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**EFFECTOS DEL TAMAÑO FOLICULAR Y EL ESTADIO DE  
MADURACIÓN SOBRE LA EXPRESIÓN DE ARNm Y DISTRIBUCIÓN  
PROTÉICA EN OVOCITOS BOVINOS MADURADOS IN VITRO**

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EFECTOS DEL TAMAÑO FOLICULAR Y EL ESTADIO DE MADURACIÓN  
SOBRE LA EXPRESIÓN DE ARNm Y DISTRIBUCIÓN PROTÉICA EN OVOCITOS  
BOVINOS MADURADOS IN VITRO

### Resumen

La capacidad de desarrollo ovocitaria es adquirida durante el desarrollo folicular y finalmente durante la maduración ovocitaria. El objetivo de esta tesis es investigar la dinámica de algunos eventos moleculares y celulares durante la maduración ovocitaria bovina comparándolos en ovocitos provenientes de folículos pequeños y grandes, los cuales presentan baja y alta capacidad de desarrollo, respectivamente. Se semi-cuantificaron, utilizando endpoint RT-PCR, las abundancias relativas de los ARNms de DYNLL1, DYNC1I1, DCTN1, PMSB1, PMSB4, PAP, Cx43, G9A, SUV39H1, DNMT1, DNMT3b y ZAR1. Por inmunofluorescencia se detectaron las distribuciones de las proteínas dynein, dynactin, 20S proteasomes ( $\alpha\beta$  subunits) e histonas modificadas (H3-K9diMe y H4-K12 acetylation). Todos los estudios fueron realizados en los estadios de GV, GVBD, MI y MII en ovocitos provenientes de folículos pequeños ( $< 2$  mm) y folículos grandes (2 - 8 mm). Intentando establecer una asociación entre sus abundancias, distribución y la alta o baja capacidad de desarrollo. Se encontraron diferencias entre las dos poblaciones de ovocitos estudiadas en siete de los doce genes analizados, esto es, diferencias en las abundancias relativas (G9A, SUV39H1, DNMT1), en las dinámicas durante la maduración (PMSB1 y ZAR1) o en ambas (DYNLL1 y DYNC1I1). Se observaron diferencias en la localización de dynein y dynactin entre las dos poblaciones de ovocitos, mientras que la distribución de los 20S proteasomes no se vió afectada por el tamaño folicular. Las modificaciones epigenéticas H3-K9diMe y H4-K12 acetylation mostraron el mismo patrón durante la maduración en ovocitos provenientes de ambos tamaños foliculares. Las diferencias encontradas entre ovocitos recuperados de folículos pequeños y grandes muestran algunos factores ooplasmáticos relacionados a la adquisición de la capacidad de desarrollo ovocitaria durante el desarrollo folicular y la maduración.

Palabras clave: tamaño folicular, maduración ovocitaria bovina, capacidad de desarrollo, abundancia relativa de ARNm, motores moleculares, proteasomas, modificaciones epigenéticas

EFFECTS OF FOLLICLE SIZE AND STAGE OF MATURATION ON mRNA  
EXPRESSION AND PROTEIN DISTRIBUTION IN BOVINE IN VITRO MATURED  
OOCYTES

**Abstract**

Oocyte developmental competence is acquired during follicular development and finally during oocyte maturation. The goal of this thesis is to investigate the dynamics of some molecular and cellular events during bovine oocyte maturation comparing with oocytes recovered from small and large follicles, low and high developmental competence, respectively. The relative abundance (RA) of RNAm of DYNLL1, DYNC1I1, DCTN1, PMSB1, PMSA4, PAP, Cx43, G9A, SUV39H1, DNMT1, DNMT3b and ZAR1 were determined by semi-quantitative endpoint RT-PCR. The distribution of cytoplasmic dynein, dynactin, 20S proteasomes ( $\alpha\beta$  subunits) and two histone modifications (H3-K9diMe and H4-K12 acetylation) was assessed by immunocytochemistry. All the studies were performed at different stages of IVM, i.e. GV, GVBD, MI and MII in oocytes collected from follicles of two different size categories, that is, < 2 mm and 2–8 mm. In order to establish a relationship between their relative abundances and distributions with oocyte developmental competence. We found differences between the two populations of oocytes in seven of a total of twelve genes analyzed, in relative abundances (G9A, SUV39H1, DNMT1), in the dynamics during oocyte maturation (PMSB1 and ZAR1) or in both of them (DYNLL1 and DYNC1I1). We detected some differences in dynein and dynactin distributions between the two types of oocytes, while no differences were observed in the localization of 20S proteasomes. Each epigenetic modification H3-K9diMe or H4-K12 acetylation showed a specific pattern and they were not affected by the follicle size. The differences that we found in oocytes recovered from small and large follicles show some ooplasmic factors involved in the acquisition of oocyte developmental competence during follicular development and oocyte maturation.

**Key words:** follicle size, bovine oocyte maturation, developmental competence, mRNA relative abundance, molecular motors, proteasomes, epigenetic modifications

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*A mi papá*  
*Ricardo A. Racedo*  
*(1939 - 1995)*

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**Lista de abreviaturas utilizadas en español y/o en inglés, nombres de genes y proteínas**

ADN	Ácido Desoxirrivo-Nucléico
ANOVA	Analysis of variances
ARNm	Ácido Ribo-Nucléico mensajero
BSA-FAF	Bovine Serum Albumin-Fatty Acid Free
BSA-Fraction V	Bovine Serum Albumin-Fraction V
cDNA	complementary Deoxyribo-Nucleic Acid
COC	Cumulus-Oocyte-Complexe
Cx43	Connexin 43
d	day
DAPI	4,6-diamino-2-phenylindole
DCTN1	dynactin 1; pGlued homolog
DMSO	dimethylsulfoxide
DNA	Deoxyribo-Nucleic Acid
DNMT1	DNA methyltransferase 1
DNMT3b	DNA methyltransferase 3b
dNTP	deoxyribo-Nucleotide Tri-Phosphate
DYNACTIN	dynactin 1; pGlued homolog protein
DYNC1I1	cytoplasmic dynein 1 intermediate chain
DYNEIN	cytoplasmic dynein 1 intermediate chain protein
DYNLL1	cytoplasmic dynein light chain LC8

## **Abreviaturas (continuación)**

EDTA	ethylenediaminetetraacetic acid
End Point RT-PCR	End Point Reverse Transcription-Polymerase Chain Reaction
ERK1 (MAPK1)	Extracellular-Regulated Kinase 1
ERK2 (MAPK2)	Extracellular-Regulated Kinase 2
fg	fentograms
G9A	HLA-B associated transcript 8
GM130	Golgi Matrix Protein of 130 KDa
GV	Germinal Vesicle
GVBD	Germinal Vesicle Break Down
h	hour
H3-K9diMe	Histone 3 – Lysine K9 dimethylation
H4-K12 acetylation	Histone 4 – Lysine K12 acetylation
hCG	human Chorionic Gonadotropin
HEPES	N-(2-hydroxyethyl) piperazine-N'-ethanesulfonic acid
HHE	Hypotaurine, Heparin, Epinephrine
ICC	Inmunocytochesmistry
IVC	In Vitro Culture
IVF	In Vitro Fertilization
IVM	In Vitro Maturation
kDa	kilodaltons

## Abreviaturas (continuación)

MI	Metaphase I
MII	Metaphase II
MAPK	Mitogen-Activated Protein Kinase
MEK1	MAPK-ERK kinase 1
MEK2	MAPK-ERK kinase 2
MPF	M-phase Promoting Factor
mRNA	messenger Ribo-Nucleic Acid
NuMA	Nuclear protein that associates with the Mitotic Apparatus
PanHistones	Core histones
PAP	Poly-A Polymerase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PMSA4	Proteasome Alfa Subunit 4
PMSB1	Proteasome Beta Subunit 1
PMSG	Pregnant Mare Serum Gonadotropin
PROTEASOMES 20S (αβ subunits)	proteasomes 20S protein subunits
PVA	polyvinil pyrrolidone
RA	Relative Abundance
RT	Reverse Transcription
RT-PCR	Reverse Transcription-Polymerase Chain Reaction

### **Abreviaturas (continuación)**

SOF	Synthetic Oviduct Fluid
SOV	Sodium Ortho-Vanadate
SUV39H1	Suppressor of Variegation 3-9 Homolog 1
TALP	Tyrode's medium with Albumin, Lactate and Pyruvate
<i>Taq</i>	Termophilus aquaticus
TCM	Tissue Culture Medium
TUBULIN	Tubulin protein
ZAR1	Zygote Arrest 1

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BOVINOS MADURADOS IN VITRO**

**Resumen ampliado**

La calidad ovocitaria es uno de los parámetros determinantes de la capacidad de desarrollo embrionario. La calidad de los ovocitos varía con el tamaño de los folículos, el cual influye sobre la maduración ovocitaria y posteriormente sobre el desarrollo. Concomitante con la foliculogénesis comienza la adquisición de la capacidad de desarrollo ovocitaria. En tanto que la maduración de los ovocitos es una secuencia de eventos celulares y moleculares, durante la cual sucede la adquisición final de la capacidad de desarrollo. El objetivo de esta tesis es investigar la dinámica de algunos eventos moleculares y celulares que ocurren durante la maduración ovocitaria comparándolos en ovocitos provenientes de folículos de diferente diámetro. Se estudió durante la maduración in vitro de ovocitos bovinos la expresión de un grupo seleccionado de ARNms y proteínas involucrados en el proceso de maduración, tales como proteínas motoras, las subunidades  $\alpha\beta$  de los proteasomas, y modificaciones epigenéticas, intentando establecer una asociación entre sus abundancias, distribución y la alta o baja capacidad de desarrollo.

Es aceptado que los ovocitos bovinos provenientes de folículos pequeños ( $< 2$  mm) poseen menor capacidad de desarrollo comparado con la población de ovocitos

recuperados de folículos grandes (2 - 8 mm). Estos grupos de ovocitos presentan diferente capacidad de desarrollo, aunque la tasa de maduración nuclear, medida como la proporción de ovocitos que alcanzan el estadio de metafase II (MII), no se ve afectada. Por lo que en esta tesis nos centramos principalmente en el estudio de la maduración citoplasmática, esto es, cambios que suceden entre el estadio de vesícula germinal (GV, por sus siglas en inglés) y la metafase II (MII) que involucran los niveles de ARNms y la distribución de proteínas.

En nuestra hipótesis, proponemos que la capacidad de desarrollo disminuida en el grupo de ovocitos recuperados de folículos pequeños está ligada a la expresión deficiente de ciertos ARNms que codifican reguladores de eventos celulares, tales como los motores moleculares (DYNLL1 [cytoplasmic dynein light chain-LC8], DYNC1I1 [cytoplasmic dynein 1 intermediate chain] y DCNT1 [dynactin], por sus siglas y nombres en inglés), los 20S proteasomas (PMSB1 [proteasome beta subunit 1] y PMSB4 [proteasomes alfa subunit 4]), la enzima responsable del proceso de poliadenilación de ARNms (PAP [poly-A polymerase]) y una proteína de comunicación intercelular (Cx43 [connexin 43]), durante la maduración ovocitaria. Para probar esta hipótesis se semi-cuantificaron los ARNms antes mencionados utilizando RT-PCR de punto final (endpoint RT-PCR, por su nombre en inglés) en distintos estadios de la maduración ovocitaria (GV, GVBD, MI y MII, por sus siglas en inglés) de ovocitos recuperados de folículos pequeños (< 2 mm) y grandes (2 - 8 mm). Hemos detectado por primera vez los transcriptos de los genes antes mencionados y hemos hallado tres ARNms que se expresan diferencialmente entre

las dos poblaciones de ovocitos durante la maduración ovocitaria. Nuestros resultados muestran que las abundancias relativas (RAs, por sus siglas en inglés) de los ARNm de los motores moleculares DYNLL1 y DYNC1I1, y la subunidad de los 20S proteasomes PMSB1, están afectados por el tamaño folicular y por el estadio de maduración. Siendo mayor la abundancia relativa (RA) de los transcriptos de DYNLL1 y DYNC1I1 en ovocitos inmaduros con alta capacidad de desarrollo o sea provenientes de folículos grandes, así como también, diverge la dinámica de los tres transcriptos durante la maduración entre ambas poblaciones de ovocitos. Estos niveles mayores de los transcriptos de DYNLL1 y DYNC1I1, y las diferencias en las dinámicas de DYNLL1, DYNC1I1 y PMSB1 durante la maduración de ovocitos provenientes de folículos de distinto tamaño podrían estar relacionados a la capacidad de desarrollo luego de la fecundación. Las diferencias encontradas entre grupos de ovocitos podrían servir como marcadores de la capacidad de desarrollo de ovocitos bovinos.

