embriões ovinos no estágio inicial de desenvolvimento. No estudo 1, as amostras foram coletadas de ovários de ovelhas adultas e as proteínas de CCOs não maturados e CCOs maturados *in vitro* foram submetidos à eletroforese unidimensional (1D) em gel de poliacrilamida contendo dodecil sulfato de sódio (SDS-PAGE) e identificadas por EM. A digestão de proteínas em gel e a análise dos peptídeos por EM revelaram 2.416 e 2.426 proteínas não-redundantes em CCOs não maturados e maturados, respectivamente, em três repetições biológicas com identificação precisa. Os dados proteômicos foram analisados de acordo com a ontologia gênica e uma rede de interação proteína-proteína e, em seguida, foram identificados vários tipos de proteínas em COC não maturados envolvidas com regulação transcricional e metabolismo energético. As proteínas identificadas em CCOs maturados estavam relacionadas principalmente à proliferação celular, e enzimas envolvidas no remodelamento da matriz extracelular, e as proteínas diferencialmente expressas foram analisadas através do False Discovery Rate (FDR <0,05), havendo diferenças significativas abundantes em 648 proteínas entre o COCs não maturado e maturado, com 385 proteínas up-regulated e 263 down-regulated. Este estudo fornece a primeira caracterização de maneira precisa em CCOs ovinos, e foi possível identificar proteínas essenciais envolvidas na maturação oocitária e desenvolvimento embrionário inicial. Para o estudo 2, as proteínas de embriões foram submetidas à eletroforese unidimensional (1D) e identificadas pela EM. A digestão de proteínas no gel e análise dos peptídeos através de EM revelou 2.292 proteínas não redundantes em embriões, em três réplicas biológicas com a identificação precisa. Os dados proteômicos foram analisados de acordo com a ontologia gênica e, em seguida, foram identificados nos embriões diversos tipos de proteínas envolvidas com regulação transcricional e metabolismo energético. Ressalta-se a presença de proteínas que estão envolvidas na proteção dos oócitos, entre outras funções, proteínas relacionadas ao metabolismo e produção de energia e proteínas que estão relacionadas à adesão celular e à organização da matriz extracelular. Este estudo fornece a primeira caracterização em embriões ovinos, sendo possível identificar proteínas essenciais implicadas no desenvolvimento embrionário como biomarcadores de qualidade de embriões.

Palavras-chave: proteínas; espectrometria de massas; folículo; fecundação *in vitro*; ovelha.

ABSTRACT

The first study aimed to describe the proteome of ovine cumulus-oocyte complex (COC) by using a mass spectrometry (MS) with a *shotgun* proteomics approach. The second study aimed to characterize the proteome of ovine embryos at early development stage. In the first study, the samples were collected from ovaries of adult ewes, and immature CCO proteins as well *in vitro* mature CCO proteins were subjected to the one-dimensional (1D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and identified by MS. In-gel protein digestion and peptide analysis with MS has revealed 2.416 and 2.426\ nonredundant proteins in immature and matured COC, respectively, within three biological replicates with accurate identification. The proteomic data were analyzed according to the gene ontology and a protein-protein interaction network, then several types of proteins involved in immature COC with transcriptional regulation and energy metabolism were identified. Proteins identified in mature COC were mainly related to cell proliferation and enzymes involved in the remodeling of the extracellular matrix, and the proteins differentially expressed were analyzed by False Discovery Rate (FDR <0.05) which were significantly different with abundance in 648 proteins between the immature and mature COC, with 385 up-regulated proteins and 263 down-regulated proteins. This study provides the first accurate characterization in ovine COCs, being possible to identify essentials proteins involved in the oocyte maturation and early embryo development. For the second study, embryo proteins were subjected to one-dimensional (1D) electrophoresis and identified by MS. In-gel protein digestion and peptide analysis with MS revealed 2,292 nonredundant proteins in embryos, within three biological replicates with accurate identification. Proteomic data were analyzed according to the gene ontology, then several types of proteins involved with transcriptional regulation and energy metabolism were identified in embryos. The presence of proteins involved in oocyte protection, among other functions, proteins related to metabolism and energy production, and proteins that are related to cell adhesion and to the organization of the extracellular matrix are emphasized. This study provides the first characterization in ovine embryos, being possible to identify essential proteins involved in embryonic development as possible embryo quality biomarkers.

Keywords: proteins; mass spectrometry; follicle; *in vitro* fertilization; sheep.

LISTA DE ABREVIATURAS E SIGLAS

1D	Unidimensional
2D	Bidimensional
ACN	Acetonitrila
ACTB	Actin cytoplasmic 1
ACTG1	Gamma-actin 1
AGC	Controle Automático de Ganho
AGPAT1	1-acyl-sn-glycerol-3-phosphate acyltransferase alpha
AIPL1	Aryl hydrocarbon receptor-interacting protein-like 1
AKT	Protein kinase B
ALB	Serum albumin
AMPc	Adenosina monofosfato cíclico
APC/C	Anaphase-promoting complex
BCIMCO	Boletim do Centro de Inteligência e Mercado de Caprinos e Ovinos
BME	Basal Medium Eagle
BMP-15	Proteína morfogenética óssea 15
BPTF	Nucleosome-remodeling factor subunit
BSA	Albumina sérica bovina
CAD	Dissociação ativada por colisão
CC	Células do cumulus
CCNB1	Ciclina B1
CCO/COC	Complexo cumulus-oócito
CDC20	Ciclo de divisão celular 20
CDC25	Ciclo de divisão celular 25
CDK	Quinase dependente de ciclina
CDK1	Quinase dependente de ciclina 1
CG	Células da granulosa
CGMs	Células da granulosa mural
CGPs/PGC	Células germinativas primordiais
CIV	Cultivo *in vitro*
CNEP1R1	Nuclear envelope phosphatase-regulatory subunit 1
CO_2	Dióxido de carbono
CREBBP	CREB binding protein

CT	Células da teca
CTDNEP1	CTD nuclear envelope phosphatase 1
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCL3	Chemokine (C-X-C motif) ligand 3
CYC	Citocromo C
DAG	Diacilglicerol
DIAPH2	Diaphanous related formin 2
DNA	Ácido desoxirribonucleico
DTT	Ditiotreitol
E_2	Estradiol
ECM/MEC	Matriz extracelular
EP300	Histona acetiltransferase p300
FA	Ácido fórmico
FDR	False discovery rate
FIV	Fecundação *in vitro*
FOXO3	Fator de Transcrição Forkhead 3
FSH	Hormônio folículo estimulante
GDF-9	Fator de crescimento e diferenciação 9
GRO1	C-X-C motif chemokine ligand 1
GVBD	Quebra da vesícula germinativa
HA	Ácido hialurônico
HDAC11	Histona deacetilase 11
HDAC8	Histona deacetilase 8
HEPES	Ácido hidroxietil piperazinoetanossulfônico
HGF	Fator de crescimento do hepatócito
HPLC	Cromatografia líquida de alta eficiência
HSP70	Heat-shock protein 70 kDa
HSP90	Heat Shock Protein 90 kDa
HSP90AA1	Heat shock protein HSP 90-alpha
HSP90α	Heat shock protein 90 alpha
HSP90β	Heat shock protein 90 beta
HSPA9	Heat-shock protein A9
HSPCA	Heat shock protein 90 alpha family class A member 1

HSR	Heat shock response
IAA	Iodoacetamida
IP3	Inositol trifosfato
IRAUy	Instituto de Reprodução Animal Uruguai
JAK-STAT	Janus Kinase/Signal transducer and activator of transcription
LC-MS/MS	Cromatografia líquida acoplada à espectrometria de massas em tandem
LH	Hormônio luteinizante
Log2	Logartimo na base 2
LPIN1	Lipina 1
LPIN2	Lipina 2
LPIN3	Lipina 3
LPL	Lipoprotein lipase
MATER	Antígeno materno requerido pelo embrião
MEM	Meio essencial mínimo
MET	Transcrição materno-embrionária
MIV/IVM	Maturação *in vitro*
MMP8	Matrix Metalloproteinase-8
MMP9	Matrix Metalloproteinase-9
MMPs	Metalloproteinases
MPF	Fator promotor da maturação
MS	Espectrometria de massas
NCE	Energia de colisão normalizada
NPM-2	Nucleoplasmina 2
P_4	Progesterona
PA	Phosphatidic acid
PADI	Peptidyl arginine deiminase
PADI6	Peptidyl arginine deiminase 6
PADI6	Peptidyl arginine deiminase 6
PBS	Tampão fosfato-salino
PDIA3	Protein disulfide-isomerase
PGF2α	Prostaglandina F2 alfa
PGL	Plasminogênio
PIV	Produção *in vitro*

PKA	Proteína quinase A
PKM	Piruvato quinase
PKM1	Piruvato quinase 1
PKM2	Piruvato quinase 2
PMK	Pyruvate kinase
PPI	Interação proteína-proteína
PRKCA	Protein kinase C
PRKCB	Protein kinase C
PTMs	Modificações pós-traducionais
PTX3	Pentraxin-related protein
RE	Retículo endoplasmático
RNAm/mRNA	Ácido ribonucleico mensageiro
RNPm	Complexo de ribonucleoproteínas mensageiros
SCMC	Subcortical maternal complex
SDS	Dodecil sulfato de sódio
SDS-PAGE	Gel de poliacrilamida contendo dodecil sulfato de sódio
SERPINE1	SERPIN domain-containing protein
SFB/FBS	Soro Fetal Bocino
SIRT5	NAD-dependent protein deacylase sirtuin-5, mitochondrial
SIRT6	NAD-dependent protein deacylase sirtuin-6, mitochondrial
SOE/ESS	Soro de ovelha em estro
SOF	Fluido sintético do oviduto
STRAP	Ferramenta de Software para Anotação Rápida de Proteínas
TCM 199	Tissue Cultured Media 199
TCM 199	Meio de cultivo de tecidos 199
TFA	Ácido trifluoroacético
TGFB1	Fator de crescimento transformante beta 1
TGF-β	Fatores de crescimento transformante beta
THBS1	Trombospondina 1
TIMP2	Tissue inhibitor of metalloproteinases 2
TIMPs	Tissue inhibitor of metalloproteinases
TNCS	Transferência nuclear de células somáticas
TNFAIP6	TNF alpha induced protein 6

TP53	Cellular tumor antigen p53
TRAs	Técnicas de Reprodução Assistida
UI/IU	Unidades internacionais
UniProtKB	UniProt Knowledgebase
VEGFC	Fator de crescimento do endotélio vascular C
VG	Vesícula germinativa
VWF	von Willebrand factor
ZAR-1	Proteína de detenção zigótica 1
ZP2	Zona pellucida glycoprotein 2
ZP3	Zona pellucida glycoprotein 3
ZP4	Zona pellucida glycoprotein 4

LISTA DE SÍMBOLOS

%	Percentagem
~	Aproximadamente
<	Menor
°	Graus
°C	Grau Celsius
µM	Micromolar
21-gauge	Gauge 21 (calibre 21)
h	hora
kV	Kilovolt
M	Molar
m/z	Massa/carga
mg	Miligrama
mL	Mililitro
mm	Milímetro
mM	Milimolar
nL	Nanolitro
pH	Potencial hidrogeniônico
S	Sul
v/v	Volume/volume
w/w	Weight by weight
µg	Micrograma
µL	Microlitro
µm	Micrômetro

SUMÁRIO

1 INTRODUÇÃO

A ovinocultura é uma atividade com grande concentração regional, onde se destacam as regiões Nordeste e Sul, na produção de lã, leite, pele e carne. No ano de 2016, conforme o Boletim do Centro de Inteligência e Mercado de Caprinos e Ovinos – BCIMCO, o Brasil possuía um rebanho de aproximadamente 18,4 milhões de ovinos (DE SOUZA *et al.*, 2017). Embora o rebanho ovino do Nordeste seja destaque no país, a produção de carne ainda não atende suficientemente à demanda interna dos estados dessa região, inviabilizando, portanto, perspectivas de expansão para mercados nas demais regiões do país ou mesmo no exterior. A baixa produção se deve a diversos fatores, dentre eles podemos destacar a baixa taxa de fertilidade desses animais, devido principalmente ao período sazonal que estes animais apresentam (dependendo da espécie e região), sendo o período de anestro uma limitação importante para os índices de fertilidade (CHEMINEAU *et al.*, 2008; MIAO *et al.*, 2016).

Dessa forma, várias Técnicas de Reprodução Assistida (TRAs) em animais de produção têm sido aplicadas nos rebanhos com intuito de aumentar a fertilidade desses animais. Essas técnicas, como por exemplo a produção *in vitro* de embriões (PIV), são responsáveis por um grande potencial de inovação na pecuária, exercendo significativo impacto na produção de alimentos e geração de riquezas para o setor do agronegócio no Brasil. Assim, há um desafio para os pesquisadores da área de reprodução animal, que é aumentar de forma progressiva a eficiência da produção de embriões a partir de fêmeas de alta produtividade. Para isto, torna-se necessário compreender a fisiologia ovariana na espécie ovina através de estudos *in vitro*, pois apesar da enorme quantidade de informações produzidas durante as duas últimas décadas, o entendimento completo dos mecanismos controladores da maturação oocitária, bem como o potencial de desenvolvimento dos embriões produzidos *in vitro*, ainda não foram alcançados.

Além das técnicas já aplicadas em reprodução animal, a análise proteômica vem se destacando na literatura, como uma abordagem inovadora para a identificação de possíveis marcadores moleculares para a maturação oocitária e potencial de desenvolvimento embrionário. Já foram descritos estudos do proteoma em oócitos de humanos (VIRANT-KLUN *et al.*, 2016), oócitos e embriões bovinos (GREALY; DISKIN; SREENAN, 1996) bem como em células do cumulus na espécie equina (WALTER *et al.*, 2014). No entanto, até o presente momento não existem relatos sobre a expressão de proteínas de complexo cumulus-oócito – CCO (maturados e não maturados) e embriões oriundos de ovelhas produzidos *in*

vitro. Assim, a caracterização das proteínas nesta espécie será capaz de contribuir positivamente para um melhor entendimento dos mecanismos que envolvem a maturação de oócitos e desenvolvimento embrionário, podendo gerar informações que irão contribuir para melhoria dos índices de produção *in vitro* (PIV) de embriões nesta espécie.

Para um maior esclarecimento da importância deste trabalho, a revisão de literatura a seguir abordará aspectos relacionados à oogênese, foliculogênese e produção *in vitro* (PIV) de embriões. Além disso, serão discutidos o papel da análise proteômica, bem como sua importância na identificação de proteínas expressas em complexos cumulus oócito e embriões ovinos, através do estudo das interações proteicas e seus mecanismos regulatórios.

2 REVISÃO DE LITERATURA

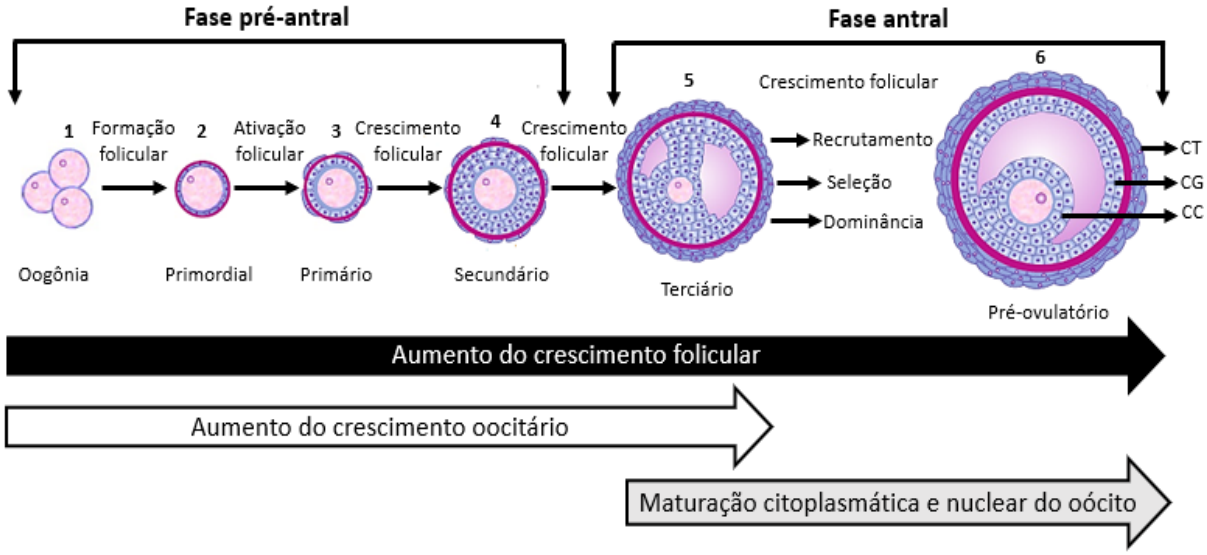
2.1 Oogênese e crescimento folicular na fase antral

No processo de formação dos oócitos (oogênese), segue-se algumas etapas, dentre as quais podemos destacar: formação das células germinativas primordiais (CGPs); migração das CGPs para as gônadas ainda indiferenciadas; colonização das gônadas pelas CGPs; proliferação das CGPs; processo de diferenciação das CGPs em oogônias; início da meiose; e primeira parada da meiose no estágio diplóteno da prófase I (VAN DEN HURK; ZHAO, 2005). Nessa fase, o oócito é circundado por uma camada de células da pré-granulosa, formando o folículo primordial, o qual se encontra quiescente no ovário constituindo a principal reserva folicular, sendo este o primeiro estágio da fase pré-antral. Posteriormente, o folículo primordial é ativado passando pelos estágios de primário e secundário, com a multiplicação das células da granulosa, aparecimento da zona pelúcia e células da teca. Ao final dessa fase, existe o aparecimento de uma cavidade repleta de líquido, denominada de antro, dando início a fase antral. A fase antral da foliculogênese está caracterizada pela presença dos folículos terciários ou antrais, os quais são morfologicamente constituídos por um oócito que é circundado por uma zona pelúcida, uma cavidade antral, diversas camadas de células da granulosa, duas camadas de células da teca, a teca interna e a teca externa, e uma membrana basal (SILVA *et al.*, 2002). O fator inicial exato para que o folículo se desenvolva do estágio pré-antral para antral ainda não está totalmente elucidado, porém, esse processo ocorre quando o oócito atinge um tamanho específico de acordo espécie, e as cavidades que se formam gradualmente se tornam confluentes para formar um grande antro. Em associação com a formação de antro, o oócito é capaz de retomar a meiose (EPPIG; O'BRIEN, 1996).

A cavidade antral é repleta de um líquido denominado fluido folicular, o qual é constituído por água, proteínas séricas, eletrólitos e altas concentrações de estradiol e inibina, ambos produzidos pelas células da granulosa (MARTINS *et al.*, 2008). Este fluido folicular atua como uma importante fonte de substâncias regulatórias, como esteroides, enzimas, glicoproteínas, lipoproteínas e fatores de crescimento (VAN DEN HURK; ZHAO, 2005). A partir deste estágio, o diâmetro do folículo aumentará notavelmente em virtude do crescimento oocitário, proliferação das células da granulosa, das células da teca e pela expansão da cavidade antral (DRIANCOURT, 2001). A produção do fluido folicular é acentuada pelo aumento gradual da vascularização dos folículos, bem como a permeabilidade dos vasos sanguíneos, os quais estão intimamente correlacionados com o crescimento

folicular antral (VAN DEN HURK; ZHAO, 2005). Em ovinos, o desenvolvimento folicular do estágio pré-antral até o estágio pré-ovulatório (conforme ilustrado na figura 1) dura aproximadamente 180 dias (~ 6 meses), sendo que ocorre o aparecimento do antro com 135 dias, levando mais 45 dias até a formação completa do folículo pré-ovulatório (CAHILL; MAULÉON, 1980).

Figura 1. Sequência esquemática do desenvolvimento folicular, crescimento oocitário, maturação citoplasmática e nuclear.



Fonte: Adaptado de Araújo *et al.*, (2014). (1) Oogônia. (2) Folículo primordial. (3) Folículo primário. (4) Folículo secundário. (5) Folículo terciário. (6) Folículo pré-ovulatório ou folículo *De Graaf*. CT: Células da teca; CG: Células da granulosa; CC: Células do cumulus. Os diferentes estágios da foliculogênese são caracterizados pelo crescimento folicular e pela proliferação e diferenciação das células da granulosa. Durante a foliculogênese, do estágio primordial ao estágio antral, os folículos aumentam de tamanho em oposição aos oócitos cujo crescimento ocorre desde o estágio primordial até o período pré-antral, quando o oócito está totalmente crescido. Desde estágio pré-antral ao estágio antral, o oócito sofre maturação, essencial para apoiar o desenvolvimento embrionário. Do estágio primordial até o final do crescimento do folículo secundário ocorre independentemente da ação das gonadotrofinas, e após esse estágio o crescimento do folículo é altamente dependente das gonadotrofinas.

No processo de desenvolvimento folicular na fase antral ocorrem três eventos principais, o recrutamento, a seleção e a dominância (VAN DEN HURK; ZHAO, 2005). Além das gonadotrofinas, algumas substâncias são produzidas localmente, dentre os quais destacamos os peptídeos, que exercem um papel essencial no processo regulatório da fase antral, tanto por meio de mecanismos endócrinos como parácrinos (FORTUNE, 2003).

Durante o recrutamento folicular, um grupo de folículos torna-se dependente das gonadotrofinas e inicia seu desenvolvimento (BARROS; FIGUEIREDO; PINHEIRO, 1995). Dos folículos que foram recrutados a partir de um determinado grupo, somente um será

selecionado para continuar se desenvolvendo (folículo dominante), mesmo em níveis baixos de FSH. Enquanto isso, os outros folículos (subordinados) começam a sofrer atresia, caracterizando esse processo como divergência folicular (GINTHER *et al.*,1996). A baixa concentração de FSH interrompe o desenvolvimento dos folículos subordinados, mas, ainda é suficiente para promover o crescimento do folículo dominante (ALVES; COSTA; GUIMARÃES, 2002).

Já foi demonstrado que a redução progressiva nos níveis de FSH e a elevação da frequência de liberação do LH são os dois principais eventos que definem a dominância folicular, sendo que o folículo dominante é o principal inibidor da secreção de FSH, e ao mesmo tempo ele tem a capacidade de manter sua sobrevivência em baixos níveis deste hormônio, pois ele apresenta uma maior quantidade de receptores para o LH. Na verdade, a divergência folicular acontece bem próxima do momento em que o FSH chega a atingir níveis basais, sendo isto crucial para a seleção de um único folículo ovulatório nas espécies monovulatórias (MIHM; EVANS, 2008).

No folículo ovulatório, durante o pico de LH, o oócito retoma a meiose, passando por alguns eventos moleculares em série, os quais estão ligados à retomada da meiose I, com consequente quebra da vesícula germinativa (GVBD), continuação da divisão meiótica e expulsão do primeiro corpúsculo polar, ocorrendo logo em seguida a segunda parada da meiose no estágio de metáfase II (MII), a qual retoma durante a fertilização (TRIPATHI; KUMAR; CHAUBE, 2010).

2.2 Complexo cumulus-oócito (CCO)

Durante a foliculogênese, as células da granulosa diferenciam-se em dois tipos de células: células cumulus (CCs) e células da granulosa mural (CGMs) (GRØNDAHL *et al.*, 2012). Ressalta-se que o folículo é envolvido por uma membrana basal (um tipo especializado de matriz extracelular) que separa as células da granulosa das células da teca (GORDON, 1994). Ainda, como resultado da formação do antro, as células da granulosa (CG) que circundam o oócito se diferencia em um tipo celular especializado denominado de células do cumulus (CC), sendo que no folículo pré-ovulatório, as CGs estarão situadas na parte periférica do folículo, enquanto que as CCs estarão circundando o oócito (WIGGLESWORTH *et al.*, 2015). Após o processo de diferenciação das células do cumulus, é estabelecida a comunicação bidirecional com o oócito (ROBKER; HENNEBOLD; RUSSELL, 2018).

As células do cumulus juntamente com o oócito formam uma estrutura chamada complexo cumulus-oócito (CCO), com estruturas especializadas que exibem projeções citoplasmáticas denominada de junções intercelulares (*gap junctions*) capazes de atravessar a zona pelúcida e chegar ao oolema (GILCHRIST; RITTER; ARMSTRONG, 2004). Evidencia-se que, as interações entre as células da granulosa e oócito ocorra desde os estágios iniciais da foliculogênese, as quais são essenciais para controlar o crescimento e diferenciação folicular, sendo mediados pelas junções *gap* ou sinalização parácrina. (DIAZ; WIGGLESWORTH; EPPIG, 2007; ZHANG *et al.*, 2010; ALAM; MIYANO, 2019).

Conforme descrito anteriormente, as células do cumulus são formadas na fase antral da foliculogênese pelo processo de diferenciação das células da granulosa. Após a diferenciação, é necessário que haja uma boa comunicação entre as células do cumulus e oócito, sendo este um fator determinante para o desenvolvimento de um oócito competente, pois, uma relação positiva das células cumulus na qualidade oocitária e, consequente, desenvolvimento embrionário inicial já fora relatado em diversas espécies de mamíferos, incluindo humanos, murinos, bovinos, caprinos e ovinos (CHA; CHIAN, 1998; KIDDER; VANDERHYDEN, 2010; DA BROI *et al.*, 2018; THOMAS *et al.*, 2004; MENCHACA *et al.*, 2012a; MENCHACA *et al.*, 2016).

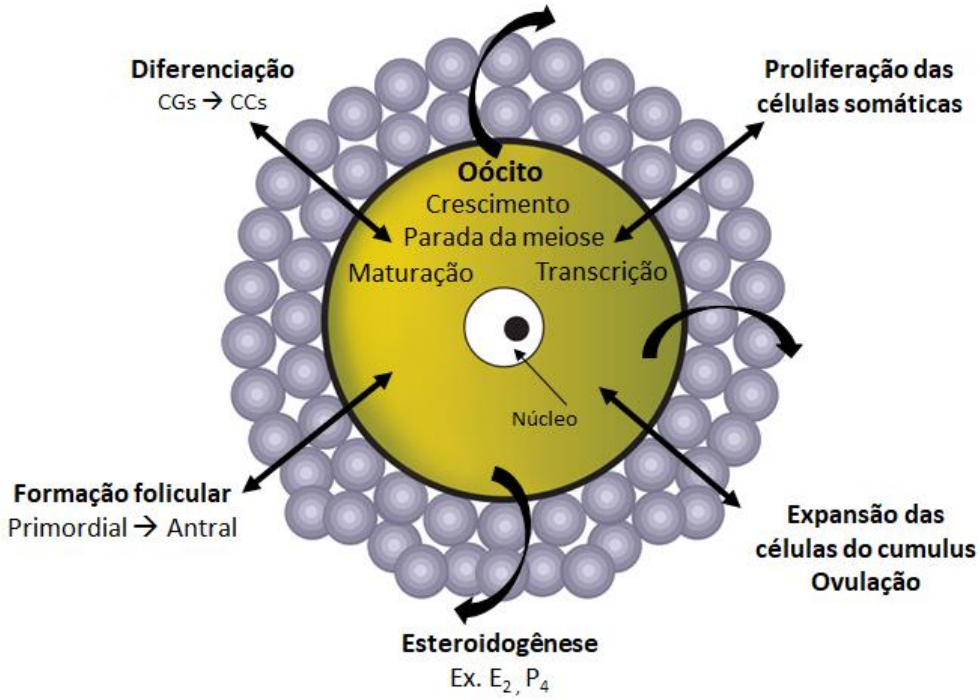
2.2.1 Comunicação bidirecional

A comunicação intercelular favorece o desenvolvimento e função dos organismos multicelulares. As três grandes formas de comunicação são parácrinas, justácrinas e endócrinas. Embora o desenvolvimento folicular ovariano envolva os três tipos de comunicação, apenas a sinalização parácrina e justácrina acontece nas células somáticas e oócito (EPPIG, 2018). A comunicação que ocorre entre as células do cumulus e o oócito acontece de maneira bidirecional, sendo essencial para o desenvolvimento das células do cumulus, bem como manter a integridade do oócito (Figura 2) (PICTON *et al.*, 2008; PARAMIO; IZQUIERDO, 2014, 2016; MENCHACA *et al.*, 2016).