Las distribuciones de las proteínas codificadas por DYNC1I1, DCNT1, PMSB1 y PMSA4 se estudiaron por inmunocitoquímica y microscopía confocal en GV, GVBD, MI y MII en ovocitos recuperados de folículos pequeños (< 2 mm) y grandes (2 - 8 mm). Las proteínas dynein, dynactin y 20S proteasomes ( $\alpha\beta$  subunits) muestran una localización específica durante cada estadio de la maduración ovocitaria, sugiriendo el sitio de acción de las mismas. Dynein está homogéneamente distribuida en el citoplasma de ovocitos en GV mientras que dynactin muestra una localización perinuclear en el mismo estadio en ambos grupos de ovocitos. Inmediatamente

luego de la primera condensación de la cromatina (GVBD), dynactin rodea al ADN e interactúa con los husos meióticos en MI y MII en ovocitos provenientes de folículos grandes. La marca de dynactin encontrada en los husos meióticos en ovocitos provenientes de folículos pequeños es suave en ambas metafases y no se observa una asociación clara con el ADN en GVBD. Dynein muestra una localización específica en MI y MII, interactuando con los husos meióticos, la presencia de dynein que se observa en el huso meiótico en MI de ovocitos provenientes de folículos pequeños es menor comparada con la que se observa en aquellos ovocitos recuperados de folículos grandes. El uso del inhibidor específico de la actividad ATPásica de dynein citoplasmática devela el rol crítico del transporte mediado por estas proteínas motoras durante la maduración ovocitaria bovina.

Los 20S proteasomes se encuentran en el núcleo de ovocitos en el estadio de GV y se asocian con el ADN durante toda la maduración ovocitaria hasta el estadio de metafase II en ambos grupos de ovocitos.

Teniendo en cuenta estudios previos y nuestros resultados podemos proponer a dynein y dynactin como reguladores importantes de eventos celulares relacionados a la adquisición final de la capacidad de desarrollo durante la maduración citoplasmática de ovocitos bovinos.

Finalmente con el objetivo de ampliar el perfil molecular que caracteriza a las dos poblaciones de ovocitos en estudio decidimos evaluar la expresión de ARNm de un grupo de genes de reprogramación y dos modificaciones de histonas, la dimetilación de las histonas H3 en la posición de la Lisina K9 (H3-K9diMe, por sus siglas en

inglés) y la acetilación de las histonas H4 en la posición de la Lisina K12 (H4-K12 acetylation, por sus siglas en inglés) en GV, GVBD, MI y MII en ovocitos provenientes de folículos pequeños (< 2 mm) y grandes (2 - 8 mm).

Las RAs de dos histona-metiltransferasas (G9A [HLA-B associated transcript 8] y SUV39H1 [suppressor of variegation 3-9 homolog 1], por sus siglas y nombres en inglés), dos ADN-metiltransferasas (DNMT1 [DNA methyltransferase 1] y DNMT3b [DNA methyltransferase 3b]) y del factor de reprogramación ZAR1 (zygote arrest 1), se semi-cuantificaron utilizando endpoint RT-PCR. Se detectaron por inmunocitoquímica y microscopía de fluorescencia las modificaciones H3-K9diMe y H4-K12 acetylation. Los resultados muestran que las abundancias relativas de los ARNm de las metiltransferas específicas de las histonas H3 en la posición K9 (G9A y SUV39H1) y la ADN metiltransferasa 1 (DNMT1), es mayor en ovocitos inmaduros recuperados de folículos grandes (2 - 8 mm). El ARNm del gen de reprogramación ZAR1 presenta una expresión dinámica durante la maduración sólo en aquellos ovocitos recuperados de folículos grandes.

H3-K9diMe se mantiene durante toda la maduración con una leve disminución de la marca luego de GVBD y H4-K12 acetylation muestra una señal fuerte en GV y GVBD con una marcada disminución de la señal luego de GVBD. Estas modificaciones no están afectadas por el tamaño folicular. Los resultados de la modificación H3-K9diMe sugieren que las diferencias encontradas en las abundancias relativas de los transcriptos de G9A y SUV39H1 entre ambas poblaciones de ovocitos no afectan el efecto final de estos genes sobre las H3-K9.

Además, el patrón en la metilación de las H3-K9 y en la acetilación de las H4-K12 que se produce en los ovocitos durante el desarrollo folicular están establecidas ya en ovocitos recuperados de folículos antrales pequeños ya que no se evidencian diferencias en el estadio de vesícula germinal entre ambas poblaciones de ovocitos. Las dinámicas encontradas en las modificaciones estudiadas en H3-K9 y H4-K12 durante la maduración ovocitaria confirman los roles de estas modificaciones epigenéticas en relación a la represión o activación de la expresión génica en cada estadio meiótico.

El presente trabajo muestra la expresión de los ARNms de doce genes seleccionados, cinco de los cuales han sido semi-cuantificados por primera vez en ovocitos (DYNLL1, DYNC1I1, DCNT1, PMSB1 y PMSA4) a través del diseño de primers y la obtención de las condiciones específicas de detección de estos transcriptos. Se encontraron diferencias entre las dos poblaciones de ovocitos estudiadas en siete de los doce genes analizados, esto es, diferencias en las abundancias relativas (G9A, SUV39H1, DNMT1), en las dinámicas durante la maduración (PMSB1 y ZAR1) o en ambas (DYNLL1 y DINC1I1). La localización de las proteínas dynein, dynactin y los 20S proteasomes ( $\alpha\beta$  subunits), se investigan en ovocitos bovinos durante el desarrollo de esta tesis, y también los patrones que presentan las modificaciones epigenéticas H3-K9diMe y H4-K12 acetylation. Se encontraron diferencias en la localización de dynein y dynactin entre las dos poblaciones de ovocitos, mientras que la distribución de los 20S proteasomes no se vió afectada por el tamaño folicular. Las modificaciones epigenéticas H3-K9diMe y H4-K12 acetylation mostraron el

mismo patrón durante la maduración de ovocitos provenientes de ambos tamaños foliculares.

Las diferencias encontradas entre ovocitos recuperados de folículos pequeños y grandes muestran algunos factores ooplasmáticos relacionados a la adquisición de la capacidad de desarrollo ovocitaria durante el desarrollo folicular y la maduración.

## **Capítulo 1**

### **Introducción**

#### Ovogénesis

La ovogénesis, proceso mediante el cual se forman las gametas femeninas en mamíferos, comprende la proliferación, el crecimiento y la maduración de los ovocitos, la formación y ruptura del folículo y la liberación de un óvulo maduro, esto es, la ovulación (Hafez, 1962).

La ovogénesis comienza durante el desarrollo fetal temprano con la formación de las células germinales primordiales (10 - 20  $\mu\text{m}$  de diámetro), estas células forman la primera población en el ovario fetal primitivo y alcanzan este sitio por movimientos ameboideos y en respuesta a diferentes sustancias como por ejemplo el factor transformante  $\beta 1$ , luego las células se dividen por mitosis para formar las células madre ováricas, las ovogonias (Picton y Gosden, 1998).

En humanos y otros especies de grandes mamíferos, el número de ovogonias, 12.5 - 25  $\mu\text{m}$  de diámetro, aumenta a través del proceso de proliferación, tienen lugar muchas rondas de división mitótica, hasta aproximadamente el día 170 de gestación en bovinos (Erickson, 1966a; Picton y Gosden, 1998). Luego del nacimiento no se formarán nuevas ovogonias (Solomon et al., 1996). Sin embargo, en ovarios juveniles y adultos de ratones se ha descripto la presencia de división activa de células madre germinales (Johnson et al., 2004).

Luego de las divisiones mitóticas, cada ovogonia aumenta su tamaño y es un ovocito primario. La zona pelúcida es sintetizada en el estadio folicular

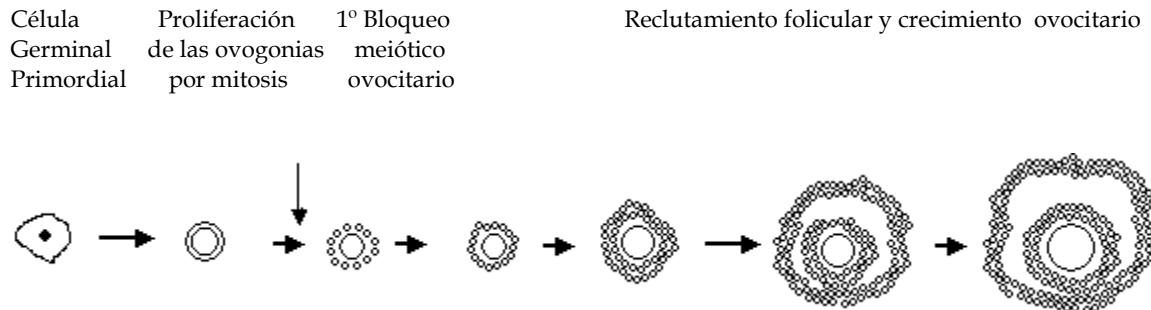
correspondiente a los folículos primordiales y está compuesta de tres glicoproteínas sulfatadas llamadas ZP1, ZP2 y ZP3 (Fair et al., 1997b; Sinowatz et al., 2001). Los ovocitos progresan hacia la profase de la primera división meiótica, la cual se inicia entre 75 y 80 días antes del nacimiento en bovinos (Erickson, 1966a). Durante la profase I, el material genético se intercambia entre cromátidas homólogas por entrecruzamiento o crossing-over, proceso mediante el cual ciertas enzimas rompen las cromátides, intercambian fragmentos, y luego reúnen las cromátides produciendo nuevas combinaciones de genes (Solomon et al., 1996). La profase meiótica comprende varios estadios intermedios: leptotene, zиготене, paquitene, diplotene y diacinesis o estadio dictiásico.

Diplotene es el estadio en el cual los ovocitos de la mayoría de los mamíferos se arrestan, para pasar a la fase dictiásica donde el ADN se descondensa formando un núcleo vesicular, la vesícula germinal (GV, por sus siglas en inglés), con abundantes organelas citoplasmáticas (Baker y Franchi, 1967; Downs, 1993b). Los ovocitos primarios arrestados son las células germinales diferenciadas y están rodeadas por una capa de células de la granulosa planas y en conjunto forman el folículo primordial (Erickson, 1966a; Braw-Tal y Yossefi, 1997; Yang et al., 1998). Al momento del nacimiento en bovinos el número de ovocitos es aproximadamente de 133.000 (Erickson, 1966b), que se encuentran arrestados en el estadio dictiásico de la profase de la primera división meiótica.

En especies monovulares, la ovogénesis consta de las etapas mencionadas anteriormente, la primera de proliferación, la segunda de crecimiento y finalmente la de maduración.

Durante la proliferación se determina el número total de células germinales de la hembra y como consecuencia los ovocitos que serán utilizados durante la vida reproductiva. El segundo período corresponde al crecimiento del ovocito. En esta etapa no sólo la célula germinal adquiere cambios sino que también las células foliculares que la rodean se dividen y dan lugar a un proceso denominado foliculogénesis. Durante ese período y de manera coordinada con la ovogénesis, el folículo primordial pasa a ser primario, luego secundario y finalmente terciario o antral (Rodríguez y Farín, 2004), figura 1.1.

## Ovogénesis y desarrollo folicular en bovinos



Característica folicular/ Ovocitaria	Folículo primordial	Folículo primario	Folículo secundario	Folículo terciario (antral)
Diámetro folicular (mm)	0,03-0,04	0,04-0,10	0,10-0,60	0,60-1,5 1,5-2 >2
Diámetro ovocitario ( $\mu\text{m}$ )	30	30	30-95	95-100 100-110 110-120
GV $\rightarrow$ GVBD	-	-	-/+	+
GVBD $\rightarrow$ MI	-	-	-	+
MI $\rightarrow$ MII	-	-	-	+
Capacidad de desarrollo	-	-	-	-

Fig. 1.1. Representación esquemática de la ovogénesis y la foliculogénesis en bovinos. A medida que aumenta el diámetro ovocitario y folicular, aumenta la adquisición de la competencia meiótica y la capacidad de desarrollo. (+) es ocurre; (-) es no ocurre; GV, vesícula germinal; GVBD, ruptura de la vesícula germinal; MI y MII, metafase I y II, respectivamente (adaptado Rodríguez y Farín, 2004).

## Foliculogénesis

### Morfología del desarrollo folicular

La foliculogénesis comienza con la formación de los folículos primordiales en el ovario fetal. Los ovocitos primarios, que se encuentran arrestados en la profase de la primera división meiótica, se rodean de una capa de células pre-granulosa planas alrededor del día 90 de gestación para formar los folículos primordiales de los ovarios (Russe, 1983). Los factores que regulan la formación de los folículos primordiales no están aún descriptos completamente. Se ha demostrado en ratones que el receptor c-kit y su ligando tienen un rol crítico en direccionar la migración y la supervivencia de las células germinales durante la vida embrionaria (Driancourt et al., 2000). Figla, también conocido como Figa, es un gen que codifica un factor de transcripción que coordina la expresión de genes estructurales de la zona pelúcida. Éste ha sido identificado como un factor importante durante la formación de los folículos primordiales (Eppig, 2001).

Los folículos primordiales, progresan a folículos primarios y luego a folículos secundarios, proceso conocido como reclutamiento inicial, y luego grupos de folículos secundarios se reclutan cíclicamente durante la vida reproductiva de las hembras. El reclutamiento inicial de folículos ocurre a través de la entrada en la fase de crecimiento, los que no entran sufren apoptosis, estos procesos remueven folículos primordiales de la población original en estado quiescente (Kaipia y Hsueh, 1997; Van Boris, 1998).

Los folículos primordiales contienen una capa única de células de pre-granulosa planas y son de un diámetro de aproximadamente 35  $\mu\text{m}$  con un ovocito de alrededor de 30  $\mu\text{m}$  de diámetro (Braw-Tal y Yossefi, 1997; Fair et al., 1997b).

La iniciación del crecimiento folicular requiere de la transición de folículos primordiales desde el estado quiescente a la fase de crecimiento y esta transición está caracterizada por tres eventos principales: el cambio en la forma de las células de la granulosa que pasan a ser cúbicas, la proliferación de las células de la granulosa y el agrandamiento de los ovocitos (Braw-Tal y Yossefi, 1997). El mecanismo por el cual los folículos primordiales son activados para iniciar el crecimiento es aún poco claro. Sin embargo, algunos factores como la proteína formadora de huesos 7 (BMP-7, por sus siglas en inglés), el factor de crecimiento insulínico tipo I (IGF-I, por sus siglas en inglés), el factor de crecimiento epidérmico (EGF, por sus siglas en inglés), la hormona inhibitoria anti-mulleriana (AMH, por sus siglas en inglés), el factor de crecimiento y diferenciación específico ovocitario 9 (GDF-9, por sus siglas en inglés), el receptor de la proteína formadora de huesos 2 (BMP-2R, por sus siglas en inglés), la proteína formadora de huesos 15 (BMP-15, por sus siglas en inglés) y el ligando c-kit están involucrados en el proceso (Fair, 2003). La entrada del folículo primordial en la fase de crecimiento se caracteriza por la conversión de las células pre-granulosa planas que rodean al ovocito en una capa de células cúbicas (Fair et al., 1997b). Este folículo se denomina ahora folículo primario (Van Boris, 1998). El diámetro alcanza

aproximadamente 55  $\mu\text{m}$ , y no hay cambios en el tamaño del ovocito (Braw-Tal y Yossefi, 1997).

En bovinos, los primeros folículos en crecimiento aparecen en la vida fetal alrededor del día 180 de gestación, siendo principalmente folículos primarios y secundarios, los primeros folículos antrales tempranos se observan al día 220 de gestación (Wandji et al., 1992). Los folículos secundarios se caracterizan por tener una doble capa de células de la granulosa y presentan células tecales ya diferenciadas subyacentes a la membrana basal (Braw-Tal y Yossefi, 1997). En los folículos primordiales la comunicación entre el ovocito y las células de la granulosa estaría mediada por una vía endocítica, evidenciado por la presencia de vesículas en el ovocito. En el folículo secundario, la comunicación es vía uniones de tipo estrechas formadas entre el ovocito y las células de la granulosa.

Los folículos terciarios están caracterizados por la formación del antro, por la presencia de capas múltiples de células de la granulosa apoyadas a una membrana basal y la presencia de las células del cúmulus rodeando al ovocito (Fair et al., 1997b). Estos folículos tienen una teca interna y una teca externa bien definidas y el ovocito está rodeado por su zona pelucida (Braw-Tal y Yossefi, 1997; Fair et al., 1997b). La formación del antro comienza en folículos de aproximadamente 0.14 – 0.28 mm. El crecimiento de los folículos antrales tempranos se puede dividir en dos fases; primero, un agrandamiento del folículo por aumento del número de células y segundo, en folículos mayores de 2.5 mm, el crecimiento folicular resulta principalmente por desarrollo del antro (Lussier et al., 1987). Las células de la

granulosa que rodean íntimamente al ovocito, constituyen el cùmulus oophorus o corona radiata (Van Boris, 1998). El ovocito alcanza un diámetro de 92  $\mu\text{m}$  en folículos antrales tempranos (Braw-Tal y Yossefi, 1997). En el bovino, el folículo preovulatorio se caracteriza por un diámetro de 15 a 20 mm, un ovocito mayor a 120  $\mu\text{m}$ , y una corona radiata expandida por la secreción de ácido hialurónico por las células del cùmulus (Eppig, 2001).

Las extensiones de la corona radiata que penetran la zona y alcanzan el ovocito se retraen y finalmente se pierden (Hyttel et al., 1997). En esta fase, se reasume la meiosis y el ovocito progres a hacia los estadios finales de la meiosis I para luego ser arrestado en la metafase II (Hyttel et al., 1986; Lussier et al., 1987). Durante la fase final de desarrollo folicular, la transcripción de genes específicos y ciertos eventos bioquímicos causan una reacción de tipo inflamatoria que resulta en un incremento en la vasodilatación y aumento del flujo sanguíneo antes de la ruptura folicular. En este momento el folículo preovulatorio est á vascularizado abundantemente y sufre proliferación celular, remodelamiento del tejido, entre otros procesos (Espey 1994; Van Boris 1998; Richards 2002). La ovulación ocurre 25 - 30 h luego del pico de LH (Kaim et al., 2003).

#### Regulación hormonal del desarrollo folicular

El crecimiento y desarrollo folicular es un proceso complejo regulado por señales extraováricas, como gonadotrofinas y hormonas metabólicas (por ejemplo la hormona de crecimiento) y factores intraováricos de acción local. El desarrollo folicular se puede clasificar en independiente de gonadotrofinas y en dependiente

de gonadotrofinas (Webb et al., 1999). En la última, la hormona folículo estimulante (FSH, por sus siglas en inglés), produce el reclutamiento folicular, ya sea reclutamiento inicial desde folículos primordiales o cíclico a partir de folículos secundarios o antrales tempranos luego de la pubertad, y la hormona luteinizante (LH, por sus siglas en inglés) se requiere para continuar el desarrollo de los folículos hacia el estadio preovulatorio y la inducción de la ovulación (Adams et al., 2008). Las hormonas FSH y LH son sintetizadas por células especializadas (gonadotropos) en el lóbulo anterior de la hipófisis y libera gonadotrofinas en respuesta a la estimulación de la glándula por el decapéptido hipotalámico liberador de gonadotrofinas (GnRH, por sus siglas en inglés) (Reichter, 1998). Las gonadotrofinas son hormonas glicoprotéicas. La FSH actúa sobre el ovario promoviendo el desarrollo de los folículos secundarios y antrales tempranos hacia folículos preovulatorios (Ying, 1988). Un aumento en la concentración de FSH plasmática genera el reclutamiento de un conjunto de folículos para que inicien su crecimiento (Adams et al., 1992). Uno de los folículos será dominante y el resto serán subordinados. Al final del proceso existen dos caminos posibles para el dominante: la ovulación o la atresia folicular. El folículo dominante eventualmente será ovulado y los subordinados luego de unos días de desarrollo, involucionarán y entrarán en atresia. El número de folículos que logra la dominancia es específico. Como consecuencia de un aumento en la concentración de estradiol e inhibina sintetizados desde el folículo dominante, se postula que los folículos subordinados quedan expuestos a bajas concentraciones de FSH y factores

inhibitorios del crecimiento sintetizados por el folículo dominante (Fortune, 1994).

Por otro lado, los folículos dominantes tienen más receptores de FSH (Adams et al., 2008). La LH está asociada con la fase de dominancia folicular (Ginther et al., 2001) y estimula la maduración final del ovocito y la ovulación (Dieleman et al., 2002).

### Crecimiento ovocitario

En los folículos secundarios, los ovocitos experimentan la etapa de mayor crecimiento, incrementando su tamaño en aproximadamente 90  $\mu\text{m}$  debido a que crecen desde un tamaño menor a 30  $\mu\text{m}$  hasta más de 120  $\mu\text{m}$  (Braw-Tal and Yossefi 1997; Hyttel et al., 1997).

Durante el crecimiento suceden un aumento en la tasa metabólica, en la síntesis de ARN y proteínas ovocitarias (Schultz, 1986). Suceden cambios en su arquitectura y aparecen nuevas estructuras como la zona pelúcida y los gránulos corticales (GCs). Concomitante con su crecimiento, los ovocitos adquieren progresivamente la competencia meiótica a través de la acumulación de factores que les permitirán reasumir la primera división meiótica, progresar hacia la segunda y detenerse en metafase para posteriormente poder ser receptivo a la fecundación (Sorensen y Wassarman, 1976; Motlik et al., 1984; De Smedt et al; 1995). Fair et al. (1995) determinaron que los ovocitos de un diámetro de 100  $\mu\text{m}$ , adquieren la capacidad para reasumir la meiosis, y a un diámetro de 110  $\mu\text{m}$  la alcanzan para completar la maduración meiótica hasta el estadio de metafase II (MII). Sin embargo, la tasa de

clivaje y de formación de blastocistos luego de la fecundación se incrementa cuando el diámetro del ovocito supera los 120  $\mu\text{m}$  (De Loos et al., 1989).