A comunicação bidirecional na transição folicular da fase pré-antral para o estágio pré-ovulatório é de fundamental importância, pois, essa cooperação é mediada pelas junções comunicantes entre o oócito e as células da granulosa (EPPIG *et al.*, 1997), onde são produzidos RNAs e proteínas essenciais para a maturação do oócito e subsequente desenvolvimento pré-implantacional (THOMAS; WALTERS; TELFER, 2003; KRISHER, 2004; VAN DEN HURK; ZHAO, 2005). Ainda, outros substratos, tais como: glicose,

aminoácidos nucleotídeos e metabólitos são transportados ao oócito via junções *gap* (EPPIG; O'BRIEN, 1996), bem como moléculas que estão envolvida na regulação da maturação meiótica do oócito (ORISAKA *et al.*, 2009). É importante ressaltar que há uma íntima ligação entre o oócito e as células somáticas que o circundam, para que haja a sincronia da aquisição da competência oocitária, e sua consequente liberação do folículo (WIGGLESWORTH *et al.*, 2013; LIMA *et al.*, 2016; ROBKER; HENNEBOLD; RUSSELL, 2018).

Figura 2: Representação esquemática da comunicação bidirecional que ocorre entre as células cumulus e o oócito.



Fonte: Adaptado de Eppig (2001). A comunicação bidirecional é essencial para o desenvolvimento de células cumulus e o desenvolvimento de um oócito viável. Os dois principais tipos de comunicação que ocorrem entre as células do cumulus e o oócito são a sinalização parácrina (setas curvas) e via junções gap (setas retas).

2.3 Competência oocitária

A competência do desenvolvimento oocitário é definida como a capacidade de um gameta feminino maturar com potencial para ser fertilizado, e sustentar o desenvolvimento embrionário até o estágio de blastocisto (CONTI; FRANCIOSI, 2018). A mesma é alcançada através de mudanças estruturais e funcionais que ocorrem de maneira gradual durante a foliculogênese, culminando com uma das etapas finais do desenvolvimento, sendo a maturação oocitária (KRISHER, 2004; JAMNONGJIT; HAMMES, 2005; SU; SUGIURA; EPPIG, 2009).

O processo de maturação do oócito envolve os aspectos de maturação citoplasmática, maturação nuclear e maturação molecular; embora possam desempenhar funções separadas, eles estão intimamente relacionados. É durante esses processos que o oócito adquire a capacidade de ser fertilizado e subsequentemente manter o desenvolvimento embrionário (KRISHER, 2004). A completa maturação nuclear não indica que o oócito esteja apto para ser fertilizado e sustentar o desenvolvimento embrionário inicial, pois ainda existem muitos processos que precisam acontecer dentro do citoplasma, independentemente da maturação nuclear (DURINZI; SANIGA; LANZENDORF, 1995). No entanto, para que esses processos complexos ocorram de maneira eficiente durante a maturação oocitária, é necessário que haja a redistribuição das organelas citoplasmáticas, o armazenamento de RNAm, proteínas e fatores de transcrição essenciais para que ocorra esse processo (FERREIRA *et al.*, 2009).

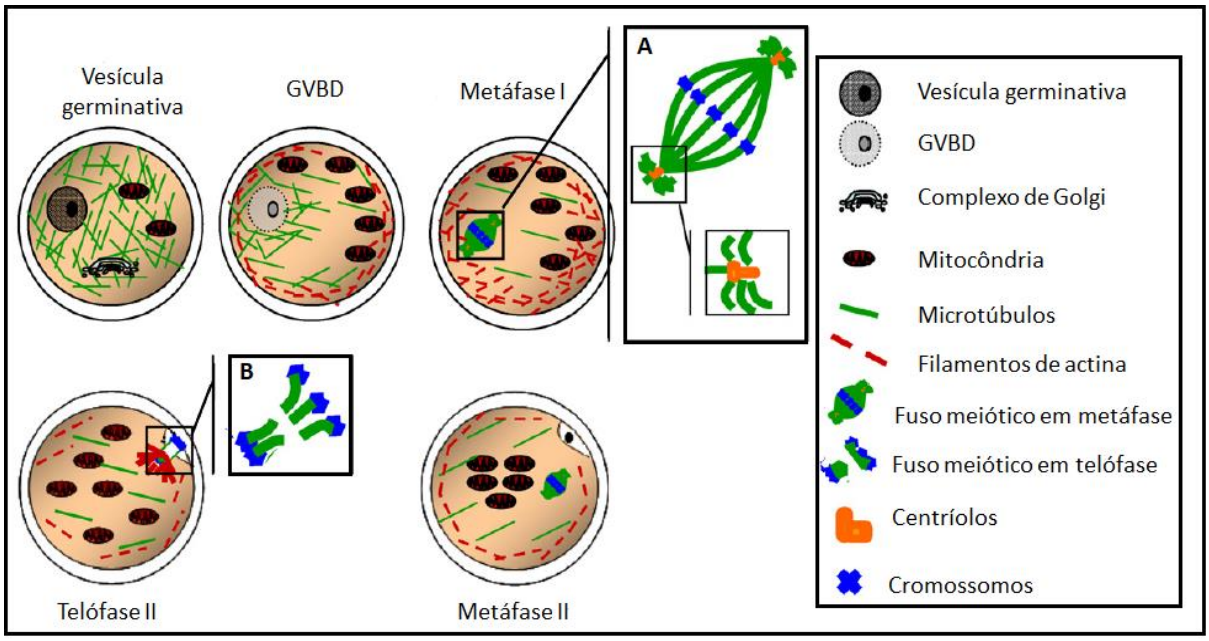
2.3.1 Maturação citoplasmática

A competência do desenvolvimento oocitário (capacidade do oócito de se desenvolver em um embrião sadio e acarretar em uma prenhez saudável) está relacionada à maturação citoplasmática do oócito, sendo que tal processo ocorre simultaneamente à maturação nuclear, embora sejam eventos independentes (YAMADA; ISAJI, 2011). Vale ressaltar que a maturação citoplasmática é influenciada pela progressão da maturação molecular e estrutural, que em estágios posteriores irão determinar a adequada fertilização e as divisões mitóticas do embrião (RYBSKA *et al.*, 2018).

O processo de redistribuição das organelas citoplasmáticas é algo que está estabelecido durante a maturação citoplasmática, onde ocorrerá várias alterações ultraestruturais relacionadas à morfologia, tais como: reorganização das organelas pela dinâmica dos microfilamentos e microtúbulos do citoesqueleto, e o reposicionamento dessas organelas dependerá das necessidades da célula durante cada estágio de desenvolvimento (FERREIRA *et al.*, 2009; MAO *et al.*, 2014), conforme ilustrado na figura 3.

Em diferentes momentos do processo de maturação, o oócito apresenta um padrão espacial de polarização, bem como várias proteínas também exibem um arranjo cortical polarizado para iniciar suas atividades específicas (CHA; CHIAN, 1998; MAO *et al.*, 2014). As organelas citoplasmáticas, tais como as mitocôndrias, o retículo endoplasmático (RE) e o complexo de Golgi alteram sua localização circundando a vesícula germinativa (PEPLING *et al.*, 2007).

Figura 3: Representação esquemática da distribuição das organelas citoplasmáticas e dinâmica de filamentos do citoesqueleto durante a maturação citoplasmática.



Fonte: Adaptado de Ferreira *et al.,* (2009). **A.** Detalhe do fuso meiótico em metáfase I e a estrutura do centríolo. **B.** Detalhe do fuso meiótico em telófase I, em que podemos ver os microtúbulos entre os conjuntos cromossômicos.

Vale ressaltar que, em oócito de humanos tem sido observado um aumento expressivo das mitocôndrias (de 6.000 para aproximadamente 193.000) com consequente aumento da atividade mitocondrial, havendo também modificações no complexo de Golgi e o acúmulos de ribossomos (REYNIER *et al.*, 2001; CUMMINS, 2004). Depois que ocorre a quebra da vesícula germinativa, as mitocôndrias se dispersam durante a extrusão do primeiro corpo polar. As alterações citoplasmáticas que ocorrem durante esse processo são: transcrição de RNAm, tradução de proteínas, modificações pós-traducionais, alterações ultra-estruturais, e remodelação da cromatina, além das modificações epigenéticas, como as alterações nos padrões de metilação do DNA e modificações de histonas (FULKA; FIRST; MOOR, 1998; WRENZYCKI; HERRMANN; NIEMANN, 2007; GOTTARDI; MINGOTI, 2009; INBAR-FEIGENBERG *et al.*, 2013).

Estudos em bovinos estabeleceram que a formação de um blastocisto viável depende dos transcritos formados durante a maturação oocitária, e que os mRNAs e proteínas envolvidos na fertilização e na regulação do desenvolvimento embrionário também são acumuladas nesta fase (DE SOUSA; WESTHUSIN; WATSON, 1998). Se a maturação citoplasmática não ocorrer com sucesso, o oócito poderá não ser fertilizado ou será incapaz de se transformar em um embrião viável (FERREIRA *et al.*, 2009; FU *et al.*, 2016a, 2016b).

Além disso, uma maturação citoplasmática inadequada do oócito poderá resultar na falha da formação do pronúcleo masculino, consequentemente causando um aumento nas anormalidades após a fertilização (CHA; CHIAN, 1998; KRISHER, 2004; HOJNIK; KOVAČIČ, 2019).

2.3.2 Maturação nuclear

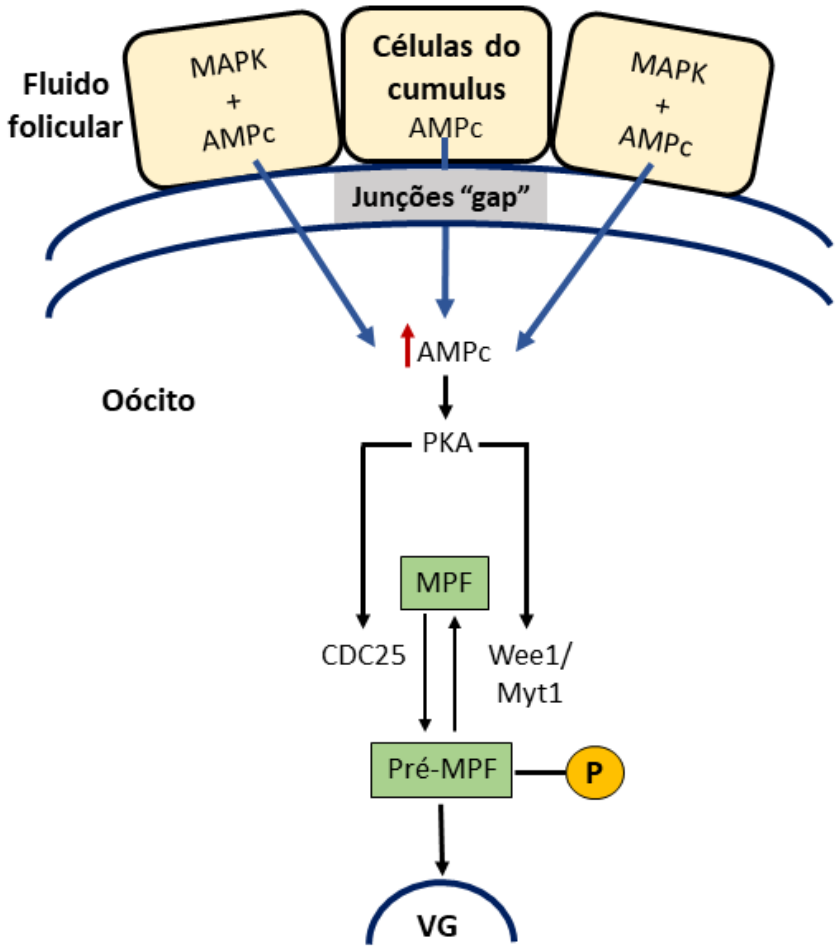
Os oócitos adquirem competência meiótica (capacidade do mesmo em retomar a meiose I e tornar-se nuclearmente maduro) durante as fases posteriores da foliculogênese, sendo observado no período da formação de antro em oócitos murinos, e posteriormente em outros mamíferos (EPPIG, 2001; FAIR, 2003; CONTI; FRANCIOSI, 2018). As mudanças que ocorrem durante a maturação nuclear incluem: transcrição e tradução de RNA, replicação e reparo do DNA, condensação cromossômica, formação dos fusos e preparação para fertilização (PICTON *et al.,* 2008; CONTI; FRANCIOSI, 2018; RYBSKA *et al.,* 2018).

A maturação oocitária é iniciada pelo aumento das gonadotrofinas no período pré-ovulatório, em especial o LH. Até o pico de LH, o oócito está parado no estágio de diplóteno da prófase I da primeira meiose, também conhecido como estágio de vesícula germinativa (VG) (PICTON; BRIGGS; GOSDEN, 1998; ZHANG; OUYANG; XIA, 2009; SÁNCHEZ; SMITZ, 2012). A retomada da meiose é caracterizada pela quebra da membrana nuclear dentro do oócito, também chamada de quebra da vesícula germinativa (GVBD). A primeira divisão (meiose I) resulta na separação de 23 cromossomos homólogos (pares) ou 46 cromossomos (2N) imediatamente antes da ovulação. Isso é caracterizado pela formação do primeiro corpúsculo polar (que contém metade dos cromossomos), e os outros 23 cromossomos (N) permanecem dentro do oócito que param na metáfase II (CHA; CHIAN, 1998; ZHANG; OUYANG; XIA, 2009; PAN; LI, 2019). Na segunda divisão (meiose II) envolve a segregação das cromátides-irmãs e isso ocorre durante a fertilização. Se uma dessas divisões não acontecer de maneira correta, poderá resultar em separação anormal dos cromossomos, sendo capaz de levar à aneuploidia, e acredita-se que 80% dos erros durante a meiose ocorram durante a meiose I (HASSOLD; HUNT, 2001; KULIEV *et al.,* 2011; POTAPOVA; GORBSKY, 2017).

A retomada da meiose ocorre devido à ação das gonadotrofinas nas células foliculares que circundam o oócito, pois, a remoção do oócito do ambiente folicular resulta na retomada da meiose de maneira espontânea (MÉDURI *et al.,* 2002; PICTON *et al.,* 2008; JAMNONGJIT; HAMMES, 2005; ZHANG; OUYANG; XIA, 2009; LIU *et al.,* 2017). Essas

gonadotrofinas induzem a formação de substâncias, como esteroides, cálcio, inositol trifosfato (IP3), adenosina monofosfato cíclico (AMPc) e purinas (YAMADA; ISAJI, 2011; SHIMADA, 2012; APPELTANT *et al.*, 2016), os quais são transportadas pelas junções *gap* até o oócito e inibem ou estimulam a maturação oocitária. Assim, uma eficiente comunicação entre oócito e células cumulus é essencial para o controle da maturação nuclear. Uma vez que o AMPc entra no oócito, ele age como um segundo mensageiro que aciona a proteína quinase A (PKA) dependente de AMPc para fosforilar o fator promotor da maturação (MPF), um complexo composto pelas subunidades CDK1 e ciclina B, via fosfatase CDC25, mantendo-a em seu estado inativo (pré-MPF) ou desfosforilando-o via quinase Wee1/Myt1, ativando-o (Figura 4). Níveis elevados de AMPc faz com que a PKA atue sobre CDC25 fosforilando o MPF e mantendo-o no estado pré-MPF, que por sua vez mantém a parada meiótica. Por outro lado, uma diminuição nos níveis de AMPc, possivelmente induzido pela perda de junções *gap* entre o oócito e suas células cumulus circundantes, causa o efeito oposto, com retomada da meiose. Isso ocorre devido a ação da PKA sobre a quinase Wee1/Myt1, o que permite a desfosforilação do MPF, fazendo com que ocorra a quebra da vesícula germinativa (GVBD) e retome a meiose. Além disso, a ativação das proteínas quinases ativadas por mitógenos (MAPK) nas células cumulus circundantes pode estar tendo um efeito. No entanto, os mecanismos exatos de como isso ocorre ou as interações entre AMPc e MAPK não estão totalmente esclarecidos (JAMNONGJIT; HAMMES, 2005; HAN; CONTI, 2006; SOLC *et al.*, 2008; ZHANG; OUYANG; XIA, 2009; OH, HAN; CONTI, 2010; PAN; LI, 2019).

Figura 4: Representação esquemática ilustrando a via de sinalização do processo de maturação oocitária no complexo cumulus-oócito



Fonte: Adaptado de Cha; Chian, 1998. O AMPc oriundo das células do cumulus pode estar entrando no oócito através das junções gap e, assim, manter o MPF fosforilado e, portanto, desativado. Uma vez que o nível de AMPc dentro do oócito diminui (ex., pela redução das junções gap entre os dois tipos de células durante a expansão do cumulus), o MPF é ativado (desfosforilado) e, portanto, causa a quebra da vesícula germinativa e consequente retomada da meiose. MAPK = Proteínas-quinase ativadas por mitógenos; AMPc = Adenosina monofosfato cíclico; PKA = Proteína quinase A; MPF = Fator promotor da maturação; CDC25 = Ciclo de divisão celular 25; Wee1/Myt1 = Proteínas quinase; VG = Vesícula germinativa;

2.3.3 Maturação molecular

A maturação molecular é um processo que ocorre durante o crescimento de oocitário (FERREIRA *et al.*, 2009). Esse processo inclui a transcrição, armazenamento e processamento dos mRNAs expressos pelos cromossomos, que posteriormente serão traduzidos em proteínas pelos ribossomos. As proteínas resultantes desses mRNAs irão auxiliar na maturação, fecundação, formação dos pronúcleos e na embriogênese inicial. Para o acúmulo de RNAm e proteínas, os oócitos necessitam de um mecanismo de transcrição que seja funcional (SIRARD, 2001; CROCOMO *et al.*, 2013).

Vale ressaltar que os mRNAs produzidos são acumulados em complexos de ribonucleoproteínas mensageiros (RNPm), enquanto que a síntese proteica geralmente é estimulada durante a maturação. No momento da quebra da vesícula germinativa *"germinal vesicle break down"* – GVBD o processo de tradução é aumentado, seguido pela poliadenilação do RNAm. Logo em seguida, ocorre um declínio na transcrição, e o RNAm poliadenilado fica acumulado até o estágio de metáfase II (TOMEK; TORNER; KANITZ, 2002; SIRARD *et al.,* 2006). Os oócitos dependem desses transcritos armazenados para continuar a meiose, sofrer divisões mitóticas após a fecundação e concluir a transição materno zigótica durante o desenvolvimento o embrionário inicial (SIRARD *et al.*, 2006). Essas altas taxas de transcrição e armazenamento da maioria dos transcritos ocorrem durante o crescimento do oócito. Durante esse período, os mRNAs são muito estáveis para permitir a acumulação (MEDVEDEV *et al.*, 2008).

2.4 Fecundação

A fecundação é caracterizada pela junção de duas células haploides extremamente especializadas, as quais compreendem os gametas masculino e feminino. Durante essa fase o oócito finaliza a meiose com a extrusão de segundo corpúsculo polar, seguido da formação do pronúcleo masculino e feminino. Após ocorrer o processo de fecundação e fusão dos pronúcleos, dar-se-á origem ao zigoto com um genoma definido (PARANJPE; VEENSTRA, 2015).

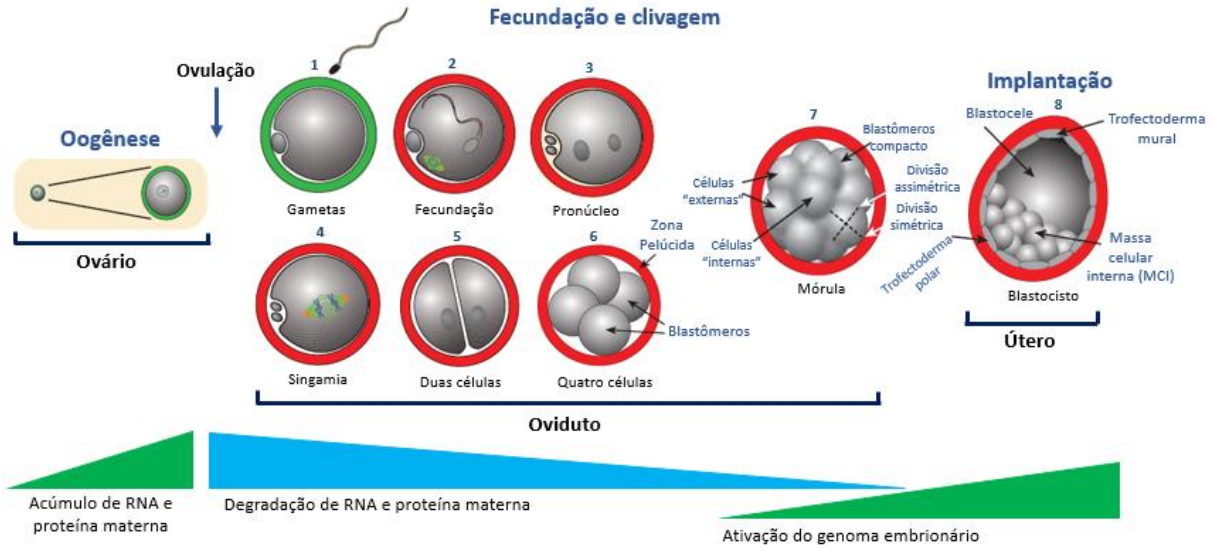
2.4.1 Desenvolvimento embrionário inicial

O desenvolvimento embrionário inicial ou período pré-implantacional está compreendido do processo de fertilização até a implantação do embrião no útero (WATSON; NATALE; BARCROFT, 2004). Vale ressaltar que nesse período do desenvolvimento embrionário, ocorre a transcrição materno-embrionária (MET), no qual os RNAs e as proteínas armazenadas no oócito são degradados de maneira gradual, e a transcrição do genoma embrionário é ativado (LEI; LU; DEAN, 2013; GRAF, *et al.*, 2014). Os transcritos e as proteínas maternas são produzidos e armazenados durante oogênese (TADROS; LIPSHITZ, 2009; HAFIDH; CAPKOVÁ; HONYS, 2011), conforme ilustrado na figura 5.

O processo de ativação do genoma do embrião é apontado como uma das fases mais relevantes do período pré-implantacional nos mamíferos, que na espécie bovina acontece

no estágio de 8-16 células (MEMILI; FIRST, 2000; GANDOLFI; GANDOLFI, 2001; BADR *et al.*, 2007). Após ocorrer a ativação do genoma embrionário, o mesmo passará pelo primeiro processo de diferenciação celular, onde as células embrionárias mais externas adquirem um formato achatado, comum às células epiteliais, porém, essa diferenciação estrutural permanecerá até que o embrião atinja o estágio de mórula (ROSSANT, 2007, 2016).

Figura 5. Representação do desenvolvimento embrionário inicial e ativação do genoma embrionário.



Fonte: Adaptado de Li; Zheng; Dean (2010). Oogênese, gametas, fecundação e diferentes estágios de desenvolvimento embrionário durante a fase pré-implantacional: (1) Gametas transcricionalmente inertes; (2-3) Fecundação, onde núcleo espermático é descondensado e reembalado com histonas armazenadas no oócito para formar o pronúcleo masculino. O ovo completa sua segunda divisão meiótica e forma o pronúcleo feminino; (4) Após a singamia e "união" dos cromossomos, a citocinese estabelece o embrião de 2 células; (5) Embrião de 2 células; (6) Embrião de 4 células; (7) Mórula compacta; (8) Blastocisto expandido. Ativação do genoma embrionário, caracterizada pela substituição gradual da atividade transcricional materna pela atividade transcricional do embrião.

No decorrer do crescimento embrionário, poderá acontecer uma sequência de alterações na configuração da cromatina, a qual está, inicialmente, associado a acetilação e metilação das histonas e a metilação do DNA genômico (NIEMANN, 2016). Vale ressaltar que os genomas materno e paterno exibem configurações da cromatina distintos e podem sofrer modificações significativas que se iniciam posteriormente a fecundação, dentre as quais podemos destacar os fatores epigenéticos, tais como as modificações das histonas e mudanças nos padrões de metilação do DNA (INBAR-FEIGENBERG *et al.*, 2013).

2.5 Produção *in vitro* de embriões (PIV)

A produção *in vitro* (PIV) de embriões vem-se destacando progressivamente no cenário mundial, representando avanços dentro das biotecnologias reprodutivas, e que podem ser empregadas em diferentes áreas de pesquisa. Essa técnica tem sido utilizada nos estudos que envolvem a embriologia básica, biologia e fisiologia do desenvolvimento, bem como sua aplicabilidade em programas de melhoramento genético e auxílio às biotecnologias emergentes, avaliação da fertilidade em machos e na reprodução assistida, tanto em humanos quanto em animais (BALDASSARE *et al.,* 2002; BALDASSARE *et al.,* 2007; DOS SANTOS-NETO, 2014).

A utilização das biotécnicas em reprodução animal têm proporcionado melhorias no que diz respeito aos animais de interesse zootécnico (SIMPLÍCIO *et al.*, 2007). Nesse âmbito, podemos incluir os pequenos ruminantes, os quais são excelentes modelos na aplicação destas tecnologias, ressaltando que o primeiro animal que fora clonado por transferência nuclear de células somáticas (TNCS), entre as espécies domésticas foi a ovina (FREITAS *et al.,* 2007).

Vale ressaltar que a PIV abrange as fases de recuperação de oócitos oriundos de folículos antrais, maturação *in vitro* dos oócitos (MIV), fecundação *in vitro* dos oócitos que foram maturados (FIV) e cultivo *in vitro* (CIV) dos zigotos até atingirem o estágio de mórula e/ou blastocisto (PARAMIO; IZQUIERDO, 2014). Após essas etapas e consequente produção de embriões, os mesmos podem ser transferidos para fêmeas receptoras ou armazenados para posterior utilização, tendo sido relatado resultados positivos por meio dessas técnicas na espécie ovina, bovina e em outras espécies (BAVISTER *et al.,* 1995; PTAK *et al.,* 1999; FREITAS *et al.,* 2007, MENCHACA *et al.,* 2016). Através dos avanços desta biotecnologia, obteve-se o nascimento dos primeiros cordeiros produzidos por FIV no Uruguai, proporcionando cada vez mais instrumentos para a multiplicação de fêmeas geneticamente superiores (MENCHACA *et al.,* 2012a), e posteriormente através da utilização desta mesma técnica foi alcançado o nascimento dos primeiros cordeiros transgênicos nascidos na América do Sul (CRISPO *et al*., 2015). No entanto, os índices de desenvolvimento embrionário ainda permanecem baixos, com média percentual variando entre 10-15% dos oócitos ovinos (BERNARDI, 2005). Além disso, foi constatado uma menor qualidade dos embriões que são produzidos *in vitro* quando comparados com embriões *in vivo*, bem como uma taxa de sobrevivência bem aquém do esperado, torando-se necessário o aperfeiçoamento da técnica (RODRIGUEZ-DORTA *et al*., 2007; PARAMIO, 2010).