La fase de crecimiento del ovocito está asociada con la formación de uniones de tipo estrechas entre el ovocito y las células del cúmulus, lo cual facilita la transferencia de señales moleculares así como también nutrientes entre el ovocito y las células del cúmulus (Eppig, 1991; Fair et al., 1997b). Durante la fase de crecimiento, suceden grandes modificaciones de las organelas del ovocito, incluyendo el retículo endoplásmico (RE) y las mitocondrias, también hay desarrollo de estructuras específicas del ovocito como los gránulos corticales (GCs). Éstos últimos tendrán un rol importante durante la fecundación, siendo responsables de bloquear la poliespermia junto con la zona pelucida. Aunque los ovocitos se encuentran arrestados en la profase de la primera división meiótica, la transcripción de ARN heterogéneo nuclear (ARNhn, los precursores de los ARNm) y ARNr y la actividad de síntesis protética continúan hasta que los ovocitos son meióticamente competentes (Fair et al., 1997a). Se ha demostrado que las cigotas heredan una reserva de macromoléculas y organelas desde el ovocito, el cual sustenta los requerimientos nutricionales, sintéticos, energéticos y regulatorios del embrión temprano (Wassermann y Albertini, 1994), estas reservas son acumuladas durante el crecimiento ovocitario y la maduración ovocitaria, aunque parte de los factores acumulados son destinados para llevar a cabo la propia maduración. Resumiendo, la etapa de crecimiento ovocitario es claramente un período

principalmente de síntesis mientras que la maduración ovocitaria es de síntesis, degradación y preparación final del citoplasma para la fecundación.

### Maduración de ovocitos

La maduración de los ovocitos es definida tradicionalmente como aquellos eventos asociados con la iniciación de la ruptura de la vesícula germinal (GVBD, por sus siglas en inglés) y la finalización de la primera división meiótica, esto es, la maduración nuclear. Una forma más apropiada de referirnos a la maduración ovocitaria sería como la sumatoria de aquellos eventos que llevan a la adquisición final de la capacidad de ser fecundado exitosamente y de iniciar el programa que dirigirá el desarrollo embrionario temprano (Leibfried-Rutledge et al., 1987). Este proceso constituye la maduración del núcleo y del citoplasma ovocitarios (Niemann y Meinecke, 1993). Durante esta etapa se pone de manifiesto la competencia meiótica y se alcanza el máximo potencial de desarrollo embrionario. La capacidad de un ovocito de madurar y de dar origen a un embrión depende de la acumulación de ARN mensajeros (ARNms) y proteínas específicas (Sirard et al., 2000), las cuales se almacenan progresivamente durante el crecimiento y la maduración ovocitaria (Wickramasinghe y Albertini, 1993; Fair et al., 1995). En el momento en que se produce la ovulación, el ovocito maduro debe contener grupos específicos de proteínas y ARNms que controlarán el desarrollo embrionario temprano, incluyendo todas las enzimas requeridas para el metabolismo ovocitario (Ménézo y Hérubel, 2002). En bovinos, la síntesis de ARN parece decrecer cuando los ovocitos alcanzan un diámetro de aproximadamente

110  $\mu\text{m}$  o el folículo alcanza un diámetro de 3 mm (Fair et al., 1995; 1996). Luego de este estadio, la síntesis de ARN heterogéneo nuclear (precursores de ARNm) se encuentra a bajos niveles (Fair et al., 1995), el cual puede ser detectado durante el estadio de GV y es posible hasta que tiene lugar la primera condensación de la cromatina (GVBD), consecuentemente se observa una reducción pronunciada en el estadio de metafase II (MII) (Memili et al., 1998; Tomek et al., 2002). Los mecanismos de regulación de la expresión de ARNms incluyen eventos pre-transcripcionales como modificaciones epigenéticas, de importancia fundamental en ovocitos y embriones tempranos (Wrenzycki et al., 2005), así como también, mecanismos de regulación post-transcripcionales. Estos últimos sirven para prevenir la degradación de los ARNms durante el almacenamiento en el ovocito y para controlar temporalmente la necesidad de utilizarlos durante la maduración ovocitaria y el desarrollo embrionario temprano. Estos mecanismos de la regulación de la expresión de los ARNms incluyen cambios en la cola de poly-A (Tomek et al., 1998, 2001; Richter 1999), ya sea alargamiento por poliadenilación o acortamiento por deadenilación ya descriptas en distintos organismos, y esenciales también en el bovino (Brevini et al., 1999; Gandolfi y Gandolfi, 2001) y regulación por micro-ARNs (Grosshans y Snack, 2002). La elongación de la cola de poli-A de los transcriptos se relaciona con la activación de los mismos para la traducción mientras que el acortamiento de la cola de poli-A se relaciona con el silenciamiento de los transcriptos (Richter, 1999). De esta forma, se permite la traducción de transcriptos de manera selectiva durante la maduración, la fecundación y el

desarrollo embrionario temprano hasta la transición materno-embriónaria (MET, por sus siglas en inglés), momento en el cual se activa el genoma embrionario (Memili y First, 1999; Tong et al., 2000). Esta fina regulación de la traducción está controlada además por fosforilación de factores iniciadores y proteínas ribosómicas (Tomek et al., 1998; 2001).

Como se mencionó anteriormente el proceso de maduración ovocitaria involucra una serie de eventos estructurales y moleculares, que conllevan a la madurez nuclear y citoplasmática y es estrictamente dependiente del ambiente folicular.

#### Maduración nuclear

Los eventos relacionados a la madurez nuclear comienzan con la ruptura de la vesícula germinal (GVBD), seguida de condensación cromosómica y formación del huso meiótico, expulsión del primer cuerpo polar y finalmente arresto de la división en metafase de la segunda división meiótica (Motlik y Fulka, 1976).

El reinicio de la meiosis *in vivo* sucede en respuesta a la estimulación por un pico endógeno de LH sobre el folículo preovulatorio. *In vitro*, la remoción del complejo ovocito-células del cúmulo (COC) del ambiente folicular reinicia espontáneamente la meiosis de aquellos ovocitos meióticamente competentes (Tsafirri et al., 1976). Este reinicio espontáneo de la meiosis se debe a que el ambiente folicular contiene factores inhibidores de la meiosis (Edwards et al., 1965), tales como el inhibidor meiótico ovocitario (OMI, por sus siglas en inglés) (Tsafirri et al., 1976), hipoxantina (Eppig et al., 1985) y AMP cíclico (Ferrel, 1999; Taieb et al., 1997). Este reinicio también puede ser inducido por gonadotrofinas

(FSH y LH) in vitro en respuesta a la señal desencadenada por la unión de estas hormonas a sus receptores en las células del cúmulo (Rodríguez y Farín, 2004). Lo anteriormente dicho se puede resumir en la figura 1.2.

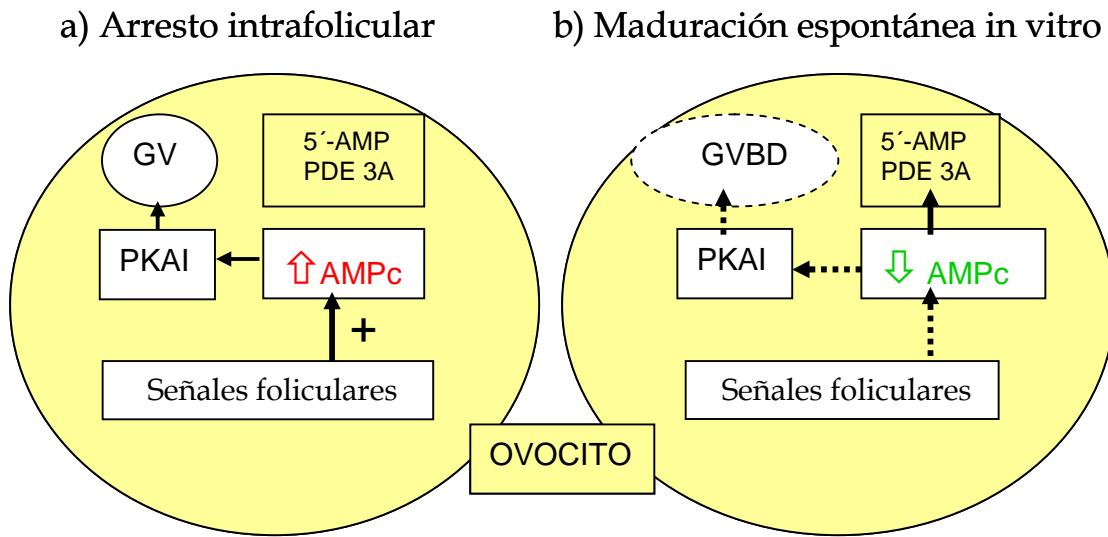


Fig. 1.2. Arresto intrafolicular y maduración espontánea in vitro. a) Los niveles elevados de AMPc mantienen el arresto meiótico del ovocito dentro del folículo. La proteína kinasa dependiente de AMPc de tipo I (PKA I, por sus siglas en inglés) y la fofodiesterasa 3A (PDE 3A, por sus siglas en inglés), están presentes en el citoplasma del ovocito. Los niveles elevados de AMPc activan a la PKA I y mantienen intacta la vesícula germinal (GV). b) Maduración espontánea como resultado de la remoción del ovocito del ambiente folicular inhibitorio. El AMPc ovocitario decrece y la PKA I deja de estar activada. La activación de PDE 3A podría mediar este paso y en consecuencia el nivel de AMPc intracelular cae, se

pierde la actividad de PKA I y puede ocurrir la ruptura de la vesícula germinal (GVBD). Líneas sólidas: activado, líneas interrumpidas: no activado (Rodríguez y Farín, 2004).

En general, el ciclo celular de las células eucariotas está regulado por dos familias de proteínas: la primera es la familia de las proteínas kinasas dependientes de ciclina (cdc, por sus siglas en inglés), las cuales inducen procesos fosforilando blancos específicos sobre serinas y treoninas. La segunda es la familia de proteínas activadoras especializadas, las ciclinas que se unen a las cdc y controlan su capacidad para fosforilar a las proteínas blanco. El ensamblaje de ciclinas y cdc, así como su activación y desensamblaje son procesos centrales que dirigen el ciclo celular. Uno de los principales protagonistas es el factor promotor de la metafase (MPF, por sus siglas en inglés) que es un heterodímero compuesto por una subunidad regulatoria, la ciclina B y una kinasa de serinas y treoninas dependiente de ciclina, el p34<sup>cdc2</sup> (Labbe et al., 1989; Pines y Hunter, 1989; Gautier et al., 1990). El MPF es el responsable de conducir la maduración del ovocito (Masui y Markert, 1971) y el proceso de mitosis en las células somáticas (Alberts et al., 1994). La reanudación de la meiosis en ovocitos de mamíferos está determinada por la activación de MPF. Esta activación requiere una determinada concentración de ciclina B (Lévesque et al., 1996), su asociación con p34<sup>cdc2</sup> (Clarke y Karsenti, 1991; Tatemoto et al., 1996) y la desfosforilación de p34<sup>cdc2</sup> (Tatemoto et al., 1996). El complejo ciclina B/ p34<sup>cdc2</sup> se encuentra fosforilado (pre-MPF) en la treonina 14 y

la tirosina 15 de p34<sup>cdc2</sup> (Gautier et al., 1988). Esta fosforilación lo mantiene inactivo y es llevada a cabo en parte por la kinasa wee1 (Parker et al., 1991; Mitra y Schultz, 1996; Charlesworth et al., 2000). La desfosforilación específica de la tirosina 15 y la treonina 14 de p34<sup>cdc2</sup> (Gautier et al., 1988; 1990; Dunphy y Newport, 1989) es mediada por los productos de los genes cdc25b y nim 1 (Russell y Nurse, 1986; Gautier et al., 1991). Una representación esquemática del proceso antes mencionado se muestra en la figura 1.3:

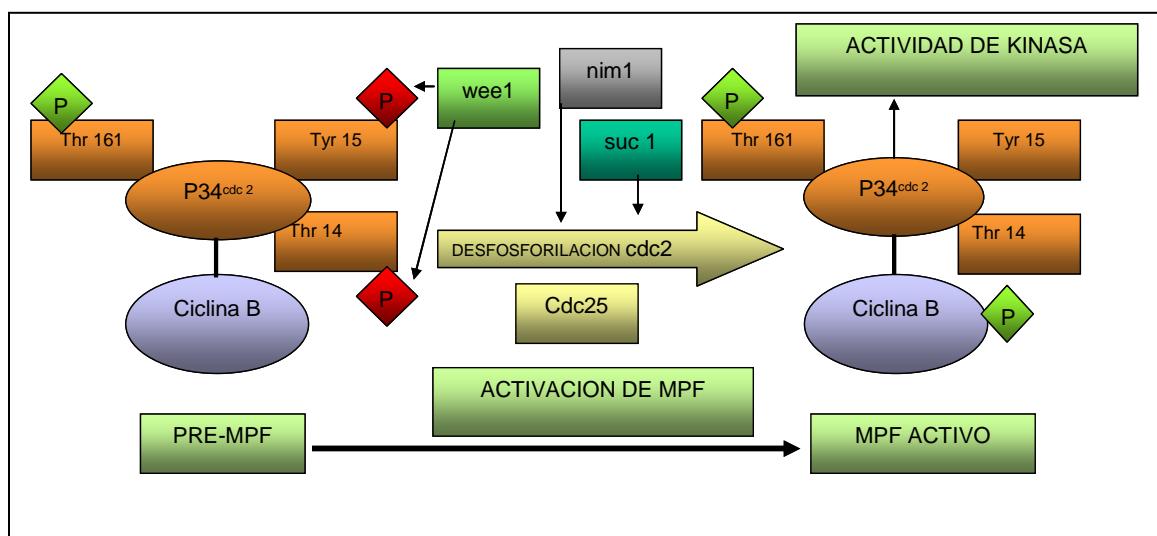


Fig. 1.3. Representación de la estructura molecular y activación del factor promotor de la metafase (MPF). El MPF está representado como un heterodímero compuesto por p34<sup>cdc2</sup> y una ciclina B. En su estado como pre-MPF, el heterodímero se encuentra fosforilado en la tirosina 15 (Tyr 15) y en la treonina 14 (Thr 14). La fosforilación en la treonina 14 y la tirosina 15 es mediada por la acción de wee1 no fosforilada. La activación de MPF es llevada a cabo primeramente por una combinación de cdc25b, nim1 y wee1. La forma fosforilada de cdc25b es la

responsable de la desfosforilación de la Tyr 15 y la Thr 14 de p34<sup>cdc2</sup>, activando a MPF. Wee1 desfosforilada previene la activación de MPF, manteniendo los residuos de Tyr 15 y Thr 14 en su forma fosforilada. Nim1 mantiene a wee1 en una forma hiperfosforilada que la hace incapaz de fosforilar los residuos de Tyr 15 y Thr 14 de p34<sup>cdc2</sup>, con lo cual previene a wee1 de fosforilar y por lo tanto inactivar a MPF. La molécula de MPF activa está desfosforilada en la Tyr 15 y la Thr 14 de p34<sup>cdc2</sup> y la ciclina está fosforilada. La forma activa de MPF posee la actividad kinasa de p34<sup>cdc2</sup>, también conocida como actividad kinasa de histonas H1 (Trounson et al., 2001).

El MPF activo cataliza la ruptura de la envoltura nuclear al fosforilar serinas de las láminas nucleares, lo cual induce su desorganización y desensamblaje (Alberts et al., 1994) entre otros eventos (figura 1.6). El MPF también fosforila directamente a las histonas H1 y a proteínas asociadas a los microtúbulos [MAPs, por sus siglas en inglés] (Alberts et al., 1994). Estas fosforilaciones por p34<sup>cdc2</sup> son esenciales para la ruptura de la vesícula germinal (GVBD) y la progresión meiótica (He et al., 1997), siendo el inicio de la traducción de los ARNm que codifican ciclina B uno de los eventos tempranos que conducen a la reanudación de la meiosis (Tremblay et al., 2005). Otra kinasa involucrada en la reanudación de la meiosis es la proteína kinasa activadora de mitógenos o kinasa regulada extracelularmente (MAPK o ERK, por sus siglas en inglés) que fosforila residuos en serina-treonina (Fissore et al., 1996). MAPK es parte de una cascada de proteínas kinasas involucrada en

distintas vías de señalización tanto en células germinales como en células somáticas. Existen dos isoformas de MAPKs en los ovocitos de mamíferos: ERK1 (p44) y ERK2 (p42) (Sun et al., 1999a). MAPK no es esencial para la ruptura de la vesícula germinal de ovocitos de ratón (Sun et al., 1999b). Luego de la ruptura de la vesícula germinal, estaría involucrada en la regulación de la dinámica de los microtúbulos, ensamblaje del huso meiótico, extrusión del primer cuerpo polar y mantenimiento de la organización en metafase (Alberts et al., 1994). MAPK interactúa con otras kinasas como p34<sup>cdc2</sup>, PKA, PKC, CaMKII y también con fosfatases (Heng-Yu Fan et al., 2004). Ambas kinasas, p34<sup>cdc2</sup> y MAPK, juegan un papel fundamental en la maduración de los ovocitos de mamíferos. En COCs bovinos cultivados *in vitro*, por ejemplo, se observa poliadenilación de ARNm que codifican activadores de MPF y MAPK entre las 9 y 12 h de iniciado el cultivo (Krischek y Meinecke, 2002). MAPK y MPF se activan, es decir, MAPK es fosforilada por una MAPK kinasa [MEK1, por sus siglas en inglés] (Pelech y Sanghera, 1992) en la treonina 183 y la tirosina 185 (Crews y Erickson, 1992) y la fosfatasa cdc25b desfosforila la tirosina 15 de MPF (Minshull et al., 1989; Dai et al., 2000; Palmer y Nebreda, 2000) al tiempo en que se produce la ruptura de la vesícula germinal (Motlik et al., 1998). Los altos niveles de MPF y MAPK son el pre-requisito para la entrada en mitosis en cualquier célula somática. En particular, el arresto ovocitario en metafase II es MPF dependiente (Tian et al., 2002). *In vivo* la activación de MPF se produce en respuesta al pico endógeno de LH (Trounson et al., 2001).

Los proteasomas tienen un rol fundamental en la regulación del ciclo celular (Goldberg 1995; Coux et al., 1996; Hershko 2005). Estudios previos demostraron la importancia de los proteasomas para completar la meiosis I por disminuir la actividad de MPF a través de la degradación de ciclinas B en ovocitos de rata (Josefsberg et al., 2000).

Otra proteína de importancia fundamental en la maduración es el producto del proto-oncogen c-mos, originalmente identificado por Frankel y Fischinger en 1976. Mos es una kinasa de serinas y treoninas de 39 KDa. Roy et al. (1990) demostraron que Mos fosforila a ciclinas B2 y por lo tanto actúa sobre la activación de MPF. Sin embargo no es esencial para la maduración de ovocitos de ratón (O'Keele et al., 1989; Araki et al., 1996). En ovocitos de *Xenopus*, funciona como activador de MAPK (Posada et al., 1993). En ovocitos bovinos, la activación de MAPK luego de inyectar ARN de Mos y detectar la proteína Mos, evidenció la presencia de una vía de transducción de señal Mos/MAPK (Fissore et al., 1996; Wu et al., 1997). Mos se asocia al factor citostático (CSF, por sus siglas en inglés), promoviendo el arresto meiótico directa o indirectamente por estabilizar la actividad de MPF durante la metafase II (Sagata et al., 1989) y además previene la progresión del ciclo celular mas allá de este estadío. Mos es sintetizado activamente en ovocitos bovinos maduros, se lo ha encontrado asociado a p34<sup>cdc2</sup> y a β-tubulina (Wu et al., 1997) lo cual sugiere que su presencia durante la maduración tendría un papel regulatorio importante. Las proteínas asociadas a los microtúbulos (MAPs), p34<sup>cdc2</sup>, MEK1 y β-tubulina son posibles sustratos de Mos (Zhou et al., 1991). En ovocitos de *Xenopus*,

de ratón y de bovinos, Mos activa a MAPK (Nebreda y Hunt, 1993; Fissore et al., 1996; Verlhac et al., 1996). Todos los cambios que ocurren a nivel nuclear y citoplasmático durante la maduración están íntimamente relacionados. La progresión de la maduración nuclear es controlada por componentes de la matriz citoplasmática (Eppig et al., 1994). La figura 1.4 resume los principales reguladores de la maduración ovocitaria y las interacciones descriptas anteriormente.

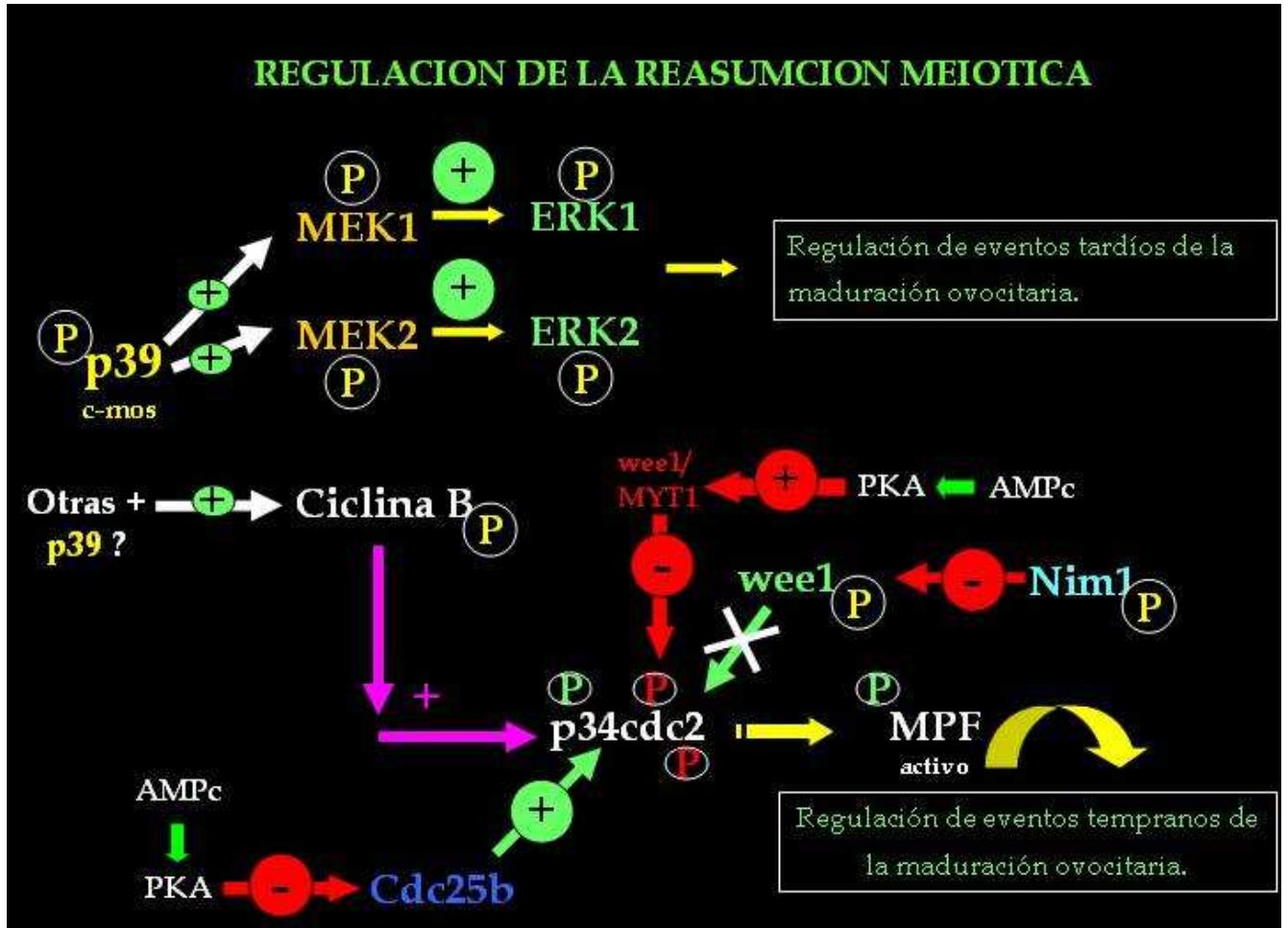


Fig. 1.4. Resumen de la regulación de la reasunción meiótica. El esquema muestra los principales reguladores de la maduración ovocitaria y sus vías de activación o inactivación descriptas. Los signos (+) y las flechas en color verde indican el efecto positivo entre los dos factores conectados, los signos (-) y las flechas en color rojo indican el efecto negativo desde el primer factor sobre el segundo. (Información reunida desde Mehlmann, 2005; Dekel, 2005; Trounson et al., 2001; Fan y Sun, 2004).

## Factores que regulan la maduración nuclear: AMPc, células del cúmulus y hormonas

El estado de arresto en el que se encuentran los ovocitos de mamíferos es consecuencia de los efectos inhibitorios de ciertos componentes presentes en el líquido folicular. En 1935 Pincus y Enzman demostraron que al remover ovocitos en el estadio de vesícula germinal del ambiente folicular, éstos iniciaban espontáneamente la maduración meiótica *in vitro*. Ha sido demostrado que dos tipos de moléculas inhibitorias son importantes en controlar la maduración meiótica, los nucleótidos cíclicos y las purinas. Se demostró en roedores (Schultz et al., 1983) así como también en bovinos (Aktas et al., 1995a; 1995b) que la adenosina monofosfato cíclica (AMPc) puede bloquear la maduración meiótica espontánea de los ovocitos en cultivo. Los análogos de AMPc, así como también, inhibidores de la enzima fosfodiesterasa [PDE, por sus siglas en inglés] (isobutil-metil-xantinas, IBMX) que previenen la degradación intracelular de AMPc, suprimen reversiblemente la ruptura de la vesícula germinal *in vitro* (Sirard y First, 1988). Estudios recientes sugieren que el mantenimiento del arresto meiótico está regulado por una intercomunicación entre AMPc, cúmulus, células de la granulosa y células tecales (Richard et al., 1997; Richard and Sirard 1998; Aktas et al., 2003). Otros nucleótidos cíclicos también podrían estar involucrados en el control de la meiosis. Los análogos de guanosina 3'-5'-monofosfato cíclica (GMPc) y la microinyección de GMPc han mostrado inhibición de la maduración ovocitaria espontánea (Tornell et al., 1990). Las purinas, tales como la hipoxantina y la

adenosina se hallaron a concentraciones milimolares en preparaciones de fluido folicular que mantiene a los ovocitos en arresto meiótico *in vitro*, probablemente a través de la supresión de la degradación de AMPc (Downs et al., 1989; 1993a; 1999). Un efecto limitado de las purinas en ovocitos bovinos también fue demostrado (Sirard, 1990).

A medida que el folículo crece y el ovocito adquiere la capacidad para madurar y desarrollar, resulta de gran importancia una estrecha comunicación entre la célula germinal y las células que la rodean [células del cúmulo] (Eppig et al., 2002). Esta comunicación es bidireccional (Eppig et al., 2002) a través de uniones de tipo estrechas que posibilitan el intercambio de factores que modulan y coordinan la maduración nuclear y citoplasmática (Carabatsos et al., 2000). Las uniones de tipo estrechas están formadas por las conexinas 43 (Cx43, por sus siglas en inglés), entre otras, y son fundamentales para mantener la comunicación entre el ovocito y las células del cúmulo y entre las células del cúmulo entre sí. Mediante esta comunicación las células del cúmulo producirían una señal de reinicio de la meiosis en el ovocito en respuesta a gonadotrofinas (Byskov et al., 1997; Rodríguez et al., 2002). El esterol activador de la meiosis (MAS, por sus siglas en inglés) es sintetizado y secretado por las células del cúmulo en respuesta a FSH (Byskov et al., 1997). Existe una fase inicial de inhibición de la meiosis en bovinos al agregar FSH al medio de maduración de los COCs, pero esta inhibición es seguida por un rápido aumento en el número de GVBD alcanzadas comparado con la maduración espontánea (Farín y Yang, 1994). Inicialmente se creía que el hecho de que la FSH

retarde la maduración nuclear aumentaría la capacidad de desarrollo de los ovocitos, ya que los eventos de la maduración citoplasmática sucederían en un período mayor (Armstrong et al., 1991). Sin embargo, Mermilliod et al. (2000) demostraron que el uso del inhibidor específico de p34<sup>cdc2</sup>, roscovitina, utilizado antes del inicio de la maduración no afectaba la capacidad de desarrollo final de los ovocitos, sugiriendo que la inhibición temporaria por FSH produce algo mas que un arresto meiótico en GV. Las células del cúmulus estimuladas por FSH aumentan sus niveles intracelulares de AMPc y consecuentemente en el ovocito, lo cual en principio inhibe la maduración por control negativo sobre MPF (Tatemoto et al., 1997). Previamente, Eppig et al. (1984) demostraron que la adenosina monofosfato en su forma cíclica (AMPc) juega un rol fundamental en el mantenimiento del arresto meiótico en el estadio de GV, por afectar tanto al ovocito como a las células del cúmulus. El AMPc es sintetizado por la enzima adenil ciclase (AC) a partir de AMP y regula a la proteína kinasa dependiente de AMPc (PKA). Existen mayoritariamente dos isoenzimas, PKA tipo I y PKA tipo II y se distinguen por sus propiedades bioquímicas y sus subunidades regulatorias. Además de la síntesis, otra forma de regulación de la concentración de AMPc intracelular está dada por las PDEs, enzimas que catalizan la reacción inversa a la AC. Se ha demostrado en todas las especies estudiadas cierta distribución de las PDEs en las células del ovario, encontrándose PDE 3 en ovocitos y PDE 4 en células somáticas (Conti et al., 2002). La maduración espontánea ocurre en respuesta a una disminución en los niveles de AMPc ovocitario a causa de la

remoción del COC de los factores foliculares inhibitorios, esta disminución sería pasiva o a través de la activación de PDE 3A presente en el citoplasma del ovocito (Conti et al., 1998), en cuanto que la maduración inducida por gonadotrofinas ocurre en respuesta a una cascada de señalización a partir de la unión de FSH a sus receptores en las células del cúmulus, cuyo primer efecto es un aumento en la concentración de AMPc intracelular que activa tanto a PKA I como a PKA II en el cúmulus (Downs y Hunzicker-Dunn, 1995; Rodriguez et al., 2002). La activación de PKA I en el cúmulus deriva en una inhibición temporaria de la maduración por mantener altos los niveles de AMPc en el ovocito independientemente de la transcripción de genes en el cúmulus. Mientras que la activación de PKA II en el cúmulus lleva a la transcripción de genes en las mismas y las proteínas sintetizadas inducen la maduración a través de la disminución de AMPc en el ovocito por PDE 3A (Rodríguez et al., 2002). Ver figura 1.5.

Una concentración elevada de AMPc en el ovocito activa la PKA I ovocitaria que mantiene la fosforilación inhibitoria sobre p34<sup>cdc2</sup> (Tatemoto et al., 1997). Cuando los niveles de AMPc disminuyen se activa MPF y se desencadena la ruptura de la vesícula germinal. Una síntesis esquemática del proceso antes descripto se muestra en la figura 1.5.

a) Fase inhibitoria de la maduración *in vitro* inducida por FSH      b) Fase estimulatoria de la maduración *in vitro* inducida por FSH

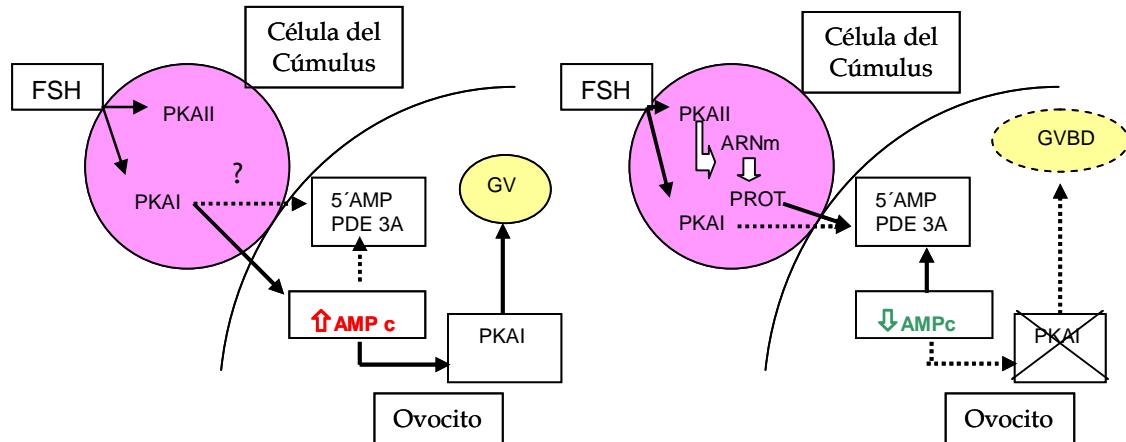


Fig. 1.5. Fases inhibitoria y estimulatoria inducidas por FSH durante la maduración *in vitro*. a) Luego de la unión de FSH a la membrana celular de las células del cúmulo aumentan los niveles de AMPc intracelulares de éstas, activándose la PKA I y la PKA II. La activación inicial de PKA I en el cúmulo resulta en altos niveles de AMPc en el ovocito. Esto inhibe temporalmente la maduración manteniendo la vesícula germinal intacta. b) La unión de FSH a sus receptores resulta en la activación de PKA II que conlleva al inicio de la transcripción de genes. Los ARNm nuevos se requieren para la ruptura de la vesícula germinal (GVBD), éstos codifican proteínas que estimulan a la PDE 3A ovocitaria, cuya actividad desfavorece el arresto meiótico temporario mediado por PKA I. Líneas sólidas: activado; líneas punteadas: no activado (Rodríguez y Farín, 2004).

### Maduración citoplasmática

La maduración citoplasmática incluye cambios en el patrón de síntesis de ARNms y proteínas y rearreglos estructurales del citoplasma ovocitario (Motlik y Kubelka, 1990). Ocurre la localización de los GCs subyacentes a la membrana plasmática en el oolema, reorganización de la corteza y el citoesqueleto asociado, redistribución del sistema de endomembranas (el retículo endosoplásmico [RE] y el aparato de Golgi) y relocalización de ARNms, evento descripto en invertebrados y anfibios (Sardet et al., 2002) y en mamíferos (Brevini et al., 2007). Las microvellosidades por su parte, se extienden dentro del espacio perivitelino recién formado (Damiani et al., 1995).

La ruptura de la vesícula germinal en bovinos requiere de síntesis protéica, la utilización de inhibidores de la síntesis protéica como cicloheximida inhibe la maduración (Sirard et al., 1989). A 6 h de iniciado el cultivo de los COCs comienza la traducción de los ARNms, previa activación por poliadenilación por la enzima poly-A polymerase [PAP, por sus siglas en inglés] (Tomek et al., 1998, 2001; Richter 1999), que codifican las proteínas necesarias para GVBD (Krischek y Meinecke, 2002), el resto de la maduración y también para etapas posteriores. El período de máxima síntesis protéica en ovocitos bovinos ocurre en las 12 horas iniciales de cultivo, período que cubre el evento de GVBD (Bin Wu et al., 1996). Más allá de la síntesis, localización y activación de las proteínas, se requiere una serie de etapas previas en relación a los ARNms ovocitarios, precursores de esas proteínas. Estas etapas son la regulación de la síntesis por mecanismos pre-

transcripcionales a nivel del ADN (modificaciones epigenéticas en determinados genes) como post-trancripcionales a nivel del ARNm (cambios en la cola de poly A y micro-ARN), la localización citoplasmática y la traducción de los ARNm ovocitarios (Brevini et al., 2007), demostrando lo complicado que es ordenar espacio-temporalmente los factores que direccionarán la maduración del ovocito y el desarrollo embrionario temprano luego de la fecundación. Un concepto relativamente nuevo surgió en la última década, con los estudios de expresión de abundancias relativas (RA, por sus siglas en inglés) de ARNm en ovocitos y embriones tempranos (Wrenzycki et al., 2007), en un esfuerzo por descubrir el perfil de expresión de ARNm y sus efectores finales, producto de los transcriptos. La localización específica de proteínas, ARNm y organelas son fundamentales durante la maduración ovocitaria y estas localizaciones son llevadas a cabo por el citoesqueleto celular y sus proteínas motoras o motores moleculares asociados. Los microfilamentos de actina y los microtúbulos de tubulina cumplen funciones específicas durante la maduración ovocitaria (Sun and Schatten, 2006; Carabatsos et al., 2000; Wang et al., 2004; FitzHarris et al., 2007; Zhang et al., 2007). Al inhibir específicamente ciertos motores moleculares asociados a microtúbulos como cytoplasmic dynein (por su nombre en inglés), se han observado desordenes citoplasmáticos y bloqueo meiótico en ovocitos de ratón (Carabatsos et al., 2000; Wang et al., 2004; FitzHarris et al., 2007).