Em condições *in vivo*, a aquisição da competência oocitária ocorre continuamente durante a foliculogênese, quando há uma perfeita sincronização entre a maturação citoplasmática e nuclear. No entanto, *in vitro*, os oócitos são recuperados de folículos que se encontram em diferentes estágios de desenvolvimento, consequentemente geram oócitos em diferentes estágios de maturação (STOUFFER; ZELINSKI-WOOTEN, 2004), que são maturados na mesma condição, portanto, a sua resposta é diferente e imprevisível (PARAMIO; IZQUIERDO, 2014). Durante o crescimento oocitário, a síntese de proteínas pelo oócito é de grande importância para a maturação e para assegurar o desenvolvimento embrionário inicial. Dentre estas proteínas, o fator de crescimento e diferenciação 9 (GDF-9) e a proteína morfogenética óssea 15 (BMP-15) são importantes reguladores da expansão e diferenciação das células do cumulus (GILCHRIST; RITTER; ARMSTRONG, 2004; HUSSEIN; THOMPSON; GILCHRIST, 2006). Além disso, outras proteínas sintetizadas pelo oócito, como por exemplo, o antígeno materno requerido pelo embrião (MATER), a proteína de detenção zigótica 1 (ZAR-1) e a nucleoplasmina (NPM-2) (TONG *et al.*, 2000; BURNS *et al.*, 2003; WU *et al.*, 2003a, WU *et al.*, 2003b) desempenham papeis vitais durante os primeiros estágios do desenvolvimento embrionário. A ausência destas proteínas em oócitos de ratas tem uma relação direta com o potencial de clivagem e progressão do embrião além do estágio de blastocisto (SÁNCHEZ *et al.*, 2009).

Faz-se necessário intensificar as melhorias nos sistemas de PIV de embriões em pequenos ruminantes, merecendo destaque os ovinos, não apenas nos conhecimentos referentes a essa biotécnica que é amplamente aplicada em bovinos, mas, atentando para as características peculiares referente à espécie, bem como às suas necessidades metabólicas (ROCHA-FRIGONI *et al.*, 2014).

2.6 Análise proteômica

A proteômica pode ser definida como a análise descritiva e quantitativa de um grupo específico de proteínas codificadas pelo genoma em circunstâncias normais ou anormais, decorrentes de processos pós-transcricionais e pós-traducionais, bem como a interação com outras proteínas e outros grupos moleculares (VALLEDOR; JORRIN, 2011). No entanto a abundância de proteínas não pode ser prevista com precisão pela quantidade de transcritos (SCHWANHAUSSER *et al.*, 2013).

Através dos estudos proteômicos, pode-se realizar uma análise comparativa, sequencial e estrutural, porém, a mesma normalmente pode dar apenas informações limitadas,

caracterizando de forma abrangente a função de uma proteína não caracterizada numa célula ou organismo, exigindo sempre investigações adicionais *in vitro* sobre determinada proteína, bem como estudos biológicos de células *in vivo* (PETSKO; RINGE, 2004), onde diferentes métodos experimentais são necessários para definir a função de uma proteína precisamente nos níveis bioquímico, celular e orgânico, a fim de caracterizá-la completamente (PETSKO; RINGE, 2004).

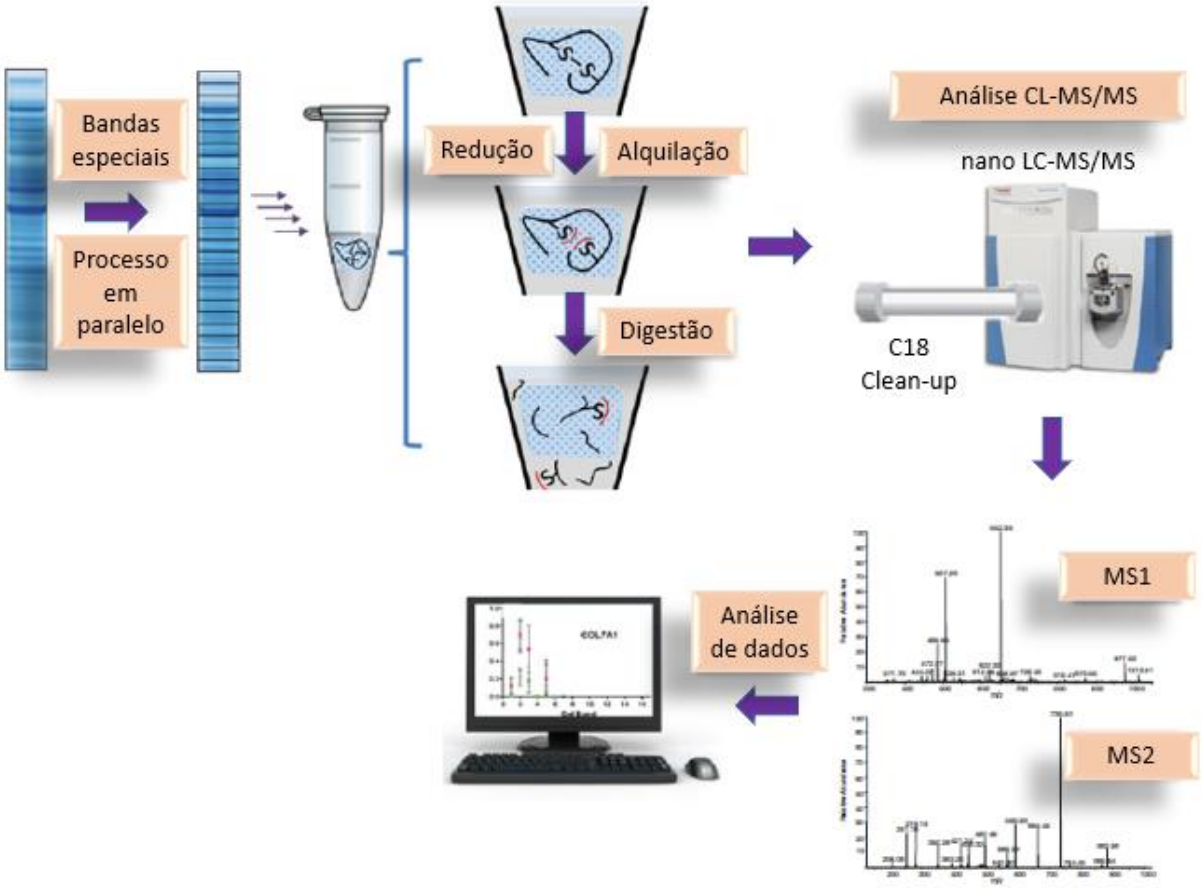
De maneira peculiar, a análise proteômica constitui-se na análise do proteoma, a qual utiliza diferentes técnicas nos processos de separação e identificação, dentre as quais destacamos a eletroforese (1D e 2D), cromatografia, espectrometria de massas e análise de bioinformática (HEIN *et al.,* 2013), sendo observado que a espectrometria de massas (MS) é uma excelente alternativa para a detecção e quantificação de proteínas, bem como a identificação de possíveis modificações pós-traducionais (PTMs) (OVERALL, 2014; SNIDER; OMARY, 2014).

Tem sido relatado a utilização destas técnicas no processo de identificação e quantificação de muitas proteínas em modelos animais no estágio de desenvolvimento embrionário (DEUTSCH *et al.,* 2014), onde geralmente as proteínas-alvo são excisadas do gel, passam por um processo de fragmentação (visando à obtenção de peptídeos), seguido pela análise dos fragmentos obtidos (de modo geral por digestão trípica) e utilizando como ferramenta principal o espectrômetro de massa, que, por sua vez, indica a massa da molécula através da mensuração da razão massa/carga do íon da molécula (SIUZDAK, 2006). Dentre as técnicas utilizadas em análise protômica, destacamos a eletroforese unidimensional em gel de poliacrilamida na presença de dodecil sulfato de sódio (1D SDS-PAGE) seguido por cromatografia líquida acoplada à espectrometria de massas em tandem (LC-MS/MS), a qual oferece uma forte abordagem analítica capaz de equilibrar as restrições do mundo real com a obtenção de uma excelente cobertura proteômica, conforme descrito na Figura 6.

Atualmente, devido ao elevado potencial que se tem através da utilização da proteômica em animais, têm sido realizadas relevantes pesquisas visando separar e identificar proteínas em amostras biológicas (ALMEIDA *et al.,* 2015). Esta técnica permitiu se obter uma visão geral das proteínas envolvidas no desenvolvimento folicular *in vitro* de ratas (ANASTÁCIO *et al.,* 2017), folículos primordiais em murinos e bubalinos (GOVINDARAJ; RAO, 2015; GOVINDARAJ *et al.,* 2016), em ovário murino (HE *et al.,* 2017), córtex ovariano em humanos (OUNI *et al.,* 2018), ovário fetal de suínos (XU *et al.,* 2015; XU *et al.,* 2017), complexo cumulus-oócito murinos e suíno (MENG *et al.,* 2007; PACZKOWSKI; KRISHER, 2010), células do cumulus de humanos e equinos (HAMAMAH *et al.,* 2006;

WALTER *et al.*, 2014; WALTER *et al.*, 2016; HUWILER *et al.*, 2016), em oócitos e zigotos de murinos (ZHANG *et al.*, 2009; PFEIFFER *et al.*, 2011; WANG *et al.*, 2010) e no fluido folicular em éguas (FAHIMINIYA *et al.*, 2011), búfalas (FU *et al.*, 2016) e ovelhas (WU *et al.*, 2018).

Figura 6. Representação esquemática do fluxo de trabalho proteômico por LC-MS/MS com o procedimento de digestão em gel.



Fonte: Adaptado de Dzieciatkowska; Hill; Hansen (2014).

3 JUSTIFICATIVA

A ovinocultura tem um relevante papel socioeconômico, principalmente na região Nordeste do Brasil, por se tratar da produção de uma de importante fonte de alimento (carne) e renda (através da venda de serviços e produtos relacionados como carne, lã e pele). Desta forma, o aprimoramento de Tecnologias de Reprodução Assistidas – TRAs como a produção *in vitro* e transferência de embriões que possibilitem uma rápida multiplicação de animais geneticamente superiores, poderão contribuir significativamente para o crescimento da pecuária.

No que se refere a produção *in vitro* de embriões, no decorrer dos anos esta biotécnica vem exercendo um papel relevante na multiplicação e conservação das espécies através da manipulação de gametas, contribuindo para a sobrevivência das populações existentes, permitindo assim otimizar o processo de multiplicação das fêmeas, podendo assim ser utilizados animais jovens, com idade mais avançada e/ou em animais prenhes, quando esta vier a ser empregada simultaneamente à recuperação de oócitos por via laparoscópica.

Apesar dos esforços voltados para aperfeiçoar as técnicas de PIV, a produção embrionária em ovinos ainda não é a ideal, sendo inferior às taxas obtidas em bovinos. Além disso, há a necessidade de melhorar a qualidade dos blastocistos produzidos *in vitro* comparados aos produzidos *in vivo*. Tal fato pode ser justificado em decorrência de uma melhor compreensão dos sistemas biológicos que estão relacionados à maturação oocitária e o desenvolvimento embrionário. Dessa forma, a análise proteômica surge como uma alternativa para a identificação de possíveis marcadores moleculares para a maturação oocitária, bem como para o desenvolvimento embrionário. Assim, a caracterização das proteínas nesta espécie será capaz de contribuir positivamente para um melhor entendimento dos mecanismos que a maturação de oócitos e desenvolvimento embrionário, podendo gerar informações que irão contribuir para melhoria dos índices de produção *in vitro* (PIV) de embriões nesta espécie, podendo servir como modelo para outras espécies.

No entanto, até o presente momento não existem relatos sobre a expressão de proteínas de complexo cumulus-oócito – CCO (maturados e não maturados) e embriões oriundos de ovelhas produzidos *in vitro*.

4 HIPÓTESE CIENTÍFICA

- ✓ Os complexos cumulus-oócito (CCOs) ovinos antes e após a maturação apresentam proteomas diferenciados.

- ✓ Os embriões ovinos produzidos *in vitro* apresentam proteomas diferenciados durante o desenvolvimento.

5 OBJETIVOS

5.1 Objetivo Geral

- ✓ Avaliar a composição proteica através da análise da proteômica de complexos cumulus-oócito (CCOs) e embriões de ovinos.

5.2 Objetivos Específicos

- ✓ Avaliar os proteomas de complexos cumulus-oócito não maturados e maturados, analisando potenciais associações destes com o desenvolvimento folicular;
- ✓ Estudar as relações entre o proteoma e aspectos qualitativos de embriões ovinos produzidos *in vitro*.

6 ARTIGO I

How *in vitro* maturation changes the proteome of ovine cumulus-oocyte complexes?

Artigo submetido para publicação no periódico *Molecular, Reproduction and Development*
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How *in vitro* maturation changes the proteome of ovine cumulus-oocyte complexes?

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Abstract

The present study evaluated the effects of *in vitro* maturation (IVM) on the proteome of cumulus-oocyte complexes (COCs) from ewes. Extracted COC proteins were analyzed by LC-MS/MS. Differences in protein abundances ($p < 0.01$) and functional enrichments in immature vs *in vitro* matured COCs were evaluated using bioinformatics tools. There were 2,550 proteins identified in the COCs, with 89 and 87 proteins exclusive to immature and mature COCs, respectively. IVM caused down-regulation of 84 and up-regulation of 34 proteins. Major up-regulated proteins in mature COCs were dopey_N domain-containing protein, structural maintenance of chromosomes protein, ubiquitin like modifier activating enzyme 2. Main down-regulated proteins in mature COCs were immunoglobulin heavy

constant mu, inter-alpha-trypsin inhibitor heavy chain 2, alpha-2-macroglobulin. Proteins exclusive to mature COCs and up-regulated after IVM related to immune response, complement cascade, vesicle mediated transport, cell cycle, extracellular matrix organization. Proteins of immature COCs and down-regulated after IVM were linked to metabolic processes, immune response, complement cascade. KEGG pathways and miRNA-regulated genes attributed to down-regulated and mature COC proteins related to complement and coagulation cascades, metabolism, humoral response, B cell-mediated immunity. Thus, IVM influenced the ovine COC proteome. This knowledge supports future development of efficient IVM protocols for *Ovis aries*.

Keywords: Proteins, ovary, follicle, reproduction, ovine.

1 INTRODUCTION

In mammalian species, the oocyte, granulosa and theca cells are closely connected and in a relationship of functional interdependence, influencing both oocyte and embryo development (Eppig & O'Brien, 1996; Sirard, 2011). During follicular growth, the granulosa cells surrounding the oocyte differentiate into cumulus cells and continue to multiply along with the cells of the granulosa murals as the follicle antrum expands (Driancourt, 2001; Rodgers & Irving-Rodgers, 2010). This arrangement indicates that cumulus cells play essential roles in oocyte development and maturation (Guo et al., 2016).

Oocyte maturation is an intricate process, which includes the meiotic resumption and progression to metaphase II (MII), release of the polar body and biochemical events in the oocyte cytoplasm and surrounding cumulus cells (Souza-Fabjan et al., 2014). Although several culture media have been used for *in vitro* maturation (IVM), the most commonly used media for oocytes from small ruminants are based on tissue culture medium 199 (TCM-199) bicarbonate-buffered with Earle's salts. These formulations contain energy and carbon sources (like glucose and glutamine) and amino acids, minerals, vitamins and are often enriched with hormones and growth factors, L-glutamine pyruvate, thiol compounds, and complex fluids such as heat-inactivated serum and follicular fluids (Paramio & Izquierdo, 2016). Despite the technical advances in the field, with 60 to 90% of ovine cumulus-oocyte complexes (COC) being successfully matured *in vitro*, usually no more than 40% of the fertilized oocytes reach the blastocyst state after IVF (Ledda et al., 2016, Abdelnour et al., 2019, Javvaji et al., 2021). In order to improve the efficiency of ovine oocyte maturation and results of *in vitro* embryo

production, researchers have tested numerous maturation media, such as IVM with defined or undefined medium (Cocero et al., 2011), supplementation with recombinant FSH (Barros et al., 2019), follicular fluid (Tian et al., 2019), kisspeptin (Byri et al., 2017) and interleukin-7 (Javvaji et al., 2019), among others. In addition, changes in the protocols involving maintenance or removal of cumulus cells (Dos Santos-Neto et al., 2020), oxygen tension (Sánchez-Ajofrín et al., 2020), follicle size (Contreras-Solis et al., 2021) have been evaluated but without groundbreaking results. The reason for this is not completely understood, but probably relates to the fact that the intricate biology of cumulus-oocyte complexes is far from being deciphered.

Understanding the cellular and molecular mechanisms that regulate the competence of oocytes and early embryonic development remains one of the major challenges of reproductive biology and medicine (Gandolfi et al., 2005, Rajput et al., 2013). In the last decades, studies have described the intricacy of the proteome of cumulus–oocyte complexes from mice, bovine and swine (Meng et al., 2007, Paczkowski & Krisher, 2010, Walter et al., 2020), and of cumulus cells from human, bovine and equine species (Hamamah et al., 2006, Peddinti et al., 2010, Walter et al., 2014, Walter et al., 2016, Huwiler et al., 2016). Results from all these investigations indicate that COC proteins influence the biological processes and quality of the oocyte. Also, new technologies and methods in mass spectrometry and bioinformatics have been used for quantification and assessment of proteins and their post-translational modifications, in support for discovery of markers of oocyte and embryo quality in different species (Orozco-Lucero & Sirard, 2014). Recently, it was observed that *in vivo* matured bovine COCs overexpress important proteins associated with distinct cellular pathways, with complement and coagulation cascades, steroid biosynthesis and ECM-formation, in comparison to *in vitro* matured COCs (Walter et al., 2020). These results indicate that conditions of the maturation significantly alter the process of protein synthesis by cumulus cells, which could have important consequences to oocyte competence (Walter et al., 2020). Given this scenario, the present study was conducted to characterize the major changes that occur in the proteome of ovine COCs subjected to *in vitro* maturation.

2 MATERIAL AND METHODS

2.1 Study design and animals

In the present study, methods in mass spectrometry and bioinformatics were used

to evaluate the major proteome of ovine COCs before and after the process of *in vitro* maturation (Figure 1).

The study was carried out in Uruguay (34°S) during the early breeding season (December) and all ovaries (bearing one or two corpora lutea) were obtained from multiparous ewes at a local slaughterhouse (Departamento de San Jacinto, Canelones, Uruguay). The animals were crosses of Merino and Corriedale breeds, with no reported inbreeding, with ages from two to five years. There were 12 sample collections at the slaughterhouse, each using ovaries from 30-50 animals. Thus, approximately 960 ovaries were used in the entire experiment, obtained from an average of 480 ewes.

The experiments were performed according to the recommendations of the Internal Animal Care Committee of Fundación IRAUy (protocol # 003/2019), which is certified by the National Council of Animal Care of Uruguay. Unless otherwise mentioned, reagents and chemicals used in the present study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Mo, USA).

2.2 Isolation and selection of ovine cumulus-oocyte complex (COCs)

Ovaries obtained at the slaughterhouse were transported (at 35°C) within 2 h to the laboratory in saline solution (NaCl 0.9%) supplemented with antibiotics. Then, COCs were aspirated from antral follicles (2 to 6 mm) using 21-gauge needles and 5-mL syringes containing 0.5 mL collection medium with HEPES (25mM)-buffered Tissue Cultured Media 199 (TCM 199), 50 IU/mL penicillin, 50 μg/mL streptomycin, 0.1% fetal bovine serum and 5 IU/mL heparin. Only COCs tightly surrounded with three or more layers of cumulus cells and homogeneous ooplasm were selected for *in vitro* maturation. COCs were recovered, separated based on morphology (grade I and II; Dadashpour Davachi et al., 2012) and washed three times in PBS. Part of the COC samples were subjected to IVM (as described below) and other COC samples were stored in 1.5 mL tubes at -80°C (Menchaca et al., 2016, Menchaca et al., 2018).

2.3 *In vitro* maturation (IVM)

In vitro maturation of the sheep oocytes was conducted as previously described in detail (Menchaca et al., 2016, Menchaca et al., 2018). Briefly, selected COCs were washed three times in TCM 199 HEPES, FBS 20% (v/v), supplemented with 50 IU/mL penicillin and

50 µg/mL streptomycin. After each collection of ovaries at the abattoir, approximately five groups of 25-30 COCs were matured *in vitro* in 100µl drops of maturation medium under mineral oil in a humidified air atmosphere (5% CO_2) for 24 h at 39°C. Maturation medium consisted of TCM supplemented with 10% (v/v) estrous sheep serum (ESS), 100 µM cysteamine, 10 µg/mL FSH and 10 µg/ml LH. After IVM, cumulus cell complexes were analyzed using a stereomicroscope (Olympus SZ61, Tokyo, Japan) and oocytes that presented the first polar body were considered mature (at MII). Oocytes without polar bodies were not used for the next phase of the experiment.

2.4 Protein extraction from immature and mature COC and SDS-PAGE

Three pools of 100 immature and three pools of 100 *in vitro* matured COCs were used for protein extraction. In summary, COC pools were homogenized separately in individual tubes with 25 µL Laemmli reducing sample buffer (0.125 M trisaminomethane at pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% (v/v) glycerol, 0.2 M dithiothreitol, and 0.02% bromophenol blue), sonicated three times for 5 minutes in an ultrasonic bath and heated for 5 min at 95°C. Then, proteins (15 µg) from all COC samples were subjected to SDS-PAGE as described elsewhere (Martins et al., 2013). An initial current of 25 mA per gel was applied for 30 minutes to make proteins slowly migrate through the stacking gel. The gel was stained with Coomassie blue R-250 and band intensities were determined by densitometry using a low molecular weight protein calibration kit (Amersham^{TM}, GE Healthcare, USA), destained after several washes in a 50% ethanol and 12% acetic acid solution, as outlined before (Martins et al., 2013, Alves et al., 2018).

2.5 In-gel tryptic digestion and protein identification *by* mass spectrometry

Coomassie blue-stained bands were excised from 1-D gels, incubated at 30ºC with 50% acetonitrile (ACN) (J. T. Baker, USA) in 100 mM ammonium bicarbonate (AMBIC) (pH 8.0) until they became colorless. Protein disulfide bonds were reduced with 10 mM dithiothreitol for 1 h at 56°C under agitation, previous to Cys alkylation with 55 mM iodoacetamide for 45 minutes in the dark. Gel bands were dehydrated with ACN and reconstituted in 50 mM AMBIC containing sequencing grade trypsin (Promega, USA) to a final protease: protein ratio of 1:20 (w/w). In-gel digestion proceeded overnight at 37ºC and tryptic peptides were eluted by adding 60% ACN / 0.1% trifluoroacetic acid (TFA) in two

steps of 1h incubation. Samples were concentrated under vacuum in a CentriVap concentrator (Labcono, USA) using C18 ZipTips. Eluted peptides were finally resuspended with 0.1% formic acid (FA) in LC/MS quality water (LiChrosolv®, Merck KGaA, Germany), as previously described in detail (Arshid et al., 2017a, Arshid et al., 2017b).

Mass spectrometry data were acquired at the Analytical Biochemistry and Proteomics Unit of the Institut Pasteur de Montevideo, Uruguay. LC-MS/MS analysis was performed with an UltiMate 3000 HPLC system (Thermo Fisher Scientific, USA) coupled to a QExactive Plus with an Easy-Spray source (Thermo Fisher Scientific, USA), as reported before (Arshid et al., 2017a, Arshid et al., 2017b). Samples were loaded into a precolumn (Acclaim PepMap TM 100, C18, 75 µm X 2 cm, 3µm particle size) and separated with an Easy-Spray analytical column (PepMap TM RSLC, C18, 75 µm X 50 cm, 2 µm particle size) at 40°C using solvent (A), made with 0.1% FA in water, and solvent (B), containing 0.1% FA in ACN. The column was equilibrated in 1% buffer B followed by a gradient elution performed as follows: 1% to 50% B during 180 min, 50% to 99% B for 15 min, 99% B for 10 min and 1% A for 9 min, with a constant flow rate of 200 nL/min. The mass spectrometer was operated in positive mode and ion spray voltage was set at 1.7 kV; capillary temperature at 250°C and S-lens RF level at 50. A top-12 data-dependent method was used for MS data acquisition. Full MS scans were acquired in a range of 200-2000 m/z with a resolution of 70000 at 200 m/z, AGC target value of 1E6 and a maximum ion injection time of 100 ms. Precursor fragmentation occurred in an HCD cell with a resolution of 17500 at 200 m/z, AGC target value of 1E4 and a maximum ion injection time of 50 ms. Normalized collision energy was used in a stepped mode (NCE 25, 30 and 35). Precursor ions with single, unassigned or eight and higher charge states were excluded. A dynamic exclusion time was set to 30s. As we used three pools of 100 mature and three pools of 100 immature COCs, and extracted proteins from those pools were extracted and analyzed separately by LC-MS/MS, there were six distinct raw files generated by the mass spectrometer.

2.6 Protein identification and data analysis

PatternLab for Proteomics 4.0 was used to process each of the six raw data files (http://www.patternlabforproteomics.org; Carvalho et al., 2016). Identification of proteins was reported assuming false discovery rate (FDR) as less than 1%, at both peptide and protein levels. Differentially abundant proteins in immature versus mature COCs were analyzed by PatternLab's T Fold module, considering a fold-change cutoff of 1.8 ($p < 0.01$) and only

proteins identified in all three replicates for each condition (immature and mature COCs).

2.7 Bioinformatics

Proteins present in all replicates of immature (three replicates) and *in vitro* matured (three replicates) COC groups were analyzed using the Software for Researching Annotations of Proteins (STRAP), an open-source application (Bhatia et al., 2019). Gene ontology (GO) terms for biological process were obtained from UniProtKB database. For the assessments of protein-protein interactions, STRING version 11.5 database was used (http://string-db.org), based on predictions of direct (physical) or indirect (functional) associations, integrating evidence from the genomic context, experiments, co-expression and literature data (Viana et al., 2018, Bezerra et al., 2019).

Functional enrichment analysis was performed considering data obtained in gene ontology, KEGG and Reactome pathways. In addition, genes that code unique proteins found in immature COCs and proteins down-regulated after IVM were screened *in silico* for possible modulation by miRNAs. Using the miRNet 2.0 server (https://www.mirnet.ca/) (Chang et al., 2020), target genes were analyzed using *Bos taurus* database, since the search mechanism was not available for *Ovis aries*. A functional enrichment analysis was performed with miRNA-modulated genes using GENE2FUNC module implemented in the FUMA (Functional Mapping and Annotation of Genome-Wide Association Studies) platform (Watanabe et al., 2017). This analysis was focused on curated pathway databases (KEGG and Reactome) and GO (biological processes) based on the Molecular Signatures Database (MSigDB) (Liberzon et al., 2015).

3 RESULTS

Analysis performed by LC-MS/MS allowed the identification of 2,550 proteins in the samples of immature and *in vitro* matured COCs from adult ewes, with 89 proteins present only in immature COCs and 87 proteins exclusively detected in the group of *in vitro* matured COCs (Figure 2a). There were 648 proteins with different abundances ($p < 0.01$) in the two categories of cumulus-oocyte complexes, as determined by Pattern Lab's T Fold module (Figure 2b). *In vitro* maturation of ovine COC resulted in down-regulation of 84 proteins and up-regulation of 22 proteins, assuming a 1.8-fold change cutoff and 0.01 p-value. Down-regulated COC proteins with the highest significant fold-changes were identified as

immunoglobulin heavy constant mu (-22.98-fold change); inter-alpha-trypsin inhibitor heavy chain 2 (-22.47) and alpha-2-macroglobulin isoform X2 (-16.64). Up-regulated proteins with the highest fold-changes were listed as dopey N domain-containing protein (4.06-fold change); structural maintenance of chromosomes protein (3.61) and ubiquitin like modifier activating enzyme 2 (2.91), among others. The complete lists of all COC proteins, exclusive proteins, as well as differentially abundant proteins both in immature vs *in vitro* matured COCs, are available in Supporting Information File, Tables S1, S2, S3, S4, S5 and S6.

As determined by the STRAP® mapping tool, important changes happened in gene ontology terms associated with the COC proteins. *In vitro* maturation increased the number of proteins involved in interaction with cells and organisms, immune system process, developmental process and regulation of biological process (Figure 3a). *In vitro* maturation of COCs reduced the number of proteins with catalytic activity (Figure 3b) and decreased the predominance of cytoplasmic, nuclear and mitochondrial proteins as well (Figure 3c).