## **Reestructuración del citoesqueleto**

La red de microfilamentos, microtúbulos y filamentos intermedios que forman el citoesqueleto sufren modificaciones y una marcada polarización durante la maduración ovocitaria. En ovocitos arrestados en algún estadio particular del ciclo celular, como metafase II en la mayoría de los mamíferos, el huso meiótico interactúa con la corteza (Maro et al., 1986). En mamíferos se ha descripto la presencia de microtúbulos en la corteza del ovocito en el estadio de vesícula germinal (Mattson y Albertini, 1990; Albertini, 1992) y ausencia en el estadio de metafase II (MII). En ovocitos de cerdo se ha confirmado que no existe una red de microtúbulos en el citoplasma en el estadio de GV (Lee et al., 2000). En el estadio dictiásico temprano, una vez formados los bivalentes y antes de la ruptura de la vesícula germinal, los microtúbulos se polimerizan en la periferia de la GV y en la corteza celular. En el estadio dictiásico tardío luego de GVBD, los microtúbulos se encuentran asociados a los cromosomas, se polarizan mientras los cromosomas se alinean en la placa ecuatorial. Durante la segregación cromosómica en anafase I y telofase I, el huso meiótico se elonga y rota. Finalmente, luego de la expulsión del primer cuerpo polar el huso se formará mediante un proceso similar al ocurrido durante la transición GV a metafase II (Lee et al., 2000). Parece ser característica la presencia de regiones ricas en actina cubriendo el huso meiótico en zonas cercanas al córtex (Evans et al., 2000).

## **Reestructuración y redistribución del aparato de Golgi, retículo endoplásmico y gránulos corticales**

El sistema de endomembranas que forman el aparato de Golgi se encuentra particionado y disperso en el estadio de vesícula germinal en ovocitos bovinos, mientras que se vesiculiza y se relocaliza en dominios ooplásmicos específicos desde el evento de GVBD hasta el estadio de metafase II (Hyttel et al., 1986; Payne y Schatten, 2003). En el caso de células somáticas se demostró que existe relación entre la fragmentación del aparato de Golgi y MPF ya que p34<sup>cdc2</sup> fosforila a una de las proteínas de la matriz del aparato de Golgi (GM130) que se asocia a los polos del huso mitótico durante la mitosis (Lowe et al., 1998; Seeman et al., 2002). Como los ovocitos de mamíferos contienen una alta concentración de ciclina B1, ciclina B2, p34<sup>cdc2</sup> y fosfatases, en estado de ARNm y proteína, es posible que estas moléculas cumplan un rol importante sobre proteínas del aparato de Golgi que conllevan a la fragmentación y relocalización de esta organela durante la meiosis (Payne y Schatten, 2003). A diferencia del aparato de Golgi, la red endomembranosa que forma el retículo endoplásmico se encuentra inicialmente concentrado alrededor de la vesícula germinal y formando parches aislados cercanos a la zona cortical. Hacia el estadío de metafase II, se relocaliza y distribuye a través del ooplasma y está ausente en la zona que rodea al huso meiótico en ovocitos en metafase II (Payne y Schatten, 2003). No se ha observado colocalización del RE y el aparato de Golgi en el estadío de vesícula germinal y durante la migración y aposición de pronúcleos luego de la fecundación;

contrariamente a lo observado en el estadio de metafase II y luego del primer clivaje embrionario (Payne y Schatten, 2003). En los mamíferos, los pequeños agregados de retículo endoplásmico cortical esparcidos en el ovocito inmaduro crecen en tamaño y número hasta el momento en que queda establecido el arresto meiótico (Shiraishi et al., 1995; Kline, 2000). El RE es el principal reservorio ovocitario de  $\text{Ca}^{++}$  y responde a IP3; la capacidad del ovocito para responder a IP3 es adquirida durante la maduración y queda establecida con el aumento del número de receptores para IP3 (IP3R, por sus siglas en inglés) presentes en esta organela. Habría relación entre los cambios de este sistema de membranas durante la maduración con la actividad de MPF y con la posibilidad de liberar  $\text{Ca}^{++}$  rápidamente en respuesta a IP3 en la fecundación (Shiraishi et al., 1995; Terasaki et al., 2001). La capacidad de liberar  $\text{Ca}^{++}$  masivamente también es un indicador de madurez citoplasmática (He et al., 1997). Contrariamente a la cantidad de información sobre la homeostasis del  $\text{Ca}^{++}$  durante la fecundación y la activación ovocitaria, el papel de este catión bivalente fue mucho menos estudiado durante la maduración ovocitaria en mamíferos (Homa, 1995). En ovocitos bovinos, el  $\text{Ca}^{++}$  parece ser necesario para la GVBD y la progresión de la meiosis (Homa, 1991). Los receptores de 1,4,5 inositol trifosfato (IP3R) y los receptores de ryanodina (RyR, por sus siglas en inglés) median la liberación intracelular de  $\text{Ca}^{++}$  en bovinos (Yue et al., 1995). La cascada mediada por IP3R libera mayoritariamente el  $\text{Ca}^{++}$  durante la maduración y la fecundación. Entonces, niveles bajos de  $\text{Ca}^{++}$  intracelular

afectan la progresión de la meiosis por inhibición de la activación de kinasas (He et al., 1997).

En ovocitos de *Xenopus* se ha demostrado recientemente que el Ca<sup>++</sup> modula tanto la maquinaria del ciclo celular como la maduración nuclear (Lu Sun et al., 2004).

El número de GCs en la corteza se incrementa continuamente durante el crecimiento del ovocito de ratón, mientras que este aumento está acotado a la etapa final del crecimiento en la mayoría de las especies estudiadas (Ducibella et al., 1994). Desde el estadio de GV hasta el de metafase I y II, los GCs se acercan a la corteza de forma progresiva junto con la migración de la vesícula germinal hacia la periferia de la célula en roedores (Ducibella et al., 1994). No existen evidencias de que los microtúbulos estén involucrados en la translocación de los GCs a la periferia del ovocito durante la maduración, pero se ha observado que los microfilamentos de actina cumplen un papel tanto en la translocación como en la secreción de los GCs. La translocación de los GCs durante la ovogénesis está regulada por un mecanismo dependiente de microfilamentos de actina (Berg y Bessel, 1997; Connors et al., 1998). Los GCs se localizan y anclan a la corteza del ovocito esperando la señal mediada por Ca<sup>++</sup> para la exocitosis masiva en el proceso de fecundación.

En conclusión, la maduración final de los ovocitos ocurre luego del pico de LH in vivo, y se caracteriza por la expansión del cúmulus, ruptura de las uniones de tipo estrechas entre el ovocito y la corona radiata, incremento del contenido de lípidos, reducción del aparato de Golgi, alineamiento de los gránulos corticales en el cortex

ovocitario, extrusión del primer cuerpo polar y arresto en metafase de la segunda división meiótica (Hyttel et al., 1986; Hyttel et al., 1989; Hyttel et al., 1997).

La figura 1.6 muestra fotografías de microscopía confocal de algunos eventos celulares que ocurren durante la maduración ovocitaria bovina realizadas en estudios preliminares.

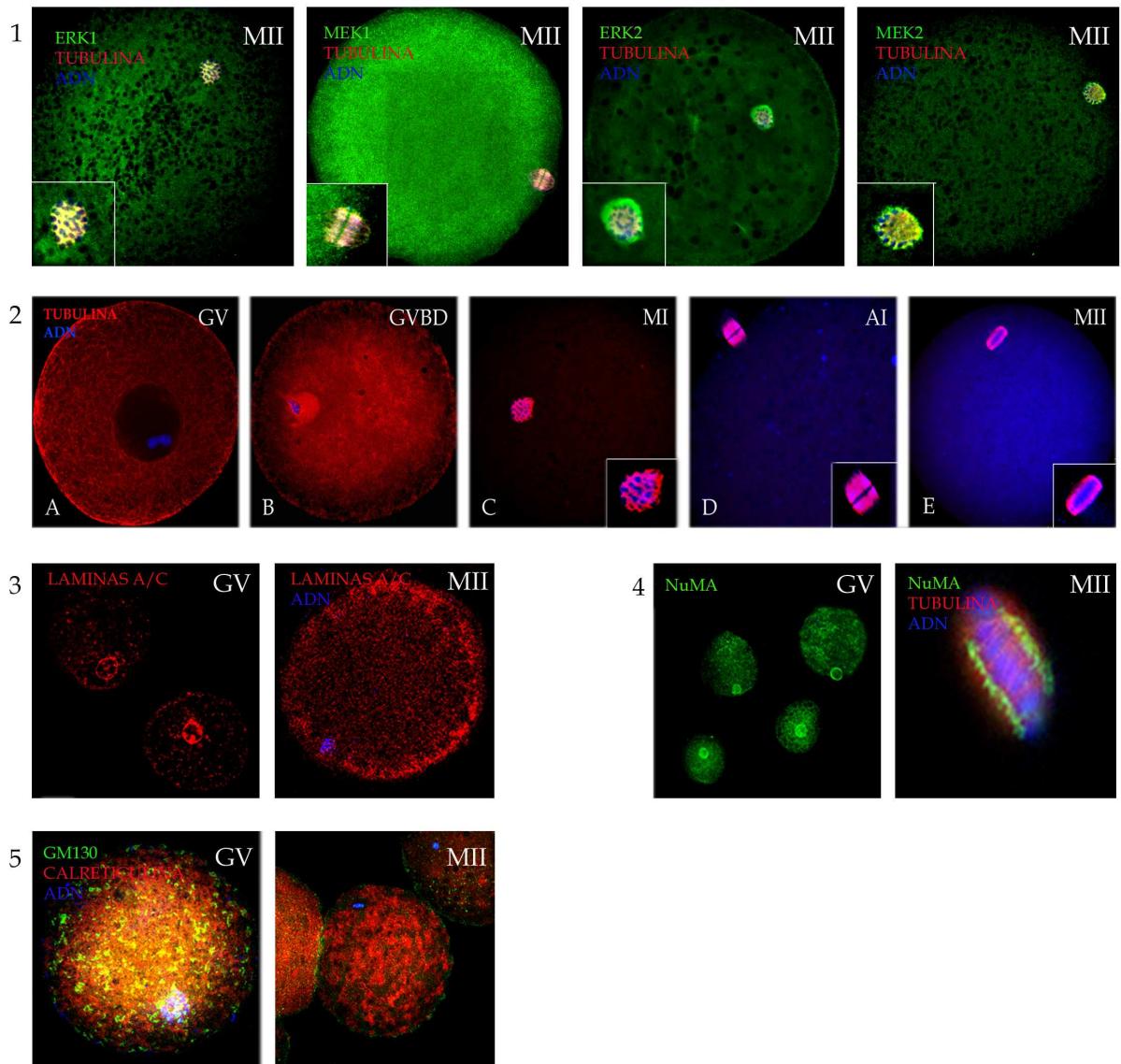


Fig. 1.6. Imágenes de microscopía confocal de eventos citoplasmáticos durante la maduración ovocitaria bovina, estudios preliminares. 1) Localización de las MAPKs (ERKs) ovocitarias y sus activadores MAPKKs (MEKs) en husos meióticos de metafases II (MII). 2) Reestructuración del citoesqueleto de tubulina durante la

maduración ovocitaria (GV, vesícula germinal; GVBD, ruptura de la vesícula germinal; MI, metafase I; AI, anafase I; MII, metafase II). 3) Láminas nucleares A/C, el desensamblaje es producto de la fosforilación por p34<sup>cdc2</sup>. 4) NuMA en la vesícula germinal de ovocitos y relocalización hacia los polos del huso meiótico en MII como resultado de la fosforilación por p34<sup>cdc2</sup>. 5) Fragmentación y redistribución del aparato de Golgi (marcador utilizado: GM130) y el retículo endoplásmico (marcador utilizado: Calreticulina), procesos que también estarían regulados por fosforilacion por p34<sup>cdc2</sup>.