Two independent protein-protein networks were generated using String server. The first network was built with the proteins that were up-regulated after IVM and proteins exclusively found in *in vitro* matured COCs (Figure 4a). In this case, the networks related to immune response and regulation of the complement cascade, vesicle-mediated transport, cell cycle control and extracellular matrix organization. A different scenario was observed for the network built with proteins down-regulated after IVM and proteins exclusively found in immature COCs (Figure 4b). Such network was associated with metabolic processes (protein, nitrogen compounds, small molecules among others), immune response and regulation of the complement cascade.

Proteins downregulated after IVM and proteins found exclusively in mature COCs were subjected to *in silico* screening for putative miRNAs that could potentially regulate the translation of the target genes. From 127 genes that encoded the query proteins, 43 genes were used to build the miRNA-gene interaction network, which were linked to 105 miRNAs (Figure 5a). miRNAs with the highest number of interactions in the network were bta-mir-2360 and bta-mir-2449. The most prevalent KEGG attributed to those 43 genes were complement and coagulation cascades and metabolic pathways. GENE2FUNC functional enrichment analysis showed that miRNA-regulated genes are mainly involved in immune system modulation by complement activation, humoral response and B-cell mediated immunity (Figure 5b-d). The complete list of miRNAs, genes and interaction values are presented in the Supporting Information File, Table S7.

4 DISCUSSION

The present study describes a comprehensive characterization of the proteome of *Ovis aries* cumulus-oocyte complexes and uniquely demonstrates that this very proteome is remarkably altered when COCs are challenged with the conditions of *in vitro* maturation. To the best of our knowledge, the current list of 2,250 proteins is the largest atlas of COC proteins for the ovine species to date.

In vitro maturation decreased the abundance of COC proteins associated with the immune system, including proteins of the complement and coagulation cascades. Complement factors are important constituents of follicular fluid of different species (Jarkovska et al., 2010, Fahiminiya et al., 2011, Ferrazza et al., 2017) and recent studies revealed that *in vitro* matured bovine and equine COCs contain less complement proteins than *in vivo* matured COCs (Walter et al., 2020; Walter et al., 2019). We found six different complement C3 isoforms downregulated after *in vitro* maturation of COCs, in agreement with Walter and coworkers (2020) who described that *in vitro* matured bovine cumulus cells had lower levels of complement C3 in comparison to *in vivo* matured cells. Our *in-silico* analyses indicated that translation of complement factors C1QA, C1QB, C1QC, C2 and C6 are potentially regulated by specific miRNAs (bta-mir-2392, bta-mir-214, bta-mir-2360, bta-mir-6535). The participation of complement C3 in sperm-egg interaction was described nearly 30 years ago (Anderson et al., 1993) but the addition of C3, C3a and C3b to IVM media did not improve *in vitro* maturation of porcine COCs, fertilization or blastocyst formation (Georgiou et al., 2011). Interestingly, *in vivo* maturation of bovine COCs not only increased the production of C3 but also led to accumulation of C3 in the follicular fluid (Walter et al., 2020), suggesting that C3 functions are probably relevant for oocyte competence.

Complement cascade pathways share proteins and functions with the coagulation cascade, which is in turn involved with ovulation and oviduct inflammatory response (Severino et al., 2013). The expression of fibrinogen gene in bovine cumulus cells increases after the LH surge, suggesting that fibrinogen plays a role in triggering the final maturation of the oocyte (Assidi et al., 2010). In the present study, alpha and beta chains of fibrinogen were identified in the proteomes of both immature and *in vitro* matured COCs, without different abundances between the two groups. Nevertheless, other proteins such as plasminogen, coagulation factor II, thrombin and antithrombin-III were downregulated after *in vitro* maturation of the *Ovis aries* COCs, strengthening the hypothesis of a key role played by coagulation cascade pathways during oocyte maturation.

Like fibrinogen, fibroblast growth factor (FGF) was present in equal abundances before and after *in vitro* COC maturation, contrasting with results from previous transcriptome studies in beef cows (Melo et al., 2017, Moorey et al., 2021). According to these reports, *FGF11* gene had greater expression in bovine cumulus cells derived from larger follicles than from smaller follicles (Melo et al., 2017, Moorey et al., 2021). In addition, FGF17 stimulated bovine cumulus expansion and increased the number of cells in the embryoblast when added to media in association with BMP-15 (Machado et al., 2015). Therefore, it is reasonable to assume that FGF is associated with COC function but *in vitro* maturation does not cause FGF upregulation in the ovine species.

The expression of SERPINs was altered in the sheep COCs after *in vitro* maturation. SERPINs comprise a functionally diverse family of protease inhibitors (Wright et al., 1993), but may also work as hormone transporters and in other mechanisms (Heit et al., 2013). In our study, SERPIN E1 was more abundant in mature COCs and this type of molecule is involved in extracellular matrix organization. On the other hand, SERPIN E2, A1, C1, G1 and D1 were downregulated after IVM of sheep COCs, and these SERPINs are associated with inflammation, complement activation, coagulation, angiogenesis and fibrinolysis. *In silico* screening showed that SERPIN D1, E1 and G1 are potentially suppressed by specific miRNAs (bta-mir-2392, bta-mir- bta-mir-2433, bta-mir-2348 and bta-mir-2366). Studies indicate that SERPIN1/2 decreased the expansion of porcine COCs and SERPIN1/2 interfered with signaling pathways regulating the production of prostaglandin E2 (Blaha et al., 2019). In another study with human COCs, the depletion or elimination of SERPIN E2 expression had no effect on cumulus expansion and oocyte maturation. However, high SERPIN E2 was associated with downregulation of *Has2* and *Vcan* expression, decreasing matrix hyaluronan contents and suppressing cumulus expansion and oocyte maturation (Lu et al., 2013). These data indicate that reduced abundance of SERPINs after *in vitro* IVM could affect blastocyst formation in the ovine species.

In vitro maturation also resulted in overexpression of TNF alpha induced protein 6 (TNFAIP6) and thrombospondin 1 (THBS1) in the *Ovis aries* COCs. TNFAIP6 interacts with proteins that mediate immune response, with metalloproteinases and enzymes involved in extracellular matrix remodeling, metalloproteinase inhibition and cell proliferation. Furthermore, THBS1 interacts with proteins related to cell proliferation, and with inhibitors of plasminogen and cell adhesion. TNFAIP6 is a component of hyaladherin superfamily (Wisniewski & Vilček, 2004) and interacts with cells of the immune system, which are known to act in decidualization, implantation of the trophoblast, migration and embryonic growth

(Kobayashi et al., 2014, Martínez-Varea et al., 2014). An interaction between TNFAIP6 and metalloproteinases (MMPs) was detected in our *in silico* analysis and many of these MMPs are associated with angiogenesis, embryo implantation and embryogenesis (Nagase et al., 2006, Brew & Nagase, 2010, Amălinei et al., 2007, Hashizume, 2007). Earlier studies described that TNFAIP6 is synthesized in rodent granulosa and cumulus cells (Fülöp et al., 1997, Yoshioka et al., 2000, Carrette et al., 2001, Mukhopadhyay et al., 2001) and animals with null mutation (by homologous recombination) in the *TNFAIP6* gene have absence or low expansion of cumulus cells (Fülöp et al., 2003, Ochsner et al., 2003a, Ochsner et al., 2003b). *In vitro* studies revealed high expression of TNFAIP6 in bovine granulosa cells (Nagyova et al., 2009), granulosa and theca cells of mares (Mondal et al., 2011) and preovulatory porcine ovary follicles (Nagyova et al., 2009), supporting TNFAIP6´s importance in preovulatory events. Thrombospondin 1, in turn, is an antiangiogenic factor, being regulated by prostaglandin F2α in cattle and sheep (Mondal et al., 2011, Zalman et al., 2012), and it plays a role in luteolysis in the bovine species (Farberov & Meidan, 2014, Farberov & Meidan, 2018). Experimental data indicate that proteins such as LPIN2, TNFAIP6 and THBS1 effectively participate in the formation, growth and release of quality oocytes.

Finally, “JAK-STAT signaling after interleukin-12 stimulation” signaling pathway was upregulated in the ovine COCs subjected to *in vitro* maturation. Induction of JAK-STAT genes and interleukin complexes may regulate COC expansion and phosphorylation of kinase cascades in mouse cumulus cells (Liu et al., 2009). According to our analyses, there were 36 COC proteins linked to JAK-STAT and HSPA9 was one of the most abundant proteins of this group. HSPA9 is a member of the Hsp70 family (Luo et al., 2010) and participate in cell cycle regulation, protection from oxidative stress and transport of cytosolic proteins into the mitochondria, among other events (Dores-Silva et al., 2015, Olive & Cowan, 2018). In mice, HSPA9 was identified only in oocytes at the germinal vesicle stage (Yu et al., 2012) and the expression of granulosa cell HSPA9 in sheep may happen to protect the entire COC complex (Fu et al., 2014, Li et al., 2016).

5 CONCLUSION

The present research relied on mass spectrometry and tools of bioinformatics to portray a comprehensive map of the COC proteome in the ovine species. The list of 2,550 proteins reveals the complexity of molecules synthesized by the cells of a mammalian COC and we provide evidence that *in vitro* maturation has a pronounced impact on the protein

profile of cumulus-oocyte complexes from ewes (Figure 1). The roles of proteins involved in the complement cascade, immune system, coagulation and inflammation and how they contribute to the acquisition of oocyte competence will allow a deeper understanding of COC physiology. Knowledge of effects of IVM on metabolic pathways and cell signaling cascades of COCs will certainly contribute to the development of more efficient IVM protocols in the future.

Conflicts of interest

The authors declare no conflicts of interest.

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FIGURES AND TABLES

Figure. 1 Overview of the experimental design and major results of the study about the effects of *in vitro* maturation on the proteome of *Ovis aries* cumulus-oocyte complexes (COC). As shown, proteins from COCs were analyzed by LC-MS/MS and bioinformatics platforms. Up and down-regulated proteins and those exclusive to mature COCs were linked to intracellular signaling, immune response, regulation of complement cascade, vesicle mediated transport, cell cycle control and ECM organization, among other events. The main template of the figure was built using Biorender platform (https://biorender.com/).

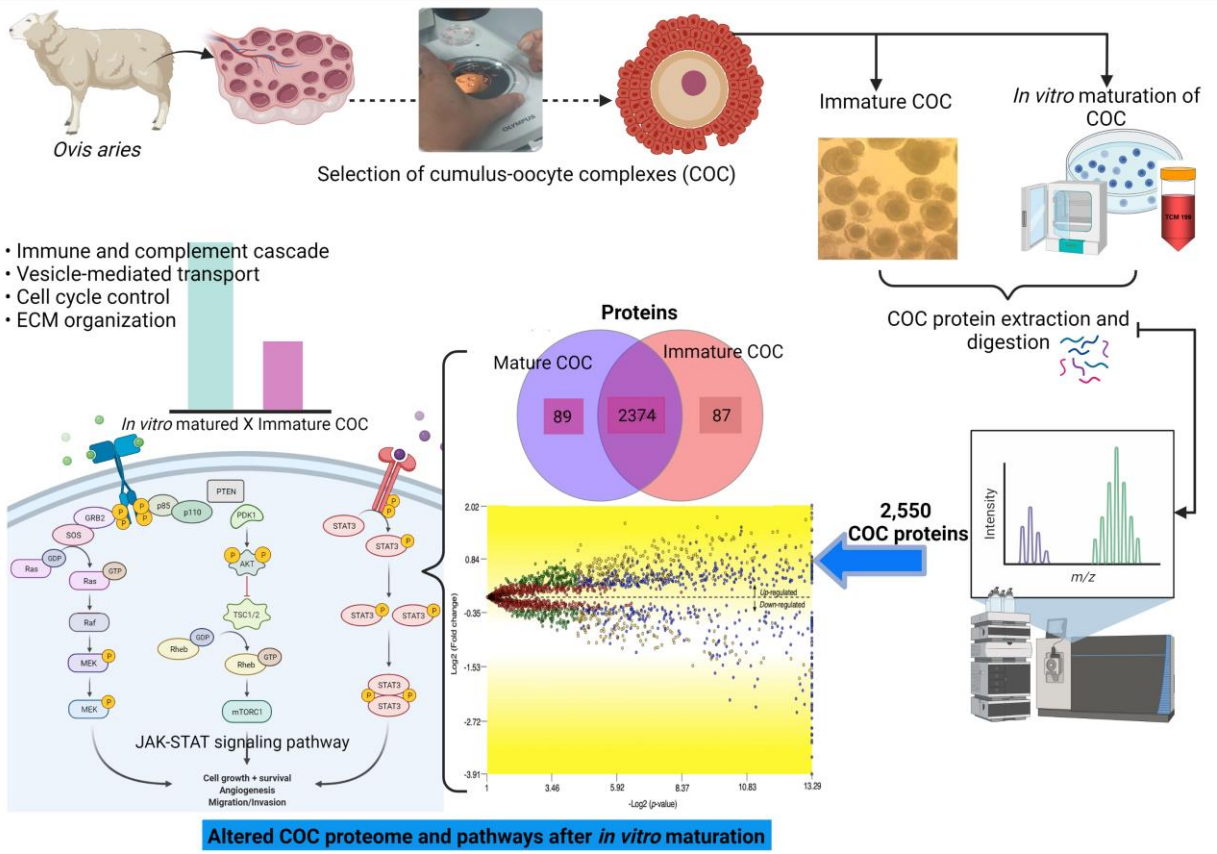


Figure. 2 Comparison of the proteomes of immature and *in vitro* matured ovine cumulus-oocyte complexes. (a) Venn diagram illustrating the distribution of the proteins in the experimental groups, as generated by InteractVenn (Heberle *et al.* 2015). (b) Vulcano plot of differentially expressed proteins generated by PatterLab. The five most abundant proteins found in immature COCs (log2 FC < -1.8) and in i*n vitro* matured COCs (log2 FC > 1.8) are indicated in tables S1 and S2, respectively.

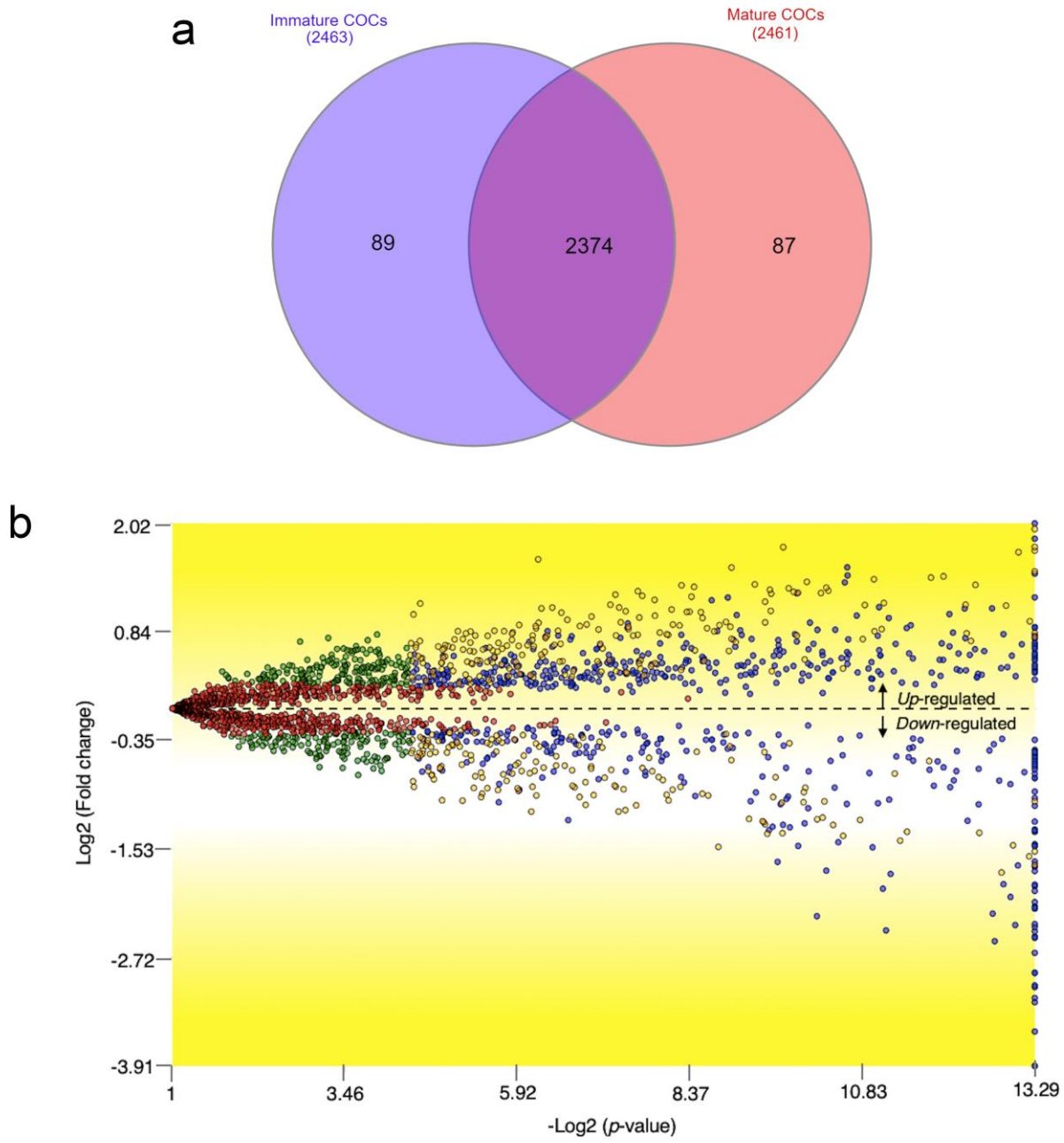


Figure. 3 Gene ontology annotations of proteins uniquely detected in the immature and *in vitro* matured ovine cumulus-oocyte complexes (COC), based on (a) biological process, (b) molecular function and (c) cellular component. Proteins identified by mass spectrometry were analyzed by STRAP software (version 1.5) and gene ontology terms were obtained from UniProtKB database.

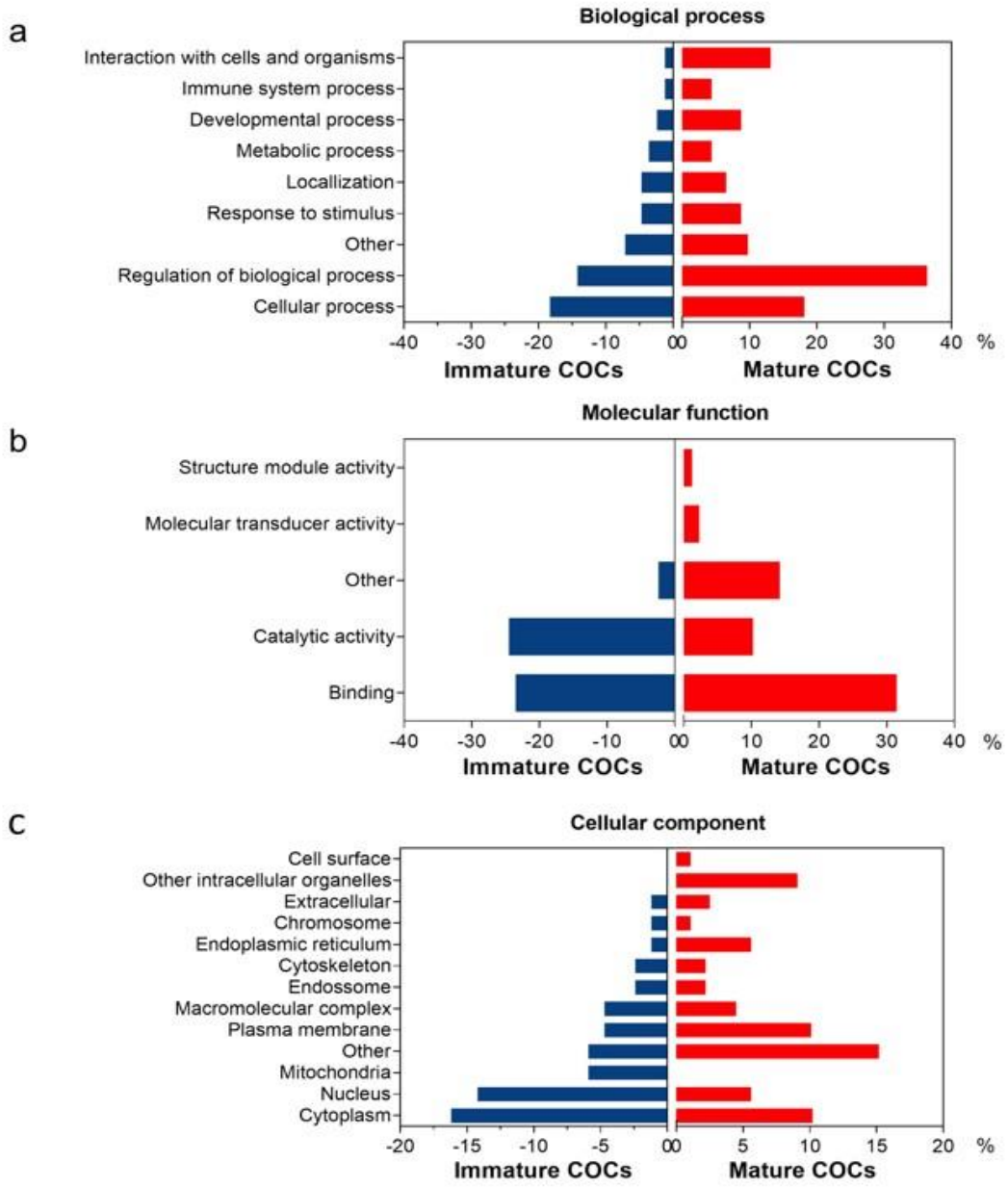


Figure. 4 *In silico* network analysis of proteins identified in immature and *in vitro* matured ovine cumulus-oocyte complexes. (a) Network built with unique and up regulated proteins after *in vitro* maturation. (b) Network built with unique proteins found in immature COCs and proteins down-regulated after *in vitro* maturation. Networks were analyzed using the STRING server (http://string-db.org). Interaction search was set at 0.7 confidence and function enrichment analyzes were performed considering GO terms, KEGG and Reactome pathways.

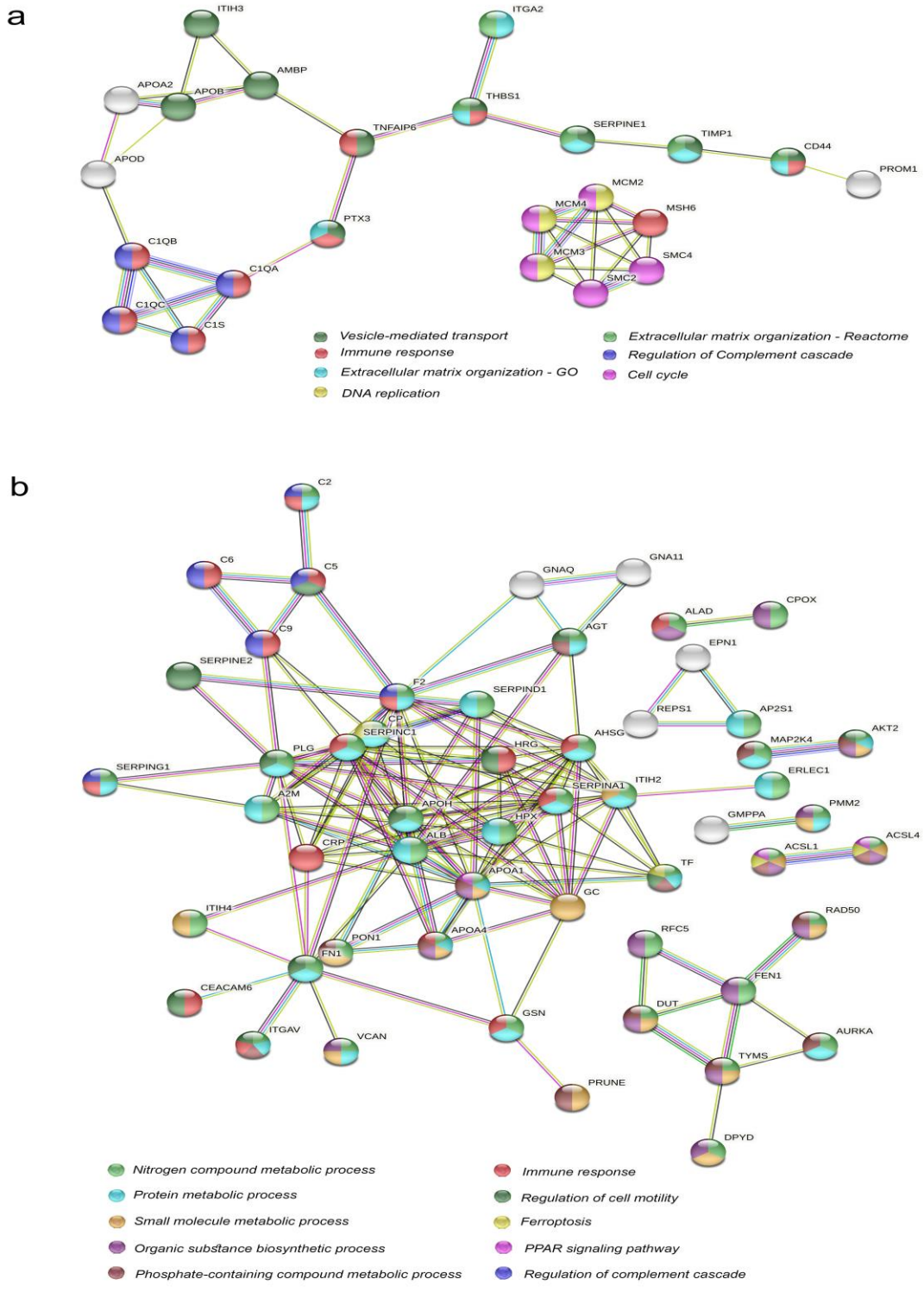
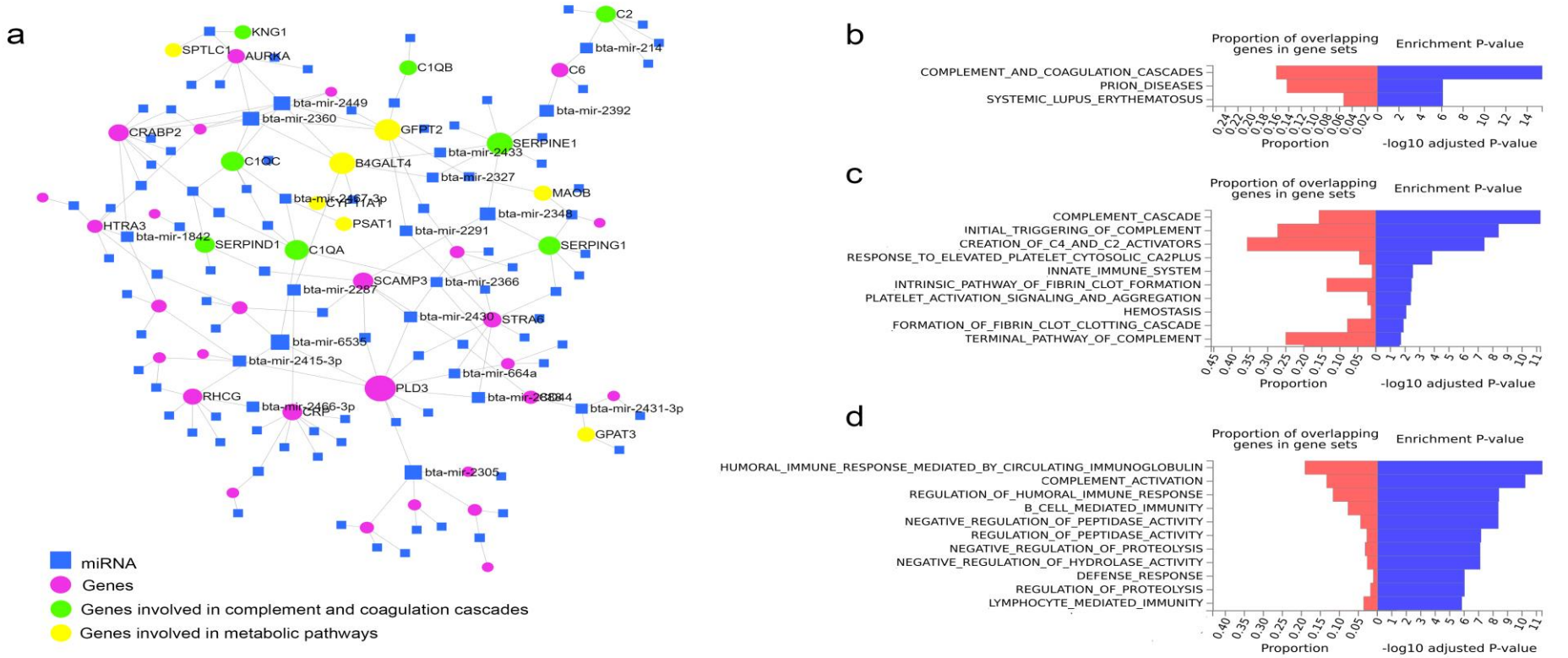


Figure 5. Network analysis and gene set enrichment analysis of miRNA associated with genes expressed only in immature and down-regulated after *in vitro* maturation of ovine cumulus-oocyte complexes. (a) miRNA-gene interaction network where miRNA (squares) are classified based on their biological processes. (b) KEGG pathways, (c) reactome and (d) gene ontology (biological process linked to genes used in the miRNA-gene interaction analysis). Schematic and analyses were obtained from miRNet server and functional enrichment analysis performed with FUMA server.



Supporting information

Table S1. List of all proteins identified in the ovine cumulus-oocyte complex proteome.

Table S2. List of the proteins detected only in immature ovine cumulus-oocyte complex.

Table S3. List of the proteins detected only in *in vitro* matured ovine cumulus-oocyte complex.

Table S4. List of the differentially abundant proteins in immature vs. *in vitro* matured ovine cumulus-oocyte complexes ($p < 0.01$; False Discovery Rate < 0.01). These proteins represent the blue dots in the volcano plot (Figure 2b).

Table S5. List of ovine cumulus-oocyte complex proteins up-regulated after *in vitro* maturation ($p < 0.01$; False Discovery Rate < 0.01).

Table S6. List of ovine cumulus-oocyte complex proteins down-regulated after *in vitro* maturation ($p < 0.01$; False Discovery Rate < 0.01).

Table S7. List of miRNAs and genes present in the miRNA-gene interaction network presented in Figure 4a, as generated by miRNET server.

Table S1. List of all proteins identified in the ovine cumulus-oocyte complex proteome.

Protein index	Protein acc. # (Uniprot)	Replicate count	Total signal	Protein description
2821	A2SW69	12	277	Annexin A2 OS=Ovis aries OX=9940 GN=ANXA2 PE=1 SV=1
1646	A6YRY8	12	149	40S ribosomal protein SA OS=Ovis aries OX=9940 GN=RPSA PE=2 SV=1
3722	A6ZE99	9	102	Perilipin OS=Ovis aries OX=9940 GN=ADFP PE=2 SV=1
3429	A7UHZ2	12	68	Proteasome 26S non-ATPase subunit 4 OS=Ovis aries OX=9940 GN=PSMD4 PE=2 SV=1
2031	A8D8X1	12	255	60S ribosomal protein L10 OS=Ovis aries OX=9940 GN=RPL10 PE=2 SV=1
10	A8DR93	12	1399	Heat shock protein 90 alpha family class A member 1 OS=Ovis aries OX=9940 GN=HSPCA PE=3 SV=1
3474	B0FYY4	12	161	Integrin beta-1 OS=Ovis aries OX=9940 GN=ITGB1 PE=2 SV=1
1514	B0FZL9	12	131	Pre-mRNA splicing factor SRP20-like protein OS=Ovis aries OX=9940 GN=SRSF3 PE=2 SV=1
976	B2LYK6	12	144	RAB7A, member RAS oncogene family OS=Ovis aries OX=9940 GN=RAB7A PE=2 SV=1
2960	B2LYL4	12	41	SNPRA OS=Ovis aries OX=9940 GN=SNRPA PE=2 SV=1
239	B2MVX2	12	136	SLC25A11 OS=Ovis aries OX=9940 GN=SLC25A11 PE=2 SV=1
3743	B6E1W2	12	124	Cyclin-dependent kinase 5 OS=Ovis aries OX=9940 GN=Cdk5 PE=2 SV=1
3588	B6UV59	12	344	Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha OS=Ovis aries OX=9940 GN=HADHA PE=2 SV=1
3814	B7TJ15	10	30	Mitogen-activated protein kinase OS=Ovis aries OX=9940 GN=MAPK14 PE=2 SV=1
2315	B7U175	12	106	Cell division cycle 2 protein isoform 1 OS=Ovis aries OX=9940 GN=CDC2 PE=2 SV=1
2125	B9VH02	12	112	Eukaryotic translation initiation factor 1A, X-linked OS=Ovis aries OX=9940 GN=EIF1AX PE=2 SV=1
2766	C0IZ95	8	17	RAB27A, member RAS oncogene family OS=Ovis aries OX=9940 GN=RAB27A PE=4 SV=1
315	C5IJ83	12	94	RAB8A, member RAS oncogene family OS=Ovis aries OX=9940 GN=RAB8A PE=2 SV=1
1132	C5IJ93	12	38	RAB9A, member RAS oncogene family OS=Ovis aries OX=9940 GN=RAB9A PE=2 SV=1
2816	C5IJ99	12	97	RHOA OS=Ovis aries OX=9940 GN=RHOA PE=2 SV=1
3229	C5ISA4	12	45	COP9 constitutive photomorphogenic subunit 7B protein-like protein OS=Ovis aries OX=9940 GN=COPS7B PE=2 SV=1

147	C5ISB1	12	135	Replication protein A subunit OS=Ovis aries OX=9940 GN=RPA1 PE=2 SV=1
311	C5IWT0	12	178	ADP-ribosylation factor 4 OS=Ovis aries OX=9940 GN=ARF4 PE=2 SV=1
200	C5IWU0	12	194	ADP-ribosylation factor 1 OS=Ovis aries OX=9940 GN=ARF1 PE=2 SV=1
3456	C5IWU4	12	54	ADP-ribosylation factor-like 3 OS=Ovis aries OX=9940 GN=ARL3 PE=2 SV=1
2094	C5IWV1	12	190	Fumarate hydratase OS=Ovis aries OX=9940 GN=FH PE=2 SV=1
143	C8BKC5	12	333	Peroxiredoxin 2 OS=Ovis aries OX=9940 GN=PRDX2 PE=2 SV=1
593	C8BKD0	6	18	Flap endonuclease 1 OS=Ovis aries OX=9940 GN=FEN1 PE=2 SV=1;
1333	C8BKD4	12	83	Chromobox 5 OS=Ovis aries OX=9940 GN=CBX5 PE=2 SV=1
3174	C8BKE1	12	58	Signal transducer and activator of transcription OS=Ovis aries OX=9940 GN=STAT1 PE=2 SV=1
1456	C9E8M7	12	98	Cytochrome b5 type A OS=Ovis aries OX=9940 GN=CYB5 PE=2 SV=1
1808	D8X187	11	89	Leukocyte elastase inhibitor OS=Ovis aries OX=9940 GN=SERPINB1 PE=2 SV=1
2456	J9UXY3	12	174	HSP10 OS=Ovis aries OX=9940 GN=HSP10 PE=3 SV=1
1667	W5NS44	12	136	hydroxysteroid 17-beta dehydrogenase 12(HSD17B12)
424	W5NS51	12	78	Protein transport protein SEC23 OS=Ovis aries OX=9940 GN=SEC23B PE=3 SV=1
693	O18751	12	54	Glycogen phosphorylase, muscle form OS=Ovis aries OX=9940 GN=PYGM PE=2 SV=3
2966	O62849	8	32	Acyl-CoA desaturase OS=Ovis aries OX=9940 GN=SCD PE=2 SV=1
3136	O78750	9	33	Cytochrome c oxidase subunit 2 OS=Ovis aries OX=9940 GN=MT-CO2 PE=1 SV=1
3524	O78752	12	24	ATP synthase subunit a OS=Ovis aries OX=9940 GN=MT-ATP6 PE=3 SV=1
3504	O78755	7	24	NADH-ubiquinone oxidoreductase chain 4 OS=Ovis aries OX=9940 GN=MT-ND4 PE=1 SV=1
2973	O78756	11	38	NADH-ubiquinone oxidoreductase chain 5 OS=Ovis aries OX=9940 GN=MT-ND5 PE=1 SV=1
3737	P02190	12	75	Myoglobin OS=Ovis aries OX=9940 GN=MB PE=1 SV=2
2075	P04074	12	387	Sodium/potassium-transporting ATPase subunit alpha-1 OS=Ovis aries OX=9940 GN=ATP1A1 PE=1 SV=1
2212	P05028	12	92	Sodium/potassium-transporting ATPase subunit beta-1 OS=Ovis aries OX=9940 GN=ATP1B1 PE=2 SV=1
2441	P09670	12	146	Superoxide dismutase [Cu-Zn] OS=Ovis aries OX=9940 GN=SOD1 PE=1 SV=2
2825	P12303	12	131	Transthyretin OS=Ovis aries OX=9940 GN=TTR PE=2 SV=1
4	P14639	12	2261	Serum albumin OS=Ovis aries OX=9940 GN=ALB PE=1 SV=1

2811	P32262	11	125	Antithrombin-III OS=Ovis aries OX=9940 GN=SERPINC1 PE=2 SV=1
2840	P50413	12	104	Thioredoxin OS=Ovis aries OX=9940 GN=TXN PE=3 SV=2
846	P52210	12	125	Fructose-bisphosphate aldolase B OS=Ovis aries OX=9940 GN=ALDOB PE=2 SV=2
858	P62262	12	370	14-3-3 protein epsilon OS=Ovis aries OX=9940 GN=YWHAE PE=1 SV=1
1244	P62896	12	74	Cytochrome c OS=Ovis aries OX=9940 GN=CYCS PE=1 SV=2
1927	P68240	8	87	Hemoglobin subunit alpha-1/2 OS=Ovis aries OX=9940 PE=1 SV=2
866	P68253	12	173	14-3-3 protein gamma (Fragments) OS=Ovis aries OX=9940 GN=YWHAG PE=1 SV=1
2198	P79202	10	105	Cholesterol side-chain cleavage enzyme, mitochondrial OS=Ovis aries OX=9940 GN=CYP11A1 PE=1 SV=1
2029	P81184	11	32	Galectin-1 OS=Ovis aries OX=9940 GN=LGALS1 PE=1 SV=2

Table S2. List of the proteins detected only in immature ovine cumulus-oocyte complex.

Protein acc. # (Uniprot)	Gene (UniProt) [a]	Replicate count	Total signal	Protein description
W5PIG7	ENO1	12	1145	Enolase 1
W5PN84	EEF2	12	760	Eukaryotic translation elongation factor 2
W5P663	ENO3	12	339	Enolase 3
W5PYM5	CCT8	12	338	Chaperonin containing TCP1 subunit 8
W5Q0L1	EEF1G	12	318	Eukaryotic translation elongation factor 1 gamma
W5NRL8	EIF3A	12	316	Eukaryotic translation initiation factor 3 subunit A
W5QGG6	CCT2	12	311	Chaperonin containing TCP1 subunit 2
W5PJX0	CCT6A	12	305	Chaperonin containing TCP1 subunit 6A
W5P5C0	ENO2	12	232	Enolase 2
W5PKK4	CCAR2	12	199	Cell cycle and apoptosis regulator 2
W5Q6E7	EIF2S3	12	143	Eukaryotic translation initiation factor 2 subunit gamma
W5P508	EIF3B	12	137	Eukaryotic translation initiation factor 3 subunit B
W5P610	CLIC1	12	125	Chloride intracellular channel protein
W5P8X9	CLIC4	12	114	Chloride intracellular channel protein
B9VH02	EIF1AX	12	112	Eukaryotic translation initiation factor 1A, X-linked
B7U175	CDC2	12	106	Cell division cycle 2 protein isoform 1
W5PKH2	CDC5L	12	105	Cell division cycle 5 like
P79202	CYP11A1	10	105	Cholesterol side-chain cleavage enzyme, mitochondrial
W5PK13	CBX3	12	100	Chromobox 3
W5QB95	EEF1B2	12	93	Eukaryotic translation elongation factor 1 beta 2
W5PFI4	CCT6B	12	90	Chaperonin containing TCP1 subunit 6B
W5PFK6	ECE1	12	90	Endothelin converting enzyme 1
W5Q9G8	ECI1	12	86	Enoyl-CoA delta isomerase
W5QJ50	EIF2S1	12	83	Eukaryotic translation initiation factor 2 subunit alpha
W5P5L6	PGS1	12	69	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase
W5Q9I0	CAPRIN1	12	68	Cell cycle associated protein 1

W5PGC1	CHMP4B	12	65	Charged multivesicular body protein 4B
W5P328	EIF2A	11	59	Eukaryotic translation initiation factor 2A
W5NZ39	(EDC4)	12	58	Enhancer of mRNA decapping 4
W5Q3Q7	CDC37	12	55	Cell division cycle 37
W5PHC4	EIF2S2	12	54	Eukaryotic translation initiation factor 2 subunit beta
W5P772	ECH1	12	52	Enoyl-CoA hydratase 1
W5P3T2	CHID1	12	51	Chitinase domain containing 1
W5QJ66	ERH	12	49	Enhancer of rudimentary homolog
W5PTS5	*N/A*	12	45	CDGSH iron sulfur domain 1
W5PCA5	CDK5RAP3	12	43	CDK5 regulatory subunit associated protein 3
W5Q313	CHDH	11	37	Choline dehydrogenase
W5Q7J3	EIF1	11	34	Eukaryotic translation initiation factor 1
W5QIN9	DUT	6	32	Deoxyuridine triphosphatase
W5NYY0	EIF2B1	12	32	Eukaryotic translation initiation factor 2B subunit alpha
W5PVL7	SIRT5	6	29	NAD-dependent protein deacylase sirtuin-5, mitochondrial
W5PK41	LPIN2	6	25	Lipin 2
W5NTH2	EIF2B3	8	25	Eukaryotic translation initiation factor 2B subunit gamma
W5PL69	BLMH	6	23	Bleomycin hydrolase
W5Q081	RAD50	5	23	RAD50 double strand break repair protein
W5QG77	CD58	9	23	CD58 molecule
W5NS21	ENOPH1	7	22	Enolase-phosphatase E1
W5PY95	*N/A*	6	21	AMH_N domain-containing protein
W5NWE0	MECR	8	21	Mitochondrial trans-2-enoyl-CoA reductase
W5PGA6	ELMO2	7	21	Engulfment and cell motility 2
W5Q050	CAMKK2	6	20	Protein kinase domain-containing protein
W5PN60	ABR	6	20	Active BCR-related
W5PC90	LOC101115083	5	20	Glutathione-dependent dehydroascorbate reductase
W5Q462	FN3KRP	6	19	Protein-ribulosamine 3-kinase
W5NZI1	RFC5	5	19	Replication factor C subunit 5
W5Q1D9	MAP2K4	6	18	Protein kinase domain-containing protein

C8BKD0	FEN1	6	18	Flap endonuclease 1
W5QDU5	RPAP3	6	18	RNA polymerase II associated protein 3 1
W5P981	AKT2	6	17	Non-specific serine/threonine protein kinase
W5Q747	CSTF3	5	17	Cleavage stimulation factor subunit 3
W5PTB7	ATG7	5	17	Autophagy related 7
W5NSD7	LOC101115543	5	17	Glutathione S-transferase omega-1-like
W5NSB6	EPN1	6	16	Epsin 1
W5PVK6	*N/A*	6	16	BH3 interacting domain death agonist
W5PMA0	AP2S1	6	16	AP complex subunit sigma
W5P931	TMEM30A	7	16	Cell cycle control protein
W5NQ72	PFAS	6	15	Phosphoribosylformylglycinamidine synthase
W5PR42	MTUS2	6	15	Microtubule associated scaffold protein 2
W5Q7Y9	DPYD	6	15	Dihydropyrimidine dehydrogenase [NADP (+)]
W5PHY3	TYMS	5	15	Thymidylate synthetase
W5PWK5	GGA1	6	14	Golgi associated, gamma adaptin ear containing, ARF binding protein 1
W5PEF4	MRI1	6	14	Methylthioribose-1-phosphate isomerase
W5QIC3	PRUNE1	5	14	Prune exopolyphosphatase 1
W5Q6R0	UBFD1	5	14	Ubiquitin family domain containing 1
W5QG46	GMPPA	5	14	GDP-mannose pyrophosphorylase A
W5NW58	HAX1	7	14	HCLS1-associated protein X-1
W5Q4U9	NUBP2	6	13	Nucleotide binding protein 2
W5PQG5	CAP2	6	13	Adenylyl cyclase-associated protein
W5Q0X2	FDFT1	5	13	Farnesyl-diphosphate farnesyltransferase 1
W5P8G0	ALAD	5	13	Delta-aminolevulinic acid dehydratase
W5PSI3	TIPRL	5	13	TOR signaling pathway regulator
W5PW31	GLDC	5	12	Glycine cleavage system P protein
W5NSG3	REPS1	5	12	RALBP1 associated Eps domain containing 1
W5PMD2	SEPT4	5	12	Septin-type G domain-containing protein
W5PK71	SUMF2	5	12	Sulfatase modifying factor 2
W5PQT2	TPRKB	5	12	TP53RK binding protein

W5P5K7	PMM2	5	11	Phosphomannomutase
W5P0K5	NUDT16L1	5	11	Nudix hydrolase 16 like 1
W5PB61	*N/A*	5	10	PITH domain containing 1

[a]Protein abbreviation is in accordance with gene abbreviation in UniProt.
N/A: notapplicable

Table S3. List of the proteins detected only in *in vitro* matured ovine cumulus-oocyte complex.

Protein acc. # (Uniprot)	Gene (UniProt) [a]	Replicate count	Total signal	Protein description
W5PGW9	TNFAIP6	6	155	TNF alpha induced protein 6
C5IWV1	FH	12	190	Fumarate hydratase
W5NQP9	ALDOC	12	349	Fructose-bisphosphate aldolase
W5NUV1	GNB1	12	111	G protein subunit beta 1
W5NVJ7	GDF3	12	102	growth differentiation factor 3
W5NW80	GAA	12	81	Glucosidase alpha, acid
W5NZ80	FAM129A	12	279	Family with sequence similarity 129 member A
W5P285	G6PD	12	230	Glucose-6-phosphate 1-dehydrogenase
W5P323	GPI	12	610	Glucose-6-phosphate isomerase
W5PH87	G3BP1	12	107	G3BP stress granule assembly factor 1
W5PKQ2	FAM213A	12	47	family with sequence similarity 213 member A
W5PPL5	GRPEL1	12	42	GrpE protein homolog
W5PS49	GNAQ	12	75	G protein subunit alpha q
W5PUP3	GDF9	12	101	Growth/differentiation factor 9
W5PXA6	FAHD2A	12	77	fumarylacetoacetate hydrolase domain containing 2A
W5Q1A2	*N/A*	12	184	GTP-binding nuclear protein Ran OS=Ovis aries OX=9940 PE=3 SV=1
W5Q260	G3BP2	12	60	G3BP stress granule assembly factor 2
W5Q302	GANAB	12	624	glucosidase II alpha subunit
W5Q438	GNB2	12	99	G protein subunit beta 2
W5QD75	GNAI3	12	137	G protein subunit alpha i3
W5QFW9	FAM162A	12	67	family with sequence similarity 162 member A
W5PAZ2	GSPT1	11	50	G1 to S phase transition 1
W5PLS7	GRB2	11	47	Growth factor receptor bound protein 2
W5PVD4	AK3	11	54	GTP:AMP phosphotransferase AK3, mitochondrial
W5Q0A8	GNPDA2	11	38	Glucosamine-6-phosphate isomerase
W5P6U1	GNB3	10	23	G protein subunit beta 3

W5Q963	FTO	10	37	FTO, alpha-ketoglutarate dependent dioxygenase
W5Q2W2	GTPBP1	9	24	GTP binding protein 1
W5Q4P9	FAM49A	9	39	family with sequence similarity 49 member A
W5P9W1	G6PC3	8	18	Glucose-6-phosphatase
W5PQR0	FAM129B	8	25	Family with sequence similarity 129 member B
W5QHP5	GNB4	8	19	G protein subunit beta 4
W5P0D0	FUNDC1)	7	14	FUN14 domain containing 1
W5PAA2	GABARAPL2	7	16	GABA type A receptor associated protein like 2
W5Q3L7	GREB1	7	34	Growth regulating estrogen receptor binding 1
Q6DKR0	SERPINA14	6	30	Uterine serpin
W5NQW4	LOC101104482	6	93	Alpha-1-macroglobulin-like
W5NSH2	ITIH3	6	150	Inter-alpha-trypsin inhibitor heavy chain 3
W5NUD6	A2ML1	6	19	Alpha-2-macroglobulin like 1
W5NV16	*N/A*	6	17	Ig-like domain-containing protein
W5NXM6	PTX3	6	80	Pentraxin 3
W5NZA9	MAOB	6	37	Amine oxidase
W5P0K3	MBNL1	6	15	Muscleblind like splicing regulator 1
W5P0Q4	LOC101102413	6	117	Haptoglobin
W5P2K6	MTDH	6	33	Metadherin
W5P3J3	C1S	6	29	Complement C1s
W5P3W5	HSD17B11	6	29	Hydroxysteroid 17-beta dehydrogenase 11
W5P812	AMBP	6	19	Alpha-1-microglobulin/bikunin precursor
W5PBY0	C4BPA	6	97	Complement component 4 binding protein alpha
W5PDH7	NPC1	6	23	NPC intracellular cholesterol transporter 1
W5PDP6	C1QB	6	25	Complement C1q B chain
W5PDQ9	C1QC	6	20	Complement C1q C chain
W5PDS4	C1QA	6	34	Complement C1q A chain
W5PFF9	*N/A*	6	50	Acyl-coenzyme A oxidase
W5PJ97	APOA2	6	23	Apolipoprotein A2
W5PP24	WLS	6	17	Wntless Wnt ligand secretion mediator

W5PPH1	RHCG	6	31	Rh family C glycoprotein
W5PPQ8	JCHAIN	6	20	Joining chain of multimeric IgA and IgM
W5PPY5	CD2AP	6	52	CD2 associated protein
W5PSV0	CDH6	6	37	Cadherin 6
W5PUG6	TIMP1	6	32	Metalloproteinase inhibitor 1
W5PUQ5	CLASP1	6	37	Cytoplasmic linker associated protein 1
W5PZF0	*N/A*	6	29	C-X-C motif chemokine
W5Q0Z0	SERPINE1	6	28	Serpin family E member 1
W5Q2E1	LUM	6	33	Lumican
W5Q349	KCTD12	6	31	Potassium channel tetramerization domain containing 12
W5Q3B6	PDE5A	6	90	Phosphodiesterase
W5Q7J0	APOB	6	66	Vitellogenin domain-containing protein
W5QDP8	FBLN1	6	88	Fibulin-1
W5QFP0	THBS1	6	356	Thrombospondin 1
W5QH45	KNG1	6	28	Kininogen 1
W5QIG4	*N/A*	6	17	BCL2 like 10 (BCL2L10)
W5NSC1	CSTF2	5	13	Cleavage stimulation factor subunit 2
W5NXJ3	LOC101112891	5	28	Complement C4-like
W5NYK7	GPAT3	5	14	Glycerol-3-phosphate acyltransferase 3
W5P1K6	SCAMP3	5	16	Secretory carrier-associated membrane protein
W5P895	CRABP2	5	14	Cellular retinoic acid binding protein 2
W5PEE6	ITGA2	5	18	Integrin subunit alpha 2
W5PG26	SAMD8	5	10	Sterile alpha motif domain containing 8
W5PH81	C7	5	15	Complement C7
W5PP81	MGAT5	5	24	Mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase
W5PS11	PSAT1	5	21	Phosphoserine aminotransferase
W5PTB1	HTRA3	5	15	HtrA serine peptidase 3
W5QA54	LOC101119629	5	10	Protein HP-25 homolog 2
W5QB87	SAMD9	5	21	Sterile alpha motif domain-containing protein 9

W5QBV7	CD44	5	14	CD44 antigen
W5QGP4	APOD	5	11	Apolipoprotein D

[a]Protein abbreviation is in accordance with gene abbreviation in UniProt.
N/A: not applicable

Table S4. List of the differentially abundant proteins in immature vs. *in vitro* matured ovine cumulus-oocyte complexes ($p < 0.01$; False

Discovery Rate < 0.01). These proteins represent the blue dots in the volcano plot (Figure 2b).

Protein acc. # (Uniprot)	Gene (UniProt)	Fold Change	(p-Value)	Signal in MATURE	Signal in IMMATURE	Protein description
W5PWT9	DOP1B	4,064955153	6,62E-05	8,15E-05	0,000331227	Protein dopey-2 OS=Ovis aries OX=9940 GN=DOPEY2 PE=4 SV=1
W5PBX7	SMC2	3,621833849	1,21E-05	7,41E-05	0,000268439	Structural maintenance of chromosomes protein OS=Ovis aries OX=9940 GN=SMC2 PE=3 SV=1
W5P353	UBA2	2,913032864	0,000633923	0,000140033	0,000407921	Ubiquitin like modifier activating enzyme 2 OS=Ovis aries OX=9940 GN=UBA2 PE=4 SV=1
W5PAN5	MCM2	2,778134713	1,69E-05	0,000173108	0,000480916	DNA replication licensing factor MCM7 OS=Ovis aries OX=9940 PE=3 SV=1
W5NVG4	SMC4	2,742397651	0,000623971	0,000129628	0,000355492	Structural maintenance of chromosomes protein OS=Ovis aries OX=9940 GN=SMC4 PE=3 SV=1
W5Q953	ERAP2	2,600696197	0,000649066	0,000147847	0,000384506	Aminopeptidase OS=Ovis aries OX=9940 GN=ERAP2 PE=3 SV=1
W5P181	NUDC	2,399682169	0,000830552	0,000107181	0,0002572	Nuclear migration protein nudC n OS=Ovis aries OX=9940 GN=NUDC PE=4 SV=1
W5PJU2	*N/A*	2,394296684	0,001070776	0,000111592	0,000267185	Aldo_ket_red domain-containing protein OS=Ovis aries OX=9940 PE=4 SV=1
W5PTC8	MCM4	2,321979733	1,00E-05	0,000260733	0,000605418	DNA helicase OS=Ovis aries OX=9940 GN=MCM4 PE=3 SV=1
W5QB02	TGM2	2,319182175	0,002107746	0,00015853	0,00036766	Transglutaminase 2 OS=Ovis aries OX=9940 GN=TGM2 PE=4 SV=1
W5P9G2	FKBP9	2,271237351	0,002404356	0,000144775	0,000328819	Peptidylprolyl isomerase OS=Ovis aries OX=9940 GN=FKBP9 PE=4 SV=1
W5PD47	HUWE1	2,155634316	0,00047294	0,000414007	0,000892449	HECT-type E3 ubiquitin transferase OS=Ovis aries OX=9940 GN=HUWE1 PE=4 SV=1

W5P9L8	TPR	2,13711066	0,000151457	0,000254337	0,000543546	Nucleoprotein TPR OS=Ovis aries OX=9940 GN=TPR PE=4 SV=1
W5P4Q3	MSH6	2,134212674	0,000192671	0,000157977	0,000337156	DNA mismatch repair protein OS=Ovis aries OX=9940 GN=MSH6 PE=3 SV=1
W5PPZ9	*N/A*	2,117177698	0,003680685	0,000118695	0,000251298	RMT2 domain-containing protein OS=Ovis aries OX=9940 PE=4 SV=1
W5PXK4	MCM3	2,045258049	0,000230329	0,000319989	0,000654459	DNA helicase OS=Ovis aries OX=9940 GN=MCM3 PE=3 SV=1
W5P2J9	*N/A*	2,030022976	0,0019022	0,000165919	0,000336819	Uncharacterized protein OS=Ovis aries OX=9940 PE=4 SV=1
W5PBY5	*N/A*	2,023373524	0,000585184	0,000150818	0,000305161	Ribonuclease inhibitor OS=Ovis aries OX=9940 PE=4 SV=1
W5Q4B7	RAP1GDS1	1,98601702	0,000421868	0,000142185	0,000282381	Uncharacterized protein OS=Ovis aries OX=9940 GN=RAP1GDS1 PE=4 SV=1
W5NS77	RNF213	1,915964205	0,000914517	0,000408482	0,000782637	Ring finger protein 213 OS=Ovis aries OX=9940 GN=RNF213 PE=4 SV=1
W5PJL1	PROM1	1,90466278	0,005549641	0,000188191	0,000358441	Prominin 1 OS=Ovis aries OX=9940 GN=PROM1 PE=4 SV=
W5P6F9	DPP3	1,879193415	0,000341545	0,000282773	0,000531385	Dipeptidyl peptidase 3 OS=Ovis aries OX=9940 GN=DPP3 PE=3 SV=1
W5QAL9	NPLOC4	1,868552251	0,008881871	0,000138083	0,000258015	NPL4 homolog, ubiquitin recognition factor OS=Ovis aries OX=9940 GN=NPLOC4 PE=4 SV=1
W5PWG1	PFKP	1,854528671	2,91E-05	0,000310228	0,000575326	6-phosphofructokinase OS=Ovis aries OX=9940 GN=PFKP PE=3 SV=1
W5QG98	PTGFRN	1,853251691	4,36E-05	0,000473859	0,000878179	Prostaglandin F2 receptor inhibitor OS=Ovis aries OX=9940 GN=PTGFRN PE=4 SV=1
W5Q6N9	AP3B1	1,843519754	4,22E-05	0,000327517	0,000603784	AP-3 complex subunit beta OS=Ovis aries OX=9940 GN=AP3B1 PE=3 SV=1
W5P564	MAP1B	1,84279084	0,005671694	0,000185323	0,000341511	Microtubule associated protein 1B OS=Ovis aries OX=9940 GN=MAP1B PE=4 SV=1
W5Q7T3	SEPT11	1,840387493	0,000359962	0,000271923	0,000500443	Septin OS=Ovis aries OX=9940 GN=SEPT11

						PE=3 SV=1

Table S5. List of ovine cumulus-oocyte complex proteins up-regulated after *in vitro* maturation ($p < 0.01$; False Discovery Rate < 0.01).

Protein acc. # (Uniprot)	Gene (UniProt)[a]	Fold Change	p-Value	Protein description
W5NXW9	IGHM	-2.298.551.634	1.00E-05	Immunoglobulin heavy constant mu
W5PW21	ITIH2	-2.247.592.548	1.00E-05	Inter-alpha-trypsin inhibitor heavy chain 2 (ITIH2)
W5NSA6	A2M	-1.664.602.945	1.00E-05	Alpha-2-macroglobulin (LOC101122940)
W5NTW3	ITIH1	-1.155.925.973	9.29E-05	Inter-alpha-trypsin inhibitor heavy chain 1 (ITIH1)
W5P1J8	LOC101113086	-1.081.527.757	0.000108179	Amine oxidase
W5NY46	PON1	-9.249.173.068	1.10E-05	Paraoxonase 1 (PON1)
A6ZE99	ADFP	-8.962.027.867	1.00E-05	Perilipin
W5NRG7	ITIH4	-8.255.267.671	1.00E-05	Inter-alpha-trypsin inhibitor heavy chain family member 4 (ITIH4)
W5PMR1	LOC101108797	-8.154.706.579	1.00E-05	GLOBIN domain-containing protein
W5QCY8	LOC101105437	-7.420.058.171	1.00E-05	Hemoglobin subunit beta (LOC101105437)
P68240	*N/A*	-7.399.564.928	1.00E-05	Hemoglobin subunit alpha-1/2
W5NWM2	APOA4	-7.354.717.251	4.78E-05	Apolipoprotein A4
W5NY95	C2	-6.368.231.262	1.00E-05	Complement C2 (C2)
W5Q981	ACSL4	-5.825.443.531	0.000146884	Acyl-CoA synthetase long-chain family member 4 (ACSL4)
W5P3R3	PLG	-5.684.413.931	1.16E-05	Plasminogen
W5P6F4	C5	-5.629.912.225	1.00E-05	Complement C5 (C5)
W5NRH2	*N/A*	-5.545.607.832	1.00E-05	Complement C3-like (LOC101103133)
W5PK78	VWA5A	-5.535.929.719	0.000253223	Von Willebrand factor A domain containing 5A (VWA5A)
W5PJQ9	NEFM	-5.401.940.165	1.00E-05	Neurofilament, medium polypeptide (NEFM)
W5PJZ1	SERPING1	-5.369.694.177	0.000430388	Serpin family G member 1
W5NYJ9	AGT	-5.236.376.533	1.00E-05	Angiotensinogen
W5QH50	HRG	-5.156.778.184	0.000121481	Histidine rich glycoprotein (HRG)
W5Q4Q3	SERPIND1	-5.118.867.019	1.78E-05	Serpin family D member 1
W5QEL0	B4GALT4	-4.822.325.981	0.00085154	Beta-1,4-galactosyltransferase 4 (B4GALT4)
W5PDE5	LOC101120001	-472.895.918	0.000149981	Zona pellucida sperm-binding protein 3 receptor-like (LOC101120001)
W5QDG8	FN1	-4.556.064.156	1.00E-05	Fibronectin 1 (FN1)
W5PGT6	C6	-4.541.128.135	4.90E-05	Complement C6 (C6)

W5NU00	*N/A*	-4.491.812.198	2.44E-05	Complement C4-A-like (LOC101123159)
W5NUX8	LOC101123672	-4.478.434.511	1.34E-05	Utrophin (UTRN)
W5Q233	VCAN	-4.335.073.918	1.00E-05	Versican (VCAN)
W5NUJ7	LOC101123419	-4.169.700.511	0.000125259	Complement C4 (LOC101123419)
D8X187	SERPINB1	-3.980.615.768	1.00E-05	Leukocyte elastase inhibitor
W5QAH3	AURKA	-3.914.953.045	0.000457119	Aurora kinase A (AURKA)
W5NXM1	*N/A*	-3.840.090.504	1.00E-05	Complement C3-like (LOC105605927)
W5NXP6	*N/A*	-3.798.036.589	5.78E-05	Complement C3-like (LOC101111946)
W5PTH1	GC	-3.783.902.991	0.000135231	GC, vitamin D binding protein (GC)
W5P1L7	F2	-3.502.298.444	0.000412571	Coagulation factor II, thrombin (F2)
P32262	SERPINC1	-342.374.916	9.18E-05	Antithrombin-III
W5PD71	CRP	-3.416.554.534	0.000780447	Pentaxin
W5QCZ9	LOC101106199	-3.318.394.603	1.00E-05	Hemoglobin fetal subunit beta (LOC101106199)
W5P627	GSN	-3.286.575.073	1.00E-05	Gelsolin (GSN)
W5P101	A1BG	-3.282.826.922	0.000115862	Alpha-1-B glycoprotein (A1BG)
W5PXU6	SERPINF1	-3.269.014.729	1.94E-05	Serpin family F member 1 (SERPINF1)
Q9XT27	CP	-3.195.513.107	0.001263184	Ceruloplasmin
W5P1Q9	STRA6	-3.188.212.062	2.99E-05	Stimulated by retinoic acid 6
W5PCL4	ACSL1	-306.434.109	0.000104462	Acyl-CoA synthetase long-chain family member 1 (ACSL1)
W5QAB1	HPX	-2.861.464.947	0.000492026	Hemopexin (HPX)
W5PS49	GNAQ	-2.840.466.146	1.00E-05	G protein subunit alpha q
W5P131	CYP11A1	-283.285.559	0.0010184	Cholesterol side-chain cleavage enzyme, mitochondrial
W5P5T4	LOC101108131	-2.822.931.787	1.00E-05	Complement C3 alpha chain
W5QGS5	SERPINE2	-2.793.697.366	1.00E-05	Serpin family E member 2 (SERPINE2)
P79202	CYP11A1	-2.755.174.949	0.000683894	Cholesterol side-chain cleavage enzyme, mitochondrial
W5NRI1	*N/A*	-2.546.752.197	1.00E-05	A2M_N_2 domain-containing protein
W5P8F9	*N/A*	-2.520.379.303	1.00E-05	BPI1 domain-containing protein
W5PH95	*N/A*	-2.511.223.844	0.001368097	Uncharacterized protein
W5NX51	APOA1	-2.509.873.752	4.77E-05	Apolipoprotein A1
W5NPK5	LOC443475	-2.500.464.477	1.00E-05	Complement C3 (LOC443475)

W5PDE3	SPTLC1	-2.460.378.833	0.001113683	Serine palmitoyltransferase long chain base subunit 1(SPTLC1)
W5Q124	LOC101119509	-2.448.907.958	0.00133111	Serpin A3-8 (LOC101119509)
W5PWE9	ALB	-2.413.514.596	0.001192513	Serum albumin
P14639	ALB	-2.385.970.638	0.000963241	Serum albumin
W5PSQ7	*N/A*	-2.354.466.505	5.78E-05	RuvB-like helicase
W5Q7I2	*N/A*	-2.341.055.514	0.000192852	Ig-like domain-containing protein
W5Q268	APOH	-2.328.230.514	0.009961055	Apolipoprotein H (APOH)
W5QC34	MAN2A1	-2.127.719.486	0.000929591	Alpha-mannosidase
W5PR52	CPNE3	-2.088.818.751	1.00E-05	Copine 3 (CPNE3)
W5PQJ5	AMACR	-2.065.900.384	0.000171617	Alpha-methylacyl-CoA racemase (AMACR)
W5PFP4	*N/A*	-2.063.912.159	0.001207353	DNA topoisomerase 1-like (LOC105604997)
W5PZS7	SERPINA1	-2.060.315.371	0.000635333	Serpin family A member 1 (SERPINA1)
W5NXP3	LOC101111083	-2.049.464.553	0.000471163	Serpin A3-5-like (LOC101111083)
W5PFC9	LOC101117129	-2.033.996.486	0.019842775	Inhibitor of carbonic anhydrase-like (LOC101117129)
W5QH56	AHSG	-2.018.251.446	0.001632078	Alpha-2-HS-glycoprotein
W5P9G8	PLD3	-1.993.484.158	0.000417188	Phospholipase D family member 3 (PLD3)
W5PHP7	*N/A*	-1.981.887.919	0.001154163	Serpin A3-7-like (LOC101117146)
W5QHZ5	*N/A*	-1.970.005.636	9.06E-05	Uncharacterized protein
W5PU75	GNA11	-1.954.787.903	0.00885122	G protein subunit alpha 11
W5Q0X5	LOC101115576	-1.947.803.911	0.001145271	Serpin A3-5 (LOC101115576)
W5P5J5	DDX21	-193.939.405	0.002815598	DExD-box helicase 21 (DDX21)
W5P1H7	ERLEC1	-1.905.441.929	0.00269495	Endoplasmic reticulum lectin 1
W5Q8F8	CPOX	-1.887.786.577	0.001549732	Coproporphyrinogen oxidase
W5NTN0	GFPT2	-1.874.801.655	7.39E-05	Glutamine-fructose-6-phosphate transaminase 2 (GFPT2)
W5PID9	C9	-1.850.067.684	0.016076861	Complement C9 (C9)
W5PF65	TF	-1.820.533.794	0.000715513	Transferrin
W5Q553	ITGAV	-1.813.130.243	0.000226025	Integrin subunit alpha V (ITGAV)

[a]Protein abbreviation is in accordance with gene abbreviation in UniProt.

N/A: not applicable

Table S6. List of ovine cumulus-oocyte complex proteins down-regulated after *in vitro* maturation ($p < 0.01$; False Discovery Rate < 0.01)

Name	Gene (UniProt)[a]	Fold Change	p-Value	Description
W5PWT9	DOP1B	4,064955153	6,62E-05	Protein dopey-2 OS=Ovis aries
W5PBX7	SMC2	3,621833849	1,21E-05	Structural maintenance of chromosomes protein
W5P353	UBA2	2,913032864	0,000633923	Ubiquitin like modifier activating enzyme 2 OS=Ovis aries
W5PAN5	MCM2	2,778134713	1,69E-05	DNA replication licensing factor MCM7 OS=Ovis aries
W5NVG4	SMC4	2,742397651	0,000623971	Structural maintenance of chromosomes protein
W5Q953	ERAP2	2,600696197	0,000649066	Aminopeptidase OS=Ovis aries
W5P181	NUDC	2,399682169	0,000830552	Nuclear migration protein nudC n OS=Ovis aries
W5PJU2	*N/A*	2,394296684	0,001070776	Aldo_ket_red domain-containing protein OS=Ovis aries
W5PTC8	MCM4	2,321979733	1,00E-05	DNA helicase OS=Ovis aries OX=9940
W5QB02	TGM2	2,319182175	0,002107746	Transglutaminase 2 OS=Ovis aries
W5P9G2	FKBP9	2,271237351	0,002404356	Peptidylprolyl isomerase OS=Ovis aries
W5PD47	HUWE1	2,155634316	0,00047294	HECT-type E3 ubiquitin transferase OS=Ovis aries
W5P9L8	TPR	2,13711066	0,000151457	Nucleoprotein TPR OS=Ovis aries
W5P4Q3	MSH6	2,134212674	0,000192671	DNA mismatch repair protein OS=Ovis aries
W5PPZ9	*N/A*	2,117177698	0,003680685	RMT2 domain-containing protein OS=Ovis aries
W5PXK4	MCM3	2,045258049	0,000230329	DNA helicase OS=Ovis aries
W5P2J9	*N/A*	2,030022976	0,0019022	Uncharacterized protein OS=Ovis aries
W5PBY5	*N/A*	2,023373524	0,000585184	Ribonuclease inhibitor OS=Ovis aries
W5Q4B7	RAP1GDS1	1,98601702	0,000421868	Uncharacterized protein OS=Ovis aries
W5NS77	RNF213	1,915964205	0,000914517	Ring finger protein 213 OS=Ovis aries
W5PJL1	PROM1	1,90466278	0,005549641	Prominin 1 OS=Ovis aries OX=9940
W5P6F9	DPP3	1,879193415	0,000341545	Dipeptidyl peptidase 3 OS=Ovis aries

aProtein abbreviation is in accordance with gene abbreviation in UniProt.
N/A: not applicable

Table S7. List of miRNAs and genes present in the miRNA-gene interaction network presented in Figure 4a, as generated by miRNET server

Id	Degree	Betweenness
CRABP2	10	1.535.060.979
PLD3	10	346.290.043
CRP	9	1.437.149.927
STRA6	8	1.207.946.206
GFPT2	8	178.623.662
SERPINE1	8	2.038.884.638
AURKA	7	9.981.428.571
C1QC	7	1.125.244.394
RHCG	7	1.342.957.497
bta-mir-2360	7	1.650.517.149
bta-mir-2449	7	1.650.517.149
HTRA3	6	7.132.472.435
SERPING1	6	908.707.164
SCAMP3	6	1.544.355.303
B4GALT4	6	186.856.438
C7	5	5.354.513.061
C2	5	578
CLASP1	5	6.852.139.727
C1QA	5	1.251.898.733
bta-mir-2305	5	1804
APOA4	4	2.104.170.332
C1S	4	3.482.549.867
AGT	4	435
bta-mir-2467-3p	4	4.865.003.521
SERPIND1	4	5.234.444.115
CSTF2	4	6.571.805.999
bta-mir-2415-3p	4	8.582.809.003
bta-mir-6535	4	2.091.633.747
APOA2	3	4.228.001.567
NPC1	3	2.233.306.704
MAOB	3	2.512.088.626
bta-mir-574	3	291
ITIH4	3	291
bta-mir-2410	3	3.035.847.866
bta-mir-2881	3	3.179.342.393
ACSL1	3	434
bta-mir-664a	3	4.440.842.631
bta-mir-2366	3	542.555.656
bta-mir-2431-3p	3	576
bta-mir-1842	3	6.946.931.476
CD44	3	7.469.217.702
bta-mir-2430	3	7.991.888.653
bta-mir-2888	3	8.366.121.577

C6	3	986
bta-mir-2348	3	1.406.795.899
bta-mir-2900	2	613.812.724
bta-mir-2897	2	1.460.614.395
bta-mir-671	2	4.601.068.931
bta-mir-2894	2	8.608.571.429
bta-mir-2455	2	146
bta-mir-149-3p	2	146
bta-mir-6523a	2	146
C1QB	2	146
CPOX	2	146
ITIH3	2	146
GPAT3	2	146
WLS	2	146
bta-mir-2439-5p	2	1.855.332.447
bta-mir-2887	2	1.955.003.521
bta-mir-2453	2	2.271.106.893
bta-mir-423-5p	2	2.290.873.196
bta-mir-6528	2	2.305.004.618
bta-mir-2454-3p	2	2.480.961.358
bta-mir-2324	2	2.816.459.212
bta-mir-2442	2	290
bta-mir-2461-5p	2	290
bta-mir-2389	2	3.080.275.215
bta-mir-3154	2	3.182.079.757
bta-mir-2382-5p	2	3.489.537.992
bta-mir-2454-5p	2	3.611.006.885
bta-mir-2320-5p	2	3.783.034.322
bta-mir-2327	2	4.866.006.608
bta-mir-2433	2	4.866.006.608
bta-mir-2291	2	7.096.653.117
bta-mir-214	2	710
bta-mir-2466-3p	2	7.109.023.078
bta-mir-2287	2	88.942.453
bta-mir-2392	2	1112
bta-mir-484	1	0
bta-mir-199a-5p	1	0
bta-mir-193a-3p	1	0
bta-mir-138	1	0
bta-mir-199b	1	0
bta-mir-423-3p	1	0
bta-mir-150	1	0
bta-let-7e	1	0
bta-mir-331-3p	1	0
bta-mir-497	1	0
bta-mir-185	1	0

bta-mir-193b	1	0
bta-mir-197	1	0
bta-mir-491	1	0
bta-mir-658	1	0
bta-mir-760-5p	1	0
bta-mir-763	1	0
bta-mir-874	1	0
bta-mir-940	1	0
bta-mir-1287	1	0
bta-mir-2292	1	0
bta-mir-2300a-5p	1	0
bta-mir-2302	1	0
bta-mir-2303	1	0
bta-mir-2306	1	0
bta-mir-1343-5p	1	0
bta-mir-2338	1	0
bta-mir-2342	1	0
bta-mir-2350	1	0
bta-mir-2353	1	0
bta-mir-2364	1	0
bta-mir-2373-5p	1	0
bta-mir-2374	1	0
bta-mir-2396	1	0
bta-mir-1584-5p	1	0
bta-mir-1584-3p	1	0
bta-mir-2412	1	0
bta-mir-2418	1	0
bta-mir-2422	1	0
bta-mir-2428	1	0
bta-mir-677	1	0
bta-mir-2441	1	0
bta-mir-2443	1	0
bta-mir-2447	1	0
bta-mir-1777a	1	0
bta-mir-2460	1	0
bta-mir-2466-5p	1	0
bta-mir-345-3p	1	0
bta-mir-2885	1	0
bta-mir-2889	1	0
bta-mir-2890	1	0
bta-mir-2902	1	0
bta-mir-409b	1	0
bta-mir-760-3p	1	0
bta-mir-3141	1	0
bta-mir-4286	1	0
bta-mir-6526	1	0

bta-mir-7862	1	0
bta-mir-7865	1	0
bta-mir-8550	1	0
bta-mir-4449	1	0
FN1	1	0
AMBP	1	0
LUM	1	0
CYP11A1	1	0
PSAT1	1	0
ITIH1	1	0
KNG1	1	0
SPTLC1	1	0
SERPINF1	1	0

7 ARTIGO II

Global proteomic analysis of pre-implantational ovine embryos produced *in-vitro*

Artigo submetido para publicação no periódico *Reproduction in Domestic Animals*
(Qualis B1 – Zootecnia e Recursos Pesqueiros)

Global proteomic analysis of pre-implantational ovine embryos produced *in-vitro*

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Abstract

The present study was conducted to characterize the major proteome of pre-implantation (D6) ovine embryos produced *in vitro*. COCs were aspirated from antral follicles (2-6 mm), matured and fertilized *in vitro*, and cultured until day six. Proteins were extracted separately from three pools of 45 embryos and separately run in SDS-PAGE. Proteins from each pool were individually subjected to in-gel digestion followed by LC-MS/MS. Three “raw. files” and protein lists were produced by Pattern Lab software but only proteins present in all three lists were used for the bioinformatics analyses. There were 2,262 proteins identified in the 6-day old ovine embryos, including albumin, zona pellucida glycoprotein 2, 3 and 4, peptidyl arginine deiminase 6, actin cytoplasmic 1, gamma-actin 1, pyruvate kinase, heat shock protein 90 and protein disulfide isomerase, among others. Major biological processes linked to the sheep embryo proteome were translation, protein transport and protein stabilization, and molecular functions, defined as ATP binding, oxygen carrier activity and oxygen binding. There were 42 enriched functional clusters according to the 2,147 genes (UniProt database). Ten selected clusters with potential association with embryo development included translation, structural constituent of ribosomes, ribosomes, nucleosomes, structural constituent

of the cytoskeleton, microtubule-based process, translation initiation factor activity, regulation of translational initiation, cell body and nucleotide biosynthetic process. The most representative KEEG pathways were ribosome, oxidative phosphorylation, glutathione metabolism, gap junction, mineral absorption, DNA replication and cGMP-PKG signaling pathway. Analyses of functional clusters clearly showed differences associated with the proteome of pre-implantation (D6) sheep embryos generated after *in vitro* fertilization in comparison with *in vivo* counterparts (Sanchez et al., 2021; DOI: 10.1111/rda.13897), confirming that the quality of *in vitro* derived blastocysts are unlike those produced *in vivo*. The present study portrays the first comprehensive overview of the proteome of pre-implantational ovine embryos grown *in vitro*.

Keywords: proteins, mass spectrometry, *in vitro* fertilization, ovine, embryo development, oocyte.

1 INTRODUCTION

Mammalian embryo development includes the formation of a zygote, activation of embryonic genome, cellular differentiation and the event of compaction, which starts five days after fertilization in the caprine, bovine and ovine species (Sakkas et al., 1989; Telford et al., 1990; van Soom et al., 1997; Schultz et al., 1999). Genome activation in the embryo is a complex process that requires well coordinated nuclear and cytoplasmic events to ensure that the two parental genomes are reprogrammed and restructured (Latham, 1999). As a result, the maternal and paternal transcripts that direct early development are replaced by transcripts from the own embryonic genome (Kaňka, 2003; Sharma, 2019).

Recently, advances in mass spectrometry and bioinformatics expanded the proteome databases related to male and female reproductive tissues and fluids. Studies have described the proteome of mouse embryos (Gao et al., 2017) and zygotes (Pfeiffer et al., 2011; Wang et al., 2010) and bovine embryos as well (Grealy et al., 1996). These data have provided vital information about the synthesis and molecular pathways of the developing embryo, that will serve, in the future, as potential markers for embryo selection and improvement of assisted reproductive technologies (Katz-Jaffe & McReynolds, 2013). Despite these promising results, the knowledge about the proteomic signatures of embryo development remains limited in the case of small ruminant species. Recently, our group described the first major proteomic profile of 6-day-old ovine embryos produced *in vivo*, reporting 667 proteins that were mainly related to functional clusters defined as energy

metabolism (TCA cycle, pyruvate and glycolysis metabolism), zona pellucida (ZP), MAPK signaling pathway, tight junction, ZP sperm binding, translation, proteasome and cell cycle, among others (Sanchez et al., 2021).

Although noteworthy research efforts have been spent over the last 30 years, *in vitro* embryo production is still unpredictable and variable in the ovine species, which imposes a significant limitation for its commercial application (Paramio & Izquierdo, 2014). These limitations may be related to the fact that embryos produced *in vitro* frequently present epigenetic alterations, mainly related to the microenvironment in which they develop (Menchaca et al., 2016; 2018). Changes in epigenetic regulation of gene expression could change the expression of proteins with key roles in embryo development. Information about proteins of *in vitro* produced embryos has not yet been reported for the ovine species, and proteomic analysis can potentially elucidate how in vitro production inflences the embryo proteome and if these changes could impart the embryo development. Therefore, this study aimed to characterize the major proteome of ovine pre-implantation (D6) embryos produced *in vitro*.

2 MATERIAL AND METHODS

2.1 Experimental design

In the present study, methods in mass spectrometry and bioinformatics were used to evaluate the major proteome of the 6-day old ovine embryo cultured *in vitro* (Figure 1). The study was carried out in Uruguay (34°S) during the early breeding season (December) and ovaries were recovered from adult, cycling multiparous crossbred ewes bearing one or two corpora lutea. Experiments were approved by the Internal Animal Care Committee of Fundación IRAUy (protocol # 003/2019), which is certified by the National Council of Animal Care of Uruguay. Unless mentioned otherwise, reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo, USA).

2.2 Selection of ovine cumulus-oocyte complex (COC)

Ovaries were collected from a local slaughterhouse and transported at 35°C within 2 h to the laboratory in saline solution with antibiotics. COCs were aspirated from antral follicles (2 to 6 mm) using a 21-gauge needle and 5-mL syringe containing 0.5 mL of HEPES

(25 mM)-buffered Tissue Cultured Media 199 (TCM 199) supplemented with 50 IU/mL Penicillin, 50 μg/mL Streptomycin, 0.1% fetal bovine serum and 5 IU/mL heparin. COCs tightly surrounded with three or more layers of cumulus cells and homogeneous ooplasm were selected for *in vitro* maturation. Grade I and II immature COCs were recovered (Dadashpour Davachi et al., 2012), washed in PBS and subjected to IVM, as described by Menchaca et al. (2016, 2018).

2.3 In vitro maturation (IVM)

In vitro maturation of ovine oocytes was carried as reported before (Menchaca et al., 2016; 2018). Briefly, selected COCs were washed in TCM 199 HEPES, fetal bovine serum 20% (v/v) supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin. Groups of 25-30 COCs were matured in 100μl-drops of maturation medium in mineral oil (humidified air atmosphere, 5% CO_2, 39°C, 24 h). Maturation medium contained TCM supplemented with 10% (v/v) estrous sheep serum (ESS), 100 μM cysteamine, 10 μg/mL FSH and 10 μg/mL LH. After IVM, cumulus cell complexes were analyzed using a stereomicroscope (Olympus SZ61, Tokyo, Japan) and the oocytes where the polar bodies were first observed were considered mature (at MII). Oocytes without polar bodies were discarded.

2.4 In vitro fertilization (IVF) and in vitro culture (IVC) of embryos

Matured oocytes were washed in fertilization medium with synthetic oviduct fluid (SOF), 10 μg/mL heparin, 1 μg/mL hypotaurine and 2% ESS (v/v). Motile spermatozoa from frozen-thawed semen were selected by swim up method (Menchaca et al., 2016). Then, $1x10^6$ spermatozoa were added to 100-μL drops of fertilization media containing 25-30 oocytes and covered with mineral oil. Fertilization was carried out during 24 h (5% CO_2, humidified atmosphere, 39°C).

Presumptive zygotes were denuded by gentle pipetting and washed in drops of development medium with SOF, bovine serum albumin (BSA) and bicarbonate buffer containing SOF supplemented with Basal Medium Eagle (BME) essential amino acids (2.5%), MEM nonessential amino acids (2.5%) and 0.4% BSA. Zygotes were transferred in groups of 25–30 to 100-μL culture droplets of development medium in mineral oil. Embryonic development was established in 5% CO_2, 5% O_2 and 90% N_2 in humidified atmosphere (39°C). The development medium was replaced on Day 3 by fresh media

(Menchaca et al., 2016). The percentage of cleaved embryos on Day 2 was recorded (2-8 cell embryos/total oocytes) and development rate on Day 6, defined by the number of morulae and blastocysts per total oocytes. At the end of the culture period (day 6), embryos were recovered, washed in PBS and stored at -80°C.

2.5 Protein extraction and one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

For protein extraction, three pools of 45 embryos were separately homogenized in individual tubes with 25 µL of Laemmli reducing sample buffer (0.125 M trisaminomethane at pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% (v/v) glycerol, 0.2 M dithiothreitol and 0.02% bromophenol blue), sonicated three times for 5 min in ultrasonic bath and heated for 5 min (95°C). An initial current of 25 mA per SDS-PAGE gel was applied for 30 minutes to make proteins migrate through the stacking gel. The gel was stained with Coomassie blue R-250, destained in 50% ethanol/12% acetic acid (Alves et al., 2018).

2.6 In-gel tryptic digestion *and protein identification* by *mass spectrometry*

Bands containing material of the three pools of embryos were individually excised from 1-D gels, incubated at 30ºC with 50% acetonitrile (ACN) (J.T. Baker, USA) in 100 mM ammonium bicarbonate (AMBIC) (pH 8.0) until they became colorless. Protein disulfide bonds reduction was performed with 10 mM dithiothreitol for 1 h at 56°C under agitation, previous to Cys alkylation with 55 mM iodoacetamide for 45 minutes in the dark. Gel bands were dehydrated with ACN and reconstituted in 50 mM AMBIC containing trypsin (Promega, USA) to a 1:20 (w/w) final protease:protein ratio. In-gel digestion proceeded overnight (37ºC) and tryptic peptides were eluted *with* 60% ACN/0.1% trifluoroacetic acid (TFA) in two steps of 1-h incubation. Samples were concentrated in a CentriVap concentrator (Labcono, USA) using C18 ZipTips. Eluted peptides were finally resuspended with 0.1% formic acid in LC/MS quality water (LiChrosolv®, Merck KGaA, Germany), as previously described (Arshid et al., 2017a; 2017b).

Mass spectrometry data were acquired at the Analytical Biochemistry and Proteomics Unit of the Institute Pasteur de Montevideo, Uruguay. LC-MS/MS analysis was performed with an UltiMate 3000 HPLC system coupled to a QExactive Plus with an Easy-Spray source (Thermo Fisher Scientific, USA), as reported before (Arshid et al., 2017a;

2017b). Digested peptides were loaded into a precolumn (Acclaim PepMap TM 100, C18, 75 µm X 2 cm, 3-µm particle size) and separated with an Easy-Spray analytical column (PepMap TM RSLC, C18, 75 µm X 50 cm, 2-µm particle size) at 40°C using a two solvent system: (A) 0.1% FA in water, (B) 0.1% FA in acetonitrile. The column was equilibrated at 1% buffer B followed by a gradient elution performed as follows: 1% to 50% B over 180 min, 50% to 99% B over 15 min, 99% B for 10 min and 1% A for 9 min, with a constant flow rate of 200 nL/min. The mass spectrometer was operated in a positive mode. Ion spray voltage was set at 1.7 kV; the capillary temperature at 250°C and S-lens RF level at 50. A top-12 data-dependent method was used for MS data acquisition. Full MS scans were acquired in a range of 200-2,000 m/z with a resolution of 70,000 at 200 m/z, AGC target value of 1E6 and a maximum ion injection time of 100 ms. Precursor fragmentation occurred in an HCD cell with a resolution of 17,500 at 200 m/z, AGC target value of 1E4 and a maximum ion injection time of 50 ms. Normalized collision energy was used in a stepped mode (NCE 25, 30 and 35). Precursor ions with single, unassigned or eight and higher charge states were excluded. A dynamic exclusion time was set to 30s.

2.7 Computational analysis

Data were generated from the mass spectrometer for the three individual pools of embryos, each formed by 45 embryos. Thus, there were three distinct “raw files” individually analyzed using PatternLab v. 4.0 (http://www.patternlabforproteomics.org; Carvalho et al., 2016) and *Ovis aries* target reverse database (http://www.uniprot.org/; 18/11/2019). Three protein lists were produced by Pattern Lab software, representing the three 45-embryo pools. Only proteins present in all three lists were used for the bioinformatics analyses. For peptide identification, m/z precursor tolerance was set at 40 ppm; methionine oxidation and cysteine carbamidomethylation were defined as variable and fixed modifications respectively. A maximum of 2 missed cleavages and 2 variable modifications per peptide were allowed, setting FDR ≤ 1% (at protein level).

2.8 Gene ontology and cluster analysis

Proteome data were analyzed using the software for functional enrichment analysis (FunRich version 3.1.4) and UniProtKB database to define gene ontology terms and biological processes (Pathan et al., 2015). Functional clusters associated with ovine embryo

proeome were analyzed through DAVID platform (https://david.ncifcrf.gov/). For this analysis, UniProt accession numbers were uploaded at DAVID platform, and clusters were defined according to enrichment scores and p-values (Huang et al., 2009). This tool was also employed to perform KEEG pathways.

STRING (http://string-db.org) version 11.0 platform was used for *in silico* analysis based on predictions collected based on direct (physical) or indirect (functional) associations, integrating evidence from the genomic context, co-expression and literature data (Viana et al., 2018). Genes defined with gene ontology terms were screened for potential modulation by miRNAs, using *Homo sapiens* dataset of mIRBase (http://www.mirbase.org); (Kozomara & Griffiths-Jones, 2011), once the search mechanisms were not available for *Ovis aries* miRNA data. To obtain the interaction between miRNAs and genes, data were submitted to miRNet 2.0 server (https://www.mirnet.ca/) (Chang et al., 2020, Sanchez et al., 2021).

3 RESULTS

There were 2,262 proteins identified in the 6-day old ovine embryos produced *in vitro*, with 2,147 proteins characterized according to the UniProt database and 115 remaining as uncharacterized (Supplementary Table 1). The 30 most abundant proteins detected in the ovine embryos are listed in Table 1 and included serum albumin (ALB), zona pellucida glycoprotein 2 (ZP2), 3 (ZP3) and 4 (ZP4), peptidyl arginine deiminase 6 (PADI6), actin cytoplasmic 1 (ACTB), gamma-actin 1 (ACTG1), pyruvate kinase (PMK), heat shock protein 90 alpha family class A member 1 (HSPCA) and protein disulfide isomerase, among others.
Major biological processes linked to the sheep embryo proteome were translation (5.2%), protein transport (3.8%) and protein stabilization (3.6%, Figure 2A). Cellular components were mainly defined as nucleus (18.2%), cytosol (16.7%) and plasma membrane (11.7%, Figure 2B) and molecular functions, as ATP binding (12.3%), oxygen carrier activity (8.0%) and oxygen binding (8.0%; Figure 2C). There were 42 enriched functional clusters according to the 2,147 genes selected in the UniProt database (Supplementary Table 2). Ten selected clusters with potential association with embryo development were listed as translation, structural constituent of ribosomes, ribosomes, nucleosomes, structural constituent of the cytoskeleton, microtubule-based process, translation initiation factor activity, regulation of translational initiation, cell body and nucleotide biosynthetic process (Table 2). The most representative KEEG pathways were ribosome, oxidative phosphorylation, glutathione metabolism, gap junction, mineral absorption, DNA replication and cGMP-PKG signaling

pathway (Table 3).

Based on the analysis carried out using miRNet server, twenty-six miRNAs were related to PKM, ACTB, ALB and ZP3 four genes, all conserved among mammalians and described in the human dataset. The interaction network (Figure 3) presented key miRNAs linked to hematopoiesis, cell cycle, angiogenesis, cell proliferation, cell death, and others. Functional analysis of the network indicated that 17 miRNAs were involved in the cell cycle pathway, 14 miRNAs in hematopoiesis, 15 in angiogenesis, 13 in cell proliferation, and 17 miRNAs involved in cell death. The complete list of miRNAs and the respective pathways is available in Supplementary Table 3.

In silico analysis of protein-protein interactions linked to selected terms indicated a highly clustered network (clustering coefficient: 0.80), containing 45 nodes with 277 edges (Figure 4). In general, the *in silico* analysis showed high confidence among proteins in the same cluster (ribosomes and translation, translation initiation factor, structural components, nucleosome, cell body, nucleotide biosynthetic processes, and others).

4 DISCUSSION

The present work reports a global proteomic profile of ovine embryos at the pre-implantation phase (Day 6) after *in vitro* fertilization. The identification of 2,262 proteins represents a major contribution for the understanding of embryo physiology at a time point proceeding hatching and implantation. Biological processes of proteins from *in vitro* cultured sheep embryos were mainly related to cell regulation processes, as previously demonstrated in human (Jensen et al., 2013), bovine (Jensen et al., 2014a, 2014b) and ovine (Sanchez et al., 2021) embryos and consistent with the catabolic and anabolic demands during the cavitation and compaction of the embryos. Regarding the molecular function, 28.3% of the sheep embryo proteins were primarily assigned to the binding category. In fact, binding proteins play an important role in transcription, translation and gene regulation in preimplantation embryos (Kwon et al., 2013).

Based on DAVID annotation, the first enriched cluster was represented by terms related to translation, followed by ribosomes and their structural components. It is well known that proper ribosome functionality ensures the development of multicellular organisms and the eukaryotic ribosomes consist of four ribosomal RNAs and 79 ribosomal proteins (Fatica & Tollervey, 2002). Protein synthesis, translation and ribosome biogenesis are energy-consuming and essential for cellular growth, proliferation and differentiation in all organisms

(Peshkin et al., 2015). Studies conducted by Kwon et al. (2018) reported that proteins of zebrafish embryos were associated with ribosomal cellular components and a large activity of translation biological processes, data that support our study about the proteome of ovine embryos. The correct function of all processes related to protein synthesis are required for proper early embryo development. Thus, dysfunctional ribosomal proteins could affect protein synthesis, leading to developmental disorders, altered tissue-specific phenotypes, ribosomopathies and cancer (Panić et al., 2006; Wang et al., 2015). Enriched clusters of sheep embryo proteins were also associated with nucleosomes and the cell body. Nucleosome organization regulates gene activity once it is positioned at transcription start sites (Lee et al., 2007) and studies show that gene packing in human and mouse sperm encodes many embryonic regulators by nucleosomes with histone modifications (Hammoud et al., 2009; Brykczynska et al., 2010). In biological processes, the nucleotide biosynthetic process was also highlighted, important for pathways involved in nucleotide formation.

Proteins grouped in KEEG pathways were also analyzed in the present study. Significant pathways associated with ribosomes, oxidative phosphorylation, glutathione metabolism and others indicate the relevance of ribosomal proteins for embryo development at a transcriptional and translational level. Different from the present data obtained with *in vitro* produced embryos, our previous study showed that the main pathways associated with *in vivo* produced sheep embryos were related to energy metabolism (TCA cycle, pyruvate, and glycolysis metabolism), zona pellucid, MAPK signaling and others (Sanchez et al., 2021). Such differences may relate to *in vitro* conditions that certainly affect embryo development and *in vitro* maintained oocytes and embryos do not have contact with the female reproductive tract and its secretions, which help to define their growth and metabolism (Lonergan et al., 2016; Paramio & Izquierdo, 2014). Significant pathways detected in the present study were linked to metabolic processes, such as oxidative phosphorylation (Cheng & Ristow, 2013) and glutathione metabolism, which is part of a defense mechanism against reactive oxygen species (ROS) and electrophiles (Pompella et al., 2003). In fact, Barros et al. (2019) identified higher levels of glutathione and ROS in large follicles in comparison with smaller follicles of the ovine species. As expected, increases in glutathione and ROS follow the development of the oocytes, their metabolic rate, and demand for more energy. In addition, pathways involved in cellular components such as gap junctions indicate great activity and high responsiveness of cells to stimulus-response, cell adhesion, differentiation and proliferation (Paes et al., 2020).

In silico analysis identified protein-protein clusters such as ribosomes and translation, translation initiation factor, structural components, nucleosome, cell body,

nucleotide biosynthetic processes, among others. The most interacting cluster is ribosomes and translation, thus demonstrating the importance of mRNA translation for development (Norris et al, 2021). During *in vitro* culture, important events such as the maternal-zygotic transition (MZT) and the small and large waves of embryo genome activation (EGA) take place (Vastenhouw et al., 2019). Studies using α-amanitin and incorporation of [^{3}H] uridine helped scientists to establish that EGA starts in 8-16 cell bovine and ovine embryos (Telford et al., 1990), in 2-4 cell buffalo embryos (Kumar et al., 2012) and when swine embryos have 4-8 cells (Memili & First, 2000).

Twenty-six miRNAs were potentially linked to genes identified in our study about the proteome of 6-day old sheep embryos. These miRNAs were interconnected with cell cycle control, cell proliferation and differentiation, hematopoiesis, angiogenesis and cell death. As well known, miRNAs play regulatory roles in animal embryo development (Chen et al., 2005) and are differentially expressed in hematopoietic organs during embryogenesis and hematopoietic lineage differentiation (Chen et al., 2005). The most significant pathways defined in the present experiment and those reported by Sanchez et al. (2021) when studied *in vitro* produced ovine embryos included cell proliferation, embryonic development, embryonic stem cell differentiation, and regulation of stem cells. Studies also suggest that miRNAs restrain cell proliferation and promote cell differentiation, presenting growth suppressive functions that induce apoptosis and cell-cycle arrest *in vitro* (Raver-Shapira et al., 2007). Embryo-maternal recognition and crosstalk are crucial events, and miRNAs alter hormone actions during implantation (Gross et al., 2017). In this regard, estrogen, progesterone and human chorionic gonadotropin receptors are modulated by miRNAs at a transcriptional and translational level (Yang & Wang, 2011) and studies conducted by Torley et al. (2011) show that miRNAs regulate genes implicated in estrogen synthesis during fetal gonad development in the ovine species.

Analyses based on functional clusters clearly show the existence of differences associated with the proteome of pre-implantation (D6) sheep embryos generated after *in vitro* fertilization in comparison with that of *in vivo* counterparts previously shown in our previous work Sanchez et al. (2021) (Table 4). In fact, such differences corroborate with experimental data as authors have previously shown that *in vitro* conditions affect the quality of the embryo and consequently its implantation potential, resulting in fragile zona pellucida (Cannon & Menino Jr., 1998), higher chromosomal abnormality (Slimane et al., 2000), reduction in total number of cells (Casillas et al., 2018) and lipid accumulation in the cytoplasm of the embryos (De Andrade Melo-Sterza & Poehland, 2021). Thus, the quality of *in vitro* derived blastocysts

are unlike those produced *in vivo* (Fazleabas et al., 2004) and the scenario depicted by the functional clusters in our study confirms this condition.

In conclusion, the present study portrays a comprehensive overview of the proteome of pre-implantational (day 6) *in vitro* produced ovine embryos. Functional clusters associated with the proteome of *in vitro* grown embryos relate to translation, ribosomes and their structural components, and ribosomal proteins. Elucidation of the proteome of developing embryos reveals crucial aspects of their metabolism, signaling pathways and physiology.

Conflicts of interest

The authors declare no conflicts of interest.

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FIGURES AND TABLES

Figure 1. Overview of the experimental design and major results of the study about the proteome of pre-implantation, 6-day old ovine blastocysts produced *in vitro*. COCs were aspirated from antral follicles, matured and fertilized *in vitro*. Presumptive zygotes were cultured for 6 days and embryo proteins were analyzed by LC-MS/MS. There were 2,262 proteins identified in the *in vitro* derived embryos and respective functional clusters were associated with translation, ribosomes, and their structural components, and ribosomal proteins, among others. The main template of the figure was built using Biorender platform (https://biorender.com/).

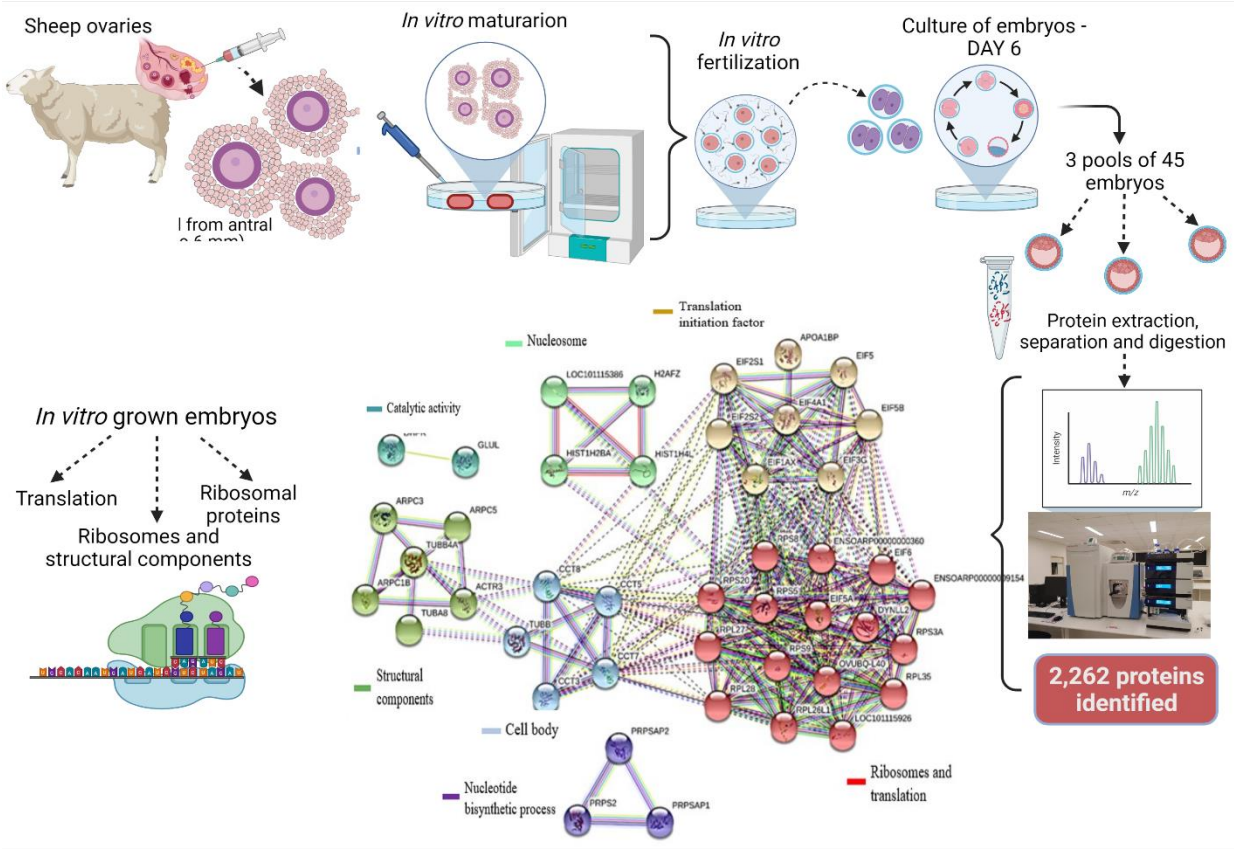
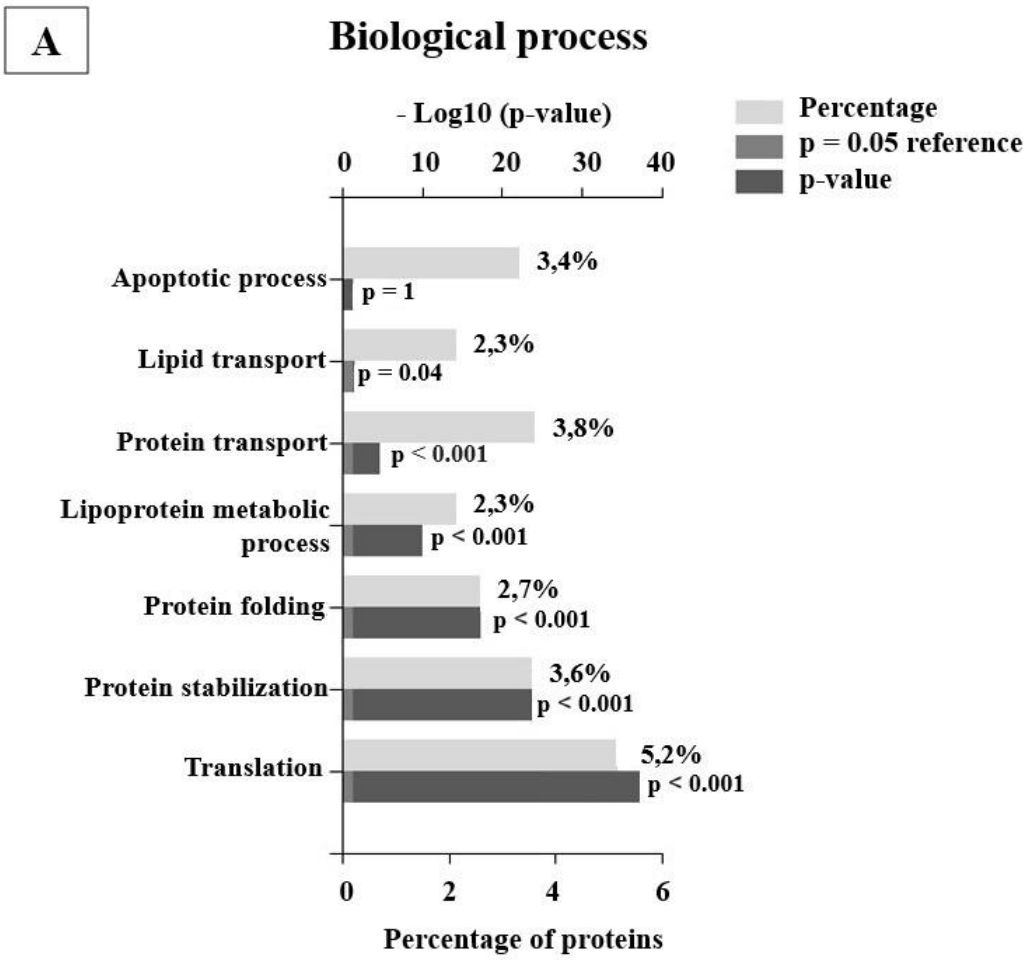
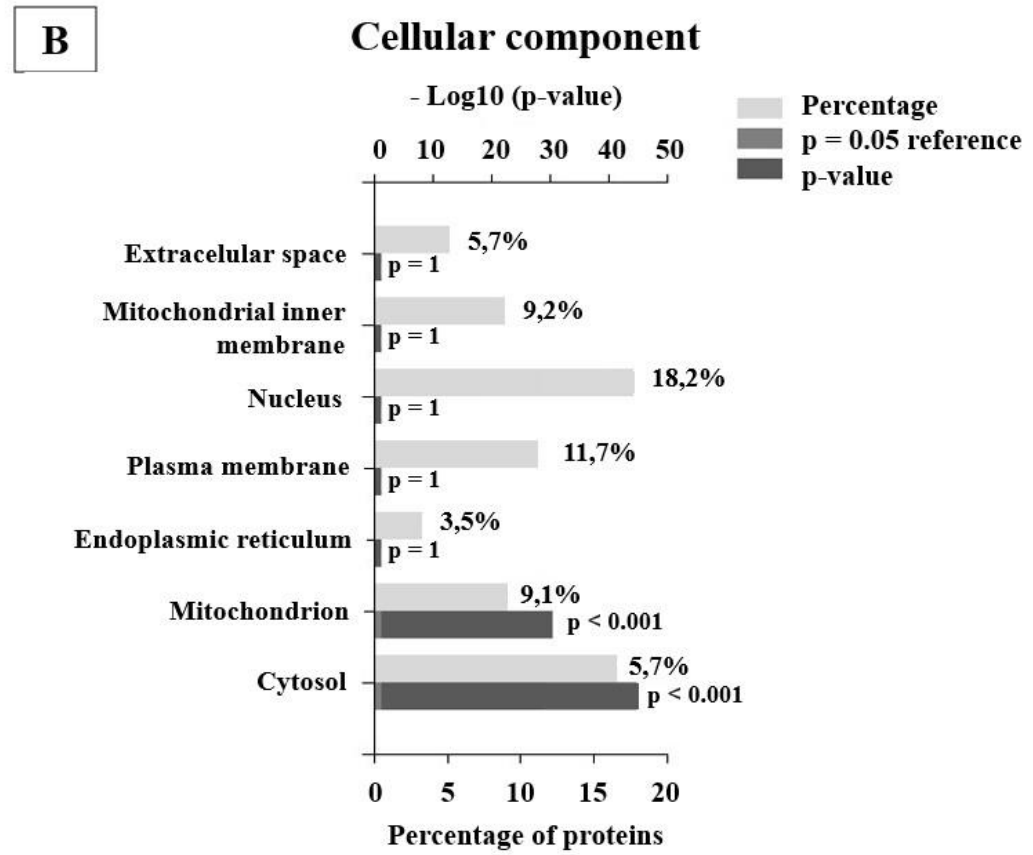


Figure 2. Gene ontology diagram showing the functional distribution of the most abundant proteins detected in 6-day old ovine blastocysts. The distribution of terms was made based on biological process (Fig. 2A), cellular component (Fig. 2B) and molecular function (Fig. 2C). Protein data were analyzed using the software for researching annotations of proteins FunRich (Functional Enrichment analysis tool, version 3.1.4). Gene ontology terms were obtained from UniProtKB database.




B
Cellular component
- Log10 (p-value)
0 10 20 30 40 50
Percentage
p = 0.05 reference
p-value
Extracelular space
5,7%
p = 1
Mitochondrial inner membrane
9,2%
p = 1
Nucleus
18,2%
p = 1
Plasma membrane
11,7%
p = 1
Endoplasmic reticulum
3,5%
p = 1
Mitochondrion
9,1%
p < 0.001
Cytosol
5,7%
p < 0.001
0 5 10 15 20
Percentage of proteins

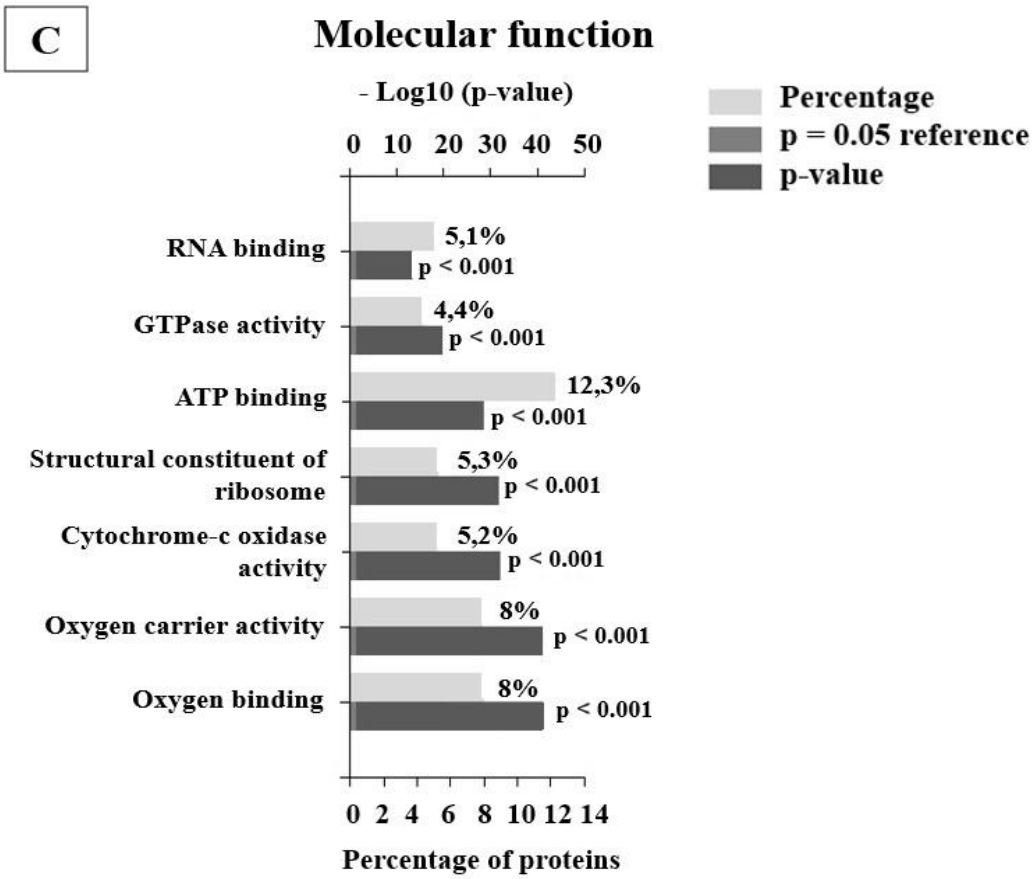

C
Molecular function
- Log10 (p-value)
0 10 20 30 40 50
Percentage
p = 0.05 reference
p-value
RNA binding
5,1%
p < 0.001
GTPase activity
4,4%
p < 0.001
ATP binding
12,3%
p < 0.001
Structural constituent of ribosome
5,3%
p < 0.001
Cytochrome-c oxidase activity
5,2%
p < 0.001
Oxygen carrier activity
8%
p < 0.001
Oxygen binding
8%
p < 0.001
0 2 4 6 8 10 12 14
Percentage of proteins

Figure 3. Network and gene set enrichment analysis of miRNA associated with the regulation of human serum albumin, zona pellucida glycoprotein 3, actin cytoplasmic 1 and pyruvate kinase, based on the highest p-value obtained from miRNet database.

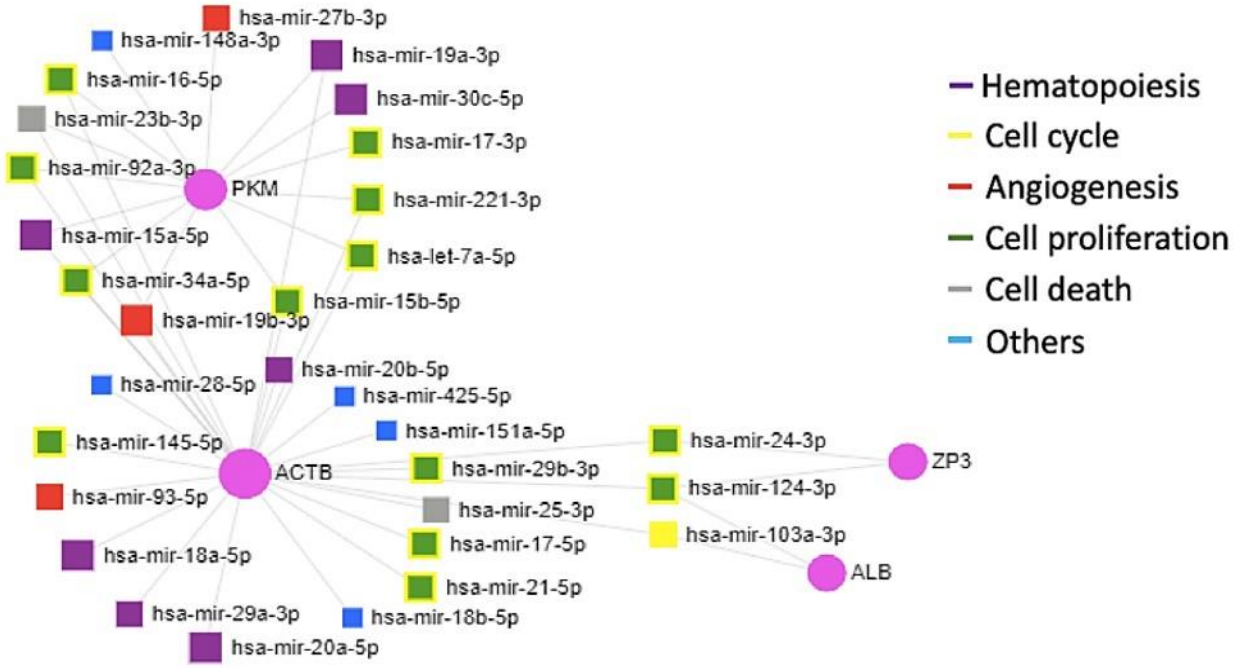


Figure 4. *In silico* analysis of protein–protein interaction considering the proteome of 6-day old ovine blastocysts produced *in vitro*. K-means clusters are involved in translation, structural components, nucleosomes, ribosomes, cell body and catalytic activity. Edge thickness is representative of confidence of the interactions (low, medium, high and highest).

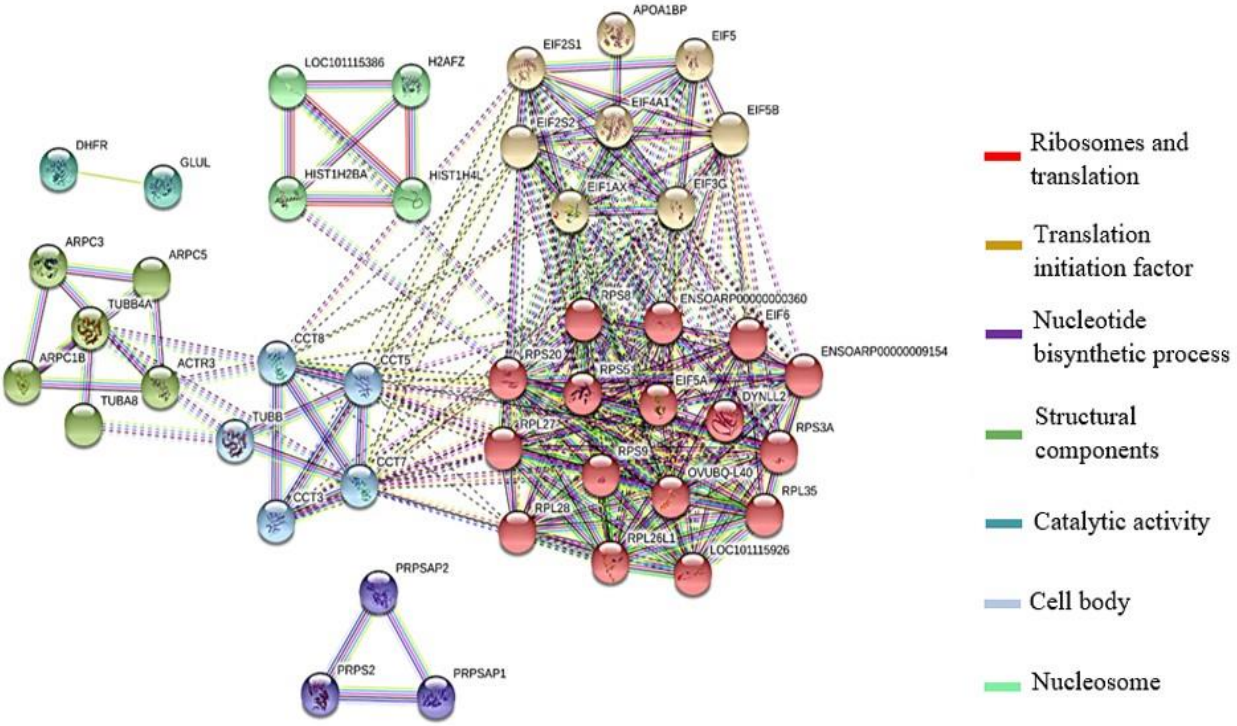


Table 1. List of the 30 most abundant proteins identified in pre-implantation, 6-day old ovine blastocysts (*Ovis aries*).

Protein accession number (Uniprot)[a]	Gene (UniProt)[a]	Lenght (aminocids)	Coverage (%)	Replicate counts	Protein description
P14639	ALB	607	0.8040	1369	Serum albumin
W5PWE9	ALB	609	0.7783	1285	Albumin
W5PWT2	ZP3	408	0.4755	1197	Zona pellucida glycoprotein 3
W5PYJ4	ZP2	714	0.5882	1069	Zona pellucida glycoprotein 2
W5PMW9	PADI6	684	0.6199	695	Peptidyl arginine deiminase 6
W5P106	ZP4	540	0.3630	670	Zona pellucida glycoprotein 4
P60713	ACTB	375	0.7387	643	Actin, cytoplasmic 1
W5QAX3	ACTG1	377	0.7347	636	Actin gamma 1
W5QC41	PKM	567	0.7213	493	Pyruvate kinase
A8DR93	HSPCA	733	0.6112	432	Heat shock protein 90 alpha family class A member 1
W5PMM7	PDIA3	449	0.7483	415	Protein disulfideN/Aisomerase
W5QFH1	ACTC1	378	0.3995	398	Actin alpha 1, cardiac muscle

W5NYJ1	ACTA1	377	0.4005	398	Actin alpha 1, skeletal muscle
W5PTR5	HSPA5	656	0.5899	397	Heat shock protein family A (Hsp70) member 5
W5NPN4	HSPA8	651	0.5376	390	Heat shock protein family A (Hsp70) member 8
W5PZK7	ACTA2	377	0.3714	374	Actin alpha 2, smooth muscle, aorta
W5PRA1	ACTG2	376	0.3723	374	Actin gamma 2, smooth muscle, enteric
W5Q1L2	HSPD1	573	0.7120	364	Heat shock protein family D (Hsp60) member 1
W5QGU0	OPA1	984	0.5396	333	OPA1, mitochondrial dynamin like GTPase
W5Q5M3	KRT18	409	0.6064	325	Keratin 18
W5PT68	FLNB	2640	0.3943	325	Filamin B
W5QHY2	LOC101101962	531	0.5932	317	Intestinal-type alkaline phosphatase-like
W5Q4W8	HSPA9	679	0.4728	306	Heat shock protein family A (Hsp70) member 9
W5PPT6	TUBB	444	0.7140	300	Tubulin beta class I
W5NT41	ACCSL	610	0.5836	294	1N/AaminocyclopropaneN/A1N/Acarboxylate synthase homolog (inactive) like
W5QBQ9	MYH9	1930	0.3218	290	Myosin heavy chain 9
W5PUT6	CLTC	1678	0.4064	279	Clathrin heavy chain
W5PHW0	HSP90AB1	724	0.5442	277	Heat shock protein 90 alpha family class B member 1

W5PN84	EEF2	974	0.4723	276	Eukaryotic translation elongation factor 2
W5P684	ACTBL2	376	0.3723	276	Actin, beta-like 2

[a] Protein abbreviation and gene name are in accordance with gene abbreviation in UniProt.

Table 2. Selected clusters associated with pre-implantation, 6-day old ovine blastocyst proteins as evaluated by Database for Annotation, Visualization and Integrated Discovery (DAVID), according p-value (-log10).

Functional annotation cluster	Protein accession number (UniProt)	Gene (UniProt)	Protein description	Size (Da)	P-value	Enrichment score
Translation	W5P114	RPS5	Ribosomal_S7 domain-containing protein	22,876	$5.45755^{(-35)}$	28.87
	W5NXM4	RPS9	40S ribosomal protein S9	22,68		
	W5PYQ7	LOC106990434	40S ribosomal protein S12	14,515		
	W5P1J6	RPL26L1	KOW domain-containing protein	17,256		
	W5NTE7	LOC114116830	60S ribosomal protein L9	21,876		
	W5P1B4	LOC101115926	60S ribosomal protein L17	21,384		
	W5P2M4	RPL27	60S ribosomal protein L27	13,223		
	W5Q8V4	RPS3A	40S ribosomal protein S3a	29,779		
Structural constituent of ribosomes	W5P114	RPS5	Ribosomal_S7 domain-containing protein	22,876	$2.49695^{(-34)}$	28.87
	W5PYQ7	LOC106990434	40S ribosomal protein S12	14,515		
	W5NTE7	LOC114116830	60S ribosomal protein L9	21,876		
	W5P1B4	LOC101115926	60S ribosomal protein L17	21,384		
	W5P2M4	RPL27	60S ribosomal protein L27	13,223		
	W5Q8V4	RPS3A	40S ribosomal protein S3a	29,779		
	W5PFD9	LOC101112702	60S ribosomal protein L35a	12,588		
	W5PZT4	RPS20	40S ribosomal protein S20	13,373		
Ribosome	W5PUU6	RPL35	60S ribosomal protein L35	12,499	$5.36825^{(-28)}$	28.87

	W5P3J0	LOC114116368	60S ribosomal protein L6	32,714		
	P0C276	UBA52	Ubiquitin-60S ribosomal protein L40	14,728		
	W5NQC0	LOC101112481	40S ribosomal protein S27	9,521		
	W5PYQ7	LOC106990434	40S ribosomal protein S12	14,515		
	W5NTE7	LOC114116830	60S ribosomal protein L9	21,876		
	W5NSP2	RPS8	40S ribosomal protein S8	25,537		
	W5P2M4	RPL27	60S ribosomal protein L27	13,223		
Nucleosome	W5PUU8	H2AC20	Histone H2A	13,988	$1.18081^{(-10)}$	7.37
	W5Q8W8	H3-3A	Histone H3	15,328		
	W5QGB5	LOC101105290	Histone H4	11,367		
	W5QFS2	LOC101115410	Histone H2B	14,500		
	W5P169	LOC101115386	Histone domain-containing protein	15,625		
	W5QFP2	H1-3	H15 domain-containing protein	22,157		
	Q6YNC8	H2AZ1	Histone H2A.Z	13,553		
Structural	W5P669	TUBB4A	Tubulin beta chain	43,194	$3.5812^{(-10)}$	6.00
constituent of	W5PW95	TUBA8	Tubulin alpha chain	54,887		
cytoskeleton	Q9MZA9	VIM	Vimentin	17,171		
	W5Q5K9	ARPC3	Actin-related protein 2/3 complex subunit 3	22,736		
	W5NVB9	ARPC5	Actin-related protein 2/3 complex subunit 5	18,296		
	W5PQ98	ACTR3	Actin-like protein 3	45,939		
	W5QBL7	ARPC1B	Actin-related protein 2/3 complex subunit	40,273		

Microtubule-based process	W5P669	TUBB4A	Tubulin beta chain	43,194	$8.78564^{(-07)}$	6.00
	W5PW95	TUBA8	Tubulin alpha chain	54,887		
	W5PEV7	DYNLL2	Dynein light chain	14,999		
	W5NVI3	N/A	Tubulin_C domain-containing protein	19,085		
Translation initiation factor activity	B9VH02	EIF1AX	Eukaryotic translation initiation factor 4C	16,460	1.43231(-07)	4.25
	W5Q0G0	EIF3G	Eukaryotic translation initiation factor 3	35,611		
	W5PHC4	EIF2S2	eIF2B_5 domain-containing protein	38,387		
	W5PQK7	EIF5A	Eukaryotic translation initiation factor 5A	17,171		
	W5PRH3	EIF6	Eukaryotic translation initiation factor 6	26,644		
	W5QJ50	EIF2S1	Eukaryotic translation initiation factor 2	36,094		
	W5P6I6	EIF5	Eukaryotic translation initiation factor 5	49,1367		
	W5PV92	EIF5B	Eukaryotic translation initiation factor 5B	138,256		
Regulation of translational initiation	W5Q0G0	EIF3G	Eukaryotic translation initiation factor 3	35,611	$5.40485^{(-07)}$	4.25
	W5PYK0	EIF4A1	RNA helicase	46,282		
	W5P6I6	EIF5	Eukaryotic translation initiation factor 5	49,136		
	W5PV92	EIF5B	Eukaryotic translation initiation factor 5B	138,256		
Cell body	W5P765	CCT3	T-complex protein 1 subunit gamma	63,600	$1.17659^{(-06)}$	3.22
	W5PXX7	CCT5	CCT-epsilon	59,615		
	W5PPT6	TUBB	Tubulin beta chain	49,671		
	W5PYM5	CCT8	CCT-theta	59,609		
	W5P816	NAXE	NAD(P)H-hydrate epimerase	32,078		

	W5PQF0	CCT7	T-complex protein 1 subunit eta	59,443		
	W5QBP6	GLUL	Glutamine synthetase	42,045		
	W5NTJ6	RPL28	60S ribosomal protein L28	15,692		
Nucleotide biosynthetic process	W5PNM5	PRPS2	Ribose-phosphate diphosphokinase	31,229	0.000146723	2.59
	W5QAZ0	PRPSAP2	Pribosyltran_N domain-containing protein	43,209		
	W5PZI9	DHFR	Dihydrofolate reductase	21,707		
	W5PC18	PRPSAP1	Phosphoribosyl pyrophosphate synthetase associated protein 1	36,408		

Table 3. KEEG pathways related to the proteome of pre-implantation, 6-day old ovine blastocysts. Analysis was conducted using DAVID server for genes.

Pathways	**Gene count**	**Percentage**	**Fold enrichment**	**p.value**	**FDR**
Ribosome	87	5.06	2.84	$4.52^{(-21)}$	$2.59^{(-19)}$
Oxidative phosphorylation	43	2.5	2.26	$2.68^{(-07)}$	$3.41^{(-06)}$
Glutathione metabolism	21	1.22	2.79	$1.90^{(-05)}$	$1.75^{(-04)}$
Gap junction	23	1.34	1.90	0.003	0.015
Mineral absorption	12	0.69	2.03	0.026	0.105
DNA replication	10	0.58	1.92	0.066	0.216
cGMP-PKG signaling pathway	30	1.74	1.34	0.079	0.253

Table 4. Functional annotation clusters associated with the proteome of pre-implantation, 6-day old ovine blastocysts produced *in vitro* and *in vivo*. The blastocysts were obtained by *in vitro* fertilization/culture, *as described in the present study,* or after artificial insemination and flush of the uterine horns, as reported by *Sanchez et al. (2021)*. Clusters were evaluated using the same software for functional enrichment analysis (FunRich version 3.1.4).

Functional annotation clusters	
***In vitro* produced embryos**	***In vivo* produced embryos**
Cell body	Binding of sperm to ZP
Microtubule-based process	Calcium/phospholipid binding
Nucleosome	Cell cycle
Nucleotide biosynthetic process	Citrate cycle (TCA Cycle)
Ribosome	Glycolysis
Regulation of translational initiation	MAPK signalling pathway
Structural constituent of ribosomes	Proteasome
Structural constituent of cytoskeleton	Pyruvate metabolismo
Translation	Tight junction
Translation initiation factor activity	Translation
	ZP

Supporting information

Table S1. Table 1. List of all proteins confidently identified in the ovine embryo proteome.

Table S2. Functional clusters based on gene ontology terms and pathway databases

Table S3. Main functions of the microRNAs involved in the regulation of ALB, ZP3, ACTB, and PKM in pre-implantation, 6-day old ovine blastocysts culture, based on the highest p-value obtained from miRNet database.

Table S1. Table 1. List of all proteins confidently identified in the ovine embryo proteome.

Name	Gene (UniProt)	Lenght	Unique peptides	Coverage	SpectrumCount	Description
P14639	ALB	607	4	0.804	1369	Serum albumin
W5PWE9	ALB	609	2	0.7783	1285	albumin (ALB)
W5PWT2	ZP3	408	30	0.4755	1197	zona pellucida glycoprotein 3(ZP3)
W5PYJ4	ZP2	714	62	0.5882	1069	zona pellucida glycoprotein 2(ZP2)
W5PMW9	PADI6	684	68	0.6199	695	peptidyl arginine deiminase 6(PADI6)
W5P106	N/A	540	2	0.363	670	zona pellucida glycoprotein 4(ZP4)
W5P104	ZP4	531	0	0.3616	668	zona pellucida glycoprotein 4(ZP4)
P60713	ACTB	375	3	0.7387	643	Actin, cytoplasmic 1
W5QAX3	N/A	377	0	0.7347	636	actin gamma 1(ACTG1)
W5NYG7	ACTB	378	0	0.7037	620	Actin, cytoplasmic 1
W5QC41	PKM	567	58	0.7213	493	Pyruvate kinase
A8DR93	HSPCA	733	42	0.6112	432	Heat shock protein 90 alpha family class A member 1
W5PMM7	PDIA3	449	46	0.7483	415	Protein disulfideN/Aisomerase
W5NYJ1	ACTA1	377	0	0.4005	398	actin, alpha 1, skeletal muscle(ACTA1)
W5QFH1	ACTC1	378	0	0.3995	398	actin, alpha, cardiac muscle 1(ACTC1)
W5PTR5	HSPA5	656	3	0.5899	397	heat shock protein family A (Hsp70) member 5(HSPA5)
W5NPN4	HSPA8	651	23	0.5376	390	heat shock protein family A (Hsp 70) member 8(HSPA8)
W5PRA1	ACTG2	376	0	0.3723	374	actin, gamma 2, smooth muscle, enteric(ACTG2)
W5PZK7	ACTA2	377	0	0.3714	374	actin, alpha 2, smooth muscle, aorta(ACTA2)
W5Q1L2	HSPD1	573	29	0.712	364	heat shock protein family D (Hsp60) member 1(HSPD1)
W5QGU0	OPA1	984	4	0.5396	333	OPA1, mitochondrial dynamin like GTPase(OPA1)
W5QGU2	OPA1	873	0	0.583	329	OPA1, mitochondrial dynamin like GTPase(OPA1)
W5PT68	FLNB	2640	75	0.3943	325	filamin B(FLNB)
W5Q5M3	KRT18	409	14	0.6064	325	keratin 18(KRT18)
W5QHY2	LOC101101962	531	20	0.5932	317	intestinal-type alkaline phosphatase-like(LOC101101962)
W5NZ09	N/A	488	42	0.7602	309	Uncharacterized protein

W5Q4W8	HSPA9	679	31	0.4728	306	heat shock protein family A (Hsp70) member 9(HSPA9)
W5PPT6	TUBB	444	6	0.714	300	tubulin beta class I(TUBB)

[a]Protein abbreviation is in accordance with gene abbreviation in UniProt.
N/A: not applicable

Table S2. Functional clusters based on gene ontology terms and pathway databases

Annotation Cluster 1	Enrichment Score: 28.87007779377153
Category	Term
GOTERM_BP_DIRECT	GO:0006412~translation
GOTERM_MF_DIRECT	GO:0003735~structural constituent of ribosome
GOTERM_CC_DIRECT	GO:0005840~ribosome
KEGG_PATHWAY	oas03010:Ribosome
Annotation Cluster 2	Enrichment Score: 7.377257292900763
Category	Term
GOTERM_CC_DIRECT	GO:0000786~nucleosome
KEGG_PATHWAY	oas05034:Alcoholism
KEGG_PATHWAY	oas05322:Systemic lupus erythematosus
Annotation Cluster 3	Enrichment Score: 6.00630833959256
Category	Term
GOTERM_MF_DIRECT	GO:0005200~structural constituent of cytoskeleton
GOTERM_BP_DIRECT	GO:0007017~microtubule-based process
KEGG_PATHWAY	oas04540:Gap junction
Annotation Cluster 4	Enrichment Score: 4.643450297970816
Category	Term
KEGG_PATHWAY	oas00190:Oxidative phosphorylation
KEGG_PATHWAY	oas05016:Huntington's disease
KEGG_PATHWAY	oas05012:Parkinson's disease
KEGG_PATHWAY	oas05010:Alzheimer's disease
GOTERM_CC_DIRECT	GO:0005747~mitochondrial respiratory chain complex I
KEGG_PATHWAY	oas04932:Non-alcoholic fatty liver disease (NAFLD)

Annotation Cluster 5	Enrichment Score: 4.256015882496883
Category	Term
GOTERM_MF_DIRECT	GO:0003743~translation initiation factor activity
GOTERM_BP_DIRECT	GO:0006446~regulation of translational initiation
GOTERM_CC_DIRECT	GO:0071541~eukaryotic translation initiation factor 3 complex, eIF3m
GOTERM_CC_DIRECT	GO:0033290~eukaryotic 48S preinitiation complex
GOTERM_CC_DIRECT	GO:0016282~eukaryotic 43S preinitiation complex
GOTERM_BP_DIRECT	GO:0001731~formation of translation preinitiation complex

Table S3. Main functions of the microRNAs involved in the regulation of ALB, ZP3, ACTB, and PKM in pre-implantation, 6-day old ovine blastocysts culture, based on the highest p-value obtained from miRNet database.

Cell cycle	**Hematopoiesis**	**Angiogenesis**	**Cell proliferation**	**Cell death**
hsa-let-7a-5p	hsa-let-7a-5p	hsa-mir-16-5p	hsa-let-7a-5p	hsa-let-7a-5p
hsa-mir-16-5p	hsa-mir-17-3p	hsa-mir-17-3p	hsa-mir-16-5p	hsa-mir-16-5p
hsa-mir-17-3p	hsa-mir-19a-3p	hsa-mir-19a-3p	hsa-mir-17-3p	hsa-mir-17-3p
hsa-mir-19b-3p	hsa-mir-92a-3p	hsa-mir-19b-3p	hsa-mir-92a-3p	hsa-mir-19b-3p
hsa-mir-92a-3p	hsa-mir-30c-5p	hsa-mir-92a-3p	hsa-mir-221-3p	hsa-mir-92a-3p
hsa-mir-221-3p	hsa-mir-221-3p	hsa-mir-221-3p	hsa-mir-124-3p	hsa-mir-30c-5p
hsa-mir-124-3p	hsa-mir-145-5p	hsa-mir-145-5p	hsa-mir-145-5p	hsa-mir-221-3p
hsa-mir-145-5p	hsa-mir-15a-5p	hsa-mir-15a-5p	hsa-mir-15b-5p	hsa-mir-145-5p
hsa-mir-15b-5p	hsa-mir-34a-5p	hsa-mir-27b-3p	hsa-mir-34a-5p	hsa-mir-23b-3p
hsa-mir-34a-5p	hsa-mir-17-5p	hsa-mir-34a-5p	hsa-mir-17-5p	hsa-mir-34a-5p
hsa-mir-103a-3p	hsa-mir-18a-5p	hsa-mir-17-5p	hsa-mir-21-5p	hsa-mir-17-5p
hsa-mir-17-5p	hsa-mir-20b-5p	hsa-mir-18a-5p	hsa-mir-24-3p	hsa-mir-18a-5p
hsa-mir-18a-5p	hsa-mir-29a-3p	hsa-mir-21-5p	hsa-mir-29b-3p	hsa-mir-21-5p
hsa-mir-21-5p	hsa-mir-20a-5p	hsa-mir-93-5p		hsa-mir-24-3p
hsa-mir-24-3p		hsa-mir-20a-5p		hsa-mir-25-3p
hsa-mir-29b-3p				hsa-mir-29b-3p
hsa-mir-20a-5p				hsa-mir-20a-5p

8 CONCLUSÕES GERAIS

- ✓ As informações obtidas através dos proteomas dos complexos cumulus-oócito não maturados e complexos cumulus-oócito maturados *in vitro*, bem como de embriões, fornecerá uma plataforma importante para nortear o progresso da fecundação *in vitro* nesta espécie.

9 PERSPECTIVAS

As informações obtidas neste trabalho poderão ser utilizadas para o aprimoramento de Tecnologias de Reprodução Assistidas – TRAs como a produção *in vitro* e transferência de embriões que possibilitem uma rápida multiplicação de animais geneticamente superiores, e poderão contribuir significativamente para o crescimento da pecuária.

No tocante à pesquisa fundamental, este trabalho poderá contribuir para elucidação dos mecanismos implicados nos processos de produção *in vitro* embriões, abrindo inúmeras possibilidades para a produção de embriões em larga escala. Além disso, poderá ser realizado uma análise epigenética, pois tem sido observado sua importância na fertilidade, e que possivelmente poderá fornecer um biomarcador para problemas potenciais com a função oocitária e o desenvolvimento embrionário inicial em ovinos.

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APÊNDICE A – LISTA DE FIGURAS

APÊNDICE B – LISTA DE TABELAS