### Maduración de ovocitos: Diferencias y similitudes entre modelos animales.

Para completar la maduración *in vitro* de manera exitosa, los ovocitos deben provenir de folículos que hayan alcanzado un tamaño mínimo, tanto en bovinos (Pavlok et al., 1992), ovinos (Szollosi et al., 1988), caprinos (Crozet et al., 1995), porcinos (Motlik et al., 1984), monos (Schramm et al., 1993; Gilchrist et al., 1995), equinos (Goudet et al., 1997) y humanos (Tsuij et al., 1985). En bovinos los folículos de 2 mm de diámetro ya contienen ovocitos capaces de sustentar el desarrollo embrionario luego de la fertilización (Pavlok et al., 1992).

La maduración *in vitro* de ovocitos provenientes de folículos preantrales seguida de fecundación y nacimientos fue exitosa únicamente en ratones. Debido a las diferencias fisiológicas entre especies poliovulares como el ratón y las monovulares como el humano (Tabla 1.1), es probable que el bovino sea un buen modelo para aproximarse a la fisiología de ovocitos humanos (Ménézo y Hérubel, 2002).

Tabla 1.1. Algunos parámetros de ovocitos y embriones de ratón, bovinos y humanos (tomado de Betteridge y Rieger, 1993).

	Humano	Bovino	Ratón
.....			
Diámetro ovocitario ( $\mu\text{m}$ )	120-150	150-180	90-100
Tiempo (hs.) para alcanzar:			
Estadío de 2 células	30	36	12
Blastocisto	120	150	70
Eclosión	150	200	100
Estadío de activación del genoma embrionario:	4 células	8 células	2 células

### Naturaleza del aporte original proyectado

Usualmente los ovocitos bovinos utilizados para la producción in vitro de embriones son obtenidos desde folículos de 2 - 8 mm. Los ovarios utilizados contienen una gran cantidad de folículos que por ser pequeños no se utilizan. Se propone un estudio celular y molecular de ovocitos bovinos con capacidad de desarrollo diferencial, esto es, ovocitos de distinta calidad con el objetivo de aumentar el conocimiento básico del proceso de maduración y la obtención de posibles marcadores del potencial de desarrollo ovocitario. El hecho de trabajar en un modelo bovino aumenta las posibilidades de aplicar la información obtenida en la clínica humana ya que se trata de una especie que comparte grandes similitudes con el humano durante el desarrollo embrionario temprano. Por otro lado es una especie ampliamente desarrollada en las técnicas de producción in vitro de embriones y se dispone de grandes cantidades de material que puede utilizarse para optimizar nuevos sistemas de producción o incluso aproximarse a la obtención de posibles marcadores de calidad ovocitaria, de utilidad tanto en especies pecuarias como en la clínica de reproducción asistida.

Se obtendrán grupos de ovocitos provenientes de folículos de distintos tamaños y se evaluará su capacidad de desarrollo luego de fecundación in vitro. Se caracterizarán aspectos moleculares como expresión de ARNm y celulares como distribución de proteínas y modificación de proteínas en los grupos de ovocitos en estudio que presenten diferente capacidad de desarrollo in vitro.

## Hipótesis

*La capacidad de desarrollo de los ovocitos bovinos provenientes de folículos de diferentes tamaños está asociada a los niveles de ARNm<sub>s</sub>, a la distribución de proteínas y a las modificaciones de histonas existentes en los distintos estadios de la maduración ovocitaria.*

## Objetivos

- I) Comparar la madurez funcional (nuclear y citoplasmática) de ovocitos bovinos provenientes de folículos pequeños (< 2 mm) y grandes (2 – 8 mm).
- II) Evaluar los efectos del tamaño folicular y el estadio meiótico sobre la expresión de ARNm<sub>s</sub> de:
  - motores moleculares: DYNLL1, DYNC1I1 y DCTN1
  - 20S proteasomas: PMSB1 y PMSB4
  - poliadenilación: PAP
  - comunicación intercelular: Cx43
  - histonas-metiltrasferasas: G9A y SUV39H1
  - ADN metiltransferasas: DNMT1 y DNMT3b
  - factor de reprogramación: ZAR1
- III) Evaluar los efectos del tamaño folicular y el estadio meiótico sobre las distribuciones protéicas de:
  - motores moleculares: dyenin (DYNC1I1) y dynactin (DCTN1)
  - 20S proteasomas ( $\alpha\beta$  subunits)
  - histonas modificadas: H3-K9diMe y H4-K12 acetylation

## **Capítulo 2**

### **Effects of follicle size and stages of maturation on mRNA expression in bovine in vitro matured oocytes**

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## Summary

A well-orchestrated expression of genes is required to ensure progression of mammalian oocytes from the first meiotic arrest to the metaphase II stage. The goal of this study was to unravel the dynamics of transcripts thought to be critically involved in oocyte maturation. The relative abundance (RA) of DYNLL1 (cytoplasmic dynein light chain LC8), DYNC1I1 (cytoplasmic dynein 1 intermediate chain), DCTN1 (dynactin 1; pGlued homolog, the activator of the cytoplasmic dynein complex 1), PMSB1 (proteasome beta subunit 1), PMSA4 (proteasome alfa subunit 4), PAP (poly-A polymerase) and Cx43 (connexin 43) were determined by semi-quantitative endpoint RT-PCR at different stages of IVM, that is, GV, GVBD, MI and MII in oocytes collected from follicles of two different size categories, that is, < 2 mm and 2–8 mm. The RA of DYNLL1 and DYNC1I1 were significantly higher in immature oocytes from bigger follicles than in oocytes from small follicles. Messenger RNA expression levels were similar for DCTN1, PMSB1, PMSA4, PAP, and Cx43 in the two groups during the maturation process. RA of DYNLL1, DYNC1I1 and PMSB1 decreased significantly during IVM in oocytes from follicles 2 to 8 mm. The RA for DYNLL1 was significantly higher in GVBD and MI in the oocytes from follicles 2 to 8 mm in size compared to the other group. The higher mRNA expression of DYNLL1 and DYNC1I1 and the diverging dynamics of DYNLL1, DYNC1I1, and PMSB1 mRNA expression during IVM in oocytes from the different follicle categories could be related to the developmental competence, that

is, development after IVF. The differences found between groups of oocytes could serve as a marker to assess the developmental competence of bovine oocytes.

## **Introduction**

The relationship between follicle size and oocyte developmental competence is well established in several species, i.e. human (Tsuji et al., 1985), pig (Motlik et al., 1984), sheep (Szollosi et al., 1988), cow (Pavlok et al., 1992), goat (Crozet et al., 1995), monkey (Schramm et al., 1993; Gilchrist et al., 1995), and horse (Goudet et al., 1997). Bovine oocytes recovered from small follicles developed to the blastocyst stage in vitro at a significantly lower frequency than their counterparts from bigger follicles (>2mm; Pavlok et al., 1992; Lonergan et al., 1994; Blondin and Sirard, 1995; Hagemann et al., 1999; Lequarre et al., 2005). Prior to maternal-embryonic transition (MET), i.e. 8- to 16-cell stage in the bovine, the oocyte is dependent on mRNA and proteins, which are accumulated during oogenesis (Memili and First, 2000). Oocytes isolated from different size follicles may thus serve as a model to study factors involved in the acquisition of developmental competence during follicular development. A well-orchestrated expression of genes is required to ensure progression of mammalian oocytes through the meiotic stages to achieve full developmental competence. De novo synthesis of RNA has been observed up to the germinal vesicle stage (GV), and ceases after GV breakdown [GVBD] (Memili et al., 1998; Tomek et al., 2002). Thus nuclear and cytoplasmic maturation are subject to regulation by the RNA pools synthesized during the growth phase and the first period of maturation.

The genes selected for this study are indicative for critical steps during the maturational process of the oocyte such as transport of molecules and organelles

(DYNLL1, DYNC1I1, DCTN1), cell cycle progression (PMSB1, PMSA4), polyadenylation (PAP) and gap junction formation (Cx43). DYNLL1 and DYNC1I1 are critical constituents of the cytoplasmic dynein 1 complex (King et al., 1998; Huang et al., 1999; Lo et al., 2001; Susalka et al., 2002), which represents the most abundant transport system of the cell (Pfister et al., 2006). A third messenger, DCTN1, is associated as protein with the cytoplasmic dynein complex 1 (Karki and Holzbaur, 1995, 1999; Vaughan and Valle, 1995; Allan, 1996; Valle and Sheetz et al., 1996; Steffen et al., 1997). These three genes are crucially involved in the meiotic progression of Xenopus and mouse oocytes (Chi-Ying et al., 1999; Wang et al., 2004). When dyneins were inhibited specifically with sodium-orthovanadate, a dynein specific inhibitor, meiosis of mouse oocytes was blocked at GV stage or in anaphase I (Wang et al., 2004); thus highlighting the significance of these proteins during oocyte maturation and the subsequent embryo development. The genes PMSB1 and PMSA4 are subunits of the 20S proteasomes complex, which is the catalytic core of the 26S proteasomes, and belongs to the ubiquitin-dependent proteolytic system required for protein degradation in all eukaryotic cells (Rock et al., 1994). PMSB1 and PMSA4 are involved in the progression of the cell cycle (Pagano et al., 1995; Tokumoto et al., 1997). PAP is an enzyme that plays a significant role in the polyadenylation process (Wahle and Keller, 1992). Translation of mRNAs present in the oocyte cytoplasm is regulated by changes in the poly-A tail (Tomek et al., 1998, 2001; Richter 1999). Finally, Cx43 is an essential part of the gap junctions between the oocyte and cumulus cells throughout oocyte maturation and between

blastomeres after fertilization. Intercellular communication via gap junctions plays a crucial role in the maintenance of homeostasis, morphogenesis, cell differentiation, and growth control in multi-cellular organisms (Oyamada et al., 2005). Here, we unravelled the expression patterns of the above genes at various time points during maturation to gain insight into the transcriptional regulation of oocyte maturation and its relationship with the developmental competence of the female gamete.

## **Materials and methods**

### ***Oocyte collection and in vitro maturation***

Bovine ovaries were collected at a local abattoir and transported to the laboratory at 25-30°C. Ovaries were washed three times in 0.9% NaCl (S5886; Sigma) supplemented with penicillin (PEN-NA, Sigma) and streptomycin (35500; Serva). Cumulus-oocyte complexes (COCs) were aspirated from follicles either < 2 mm or 2 - 8 mm in size using an 18-gauge needle attached to a 5-ml syringe. The COCs were selected under a stereomicroscope and only oocytes with at least three layers of compact cumulus cells with a homogeneous granulated cytoplasm were used for this study. The COCs were maintained in Dulbecco's PBS medium (D5773; Sigma) supplemented with 50 IU/ml penicillin (PEN-NA, Sigma), 50 µg/ml streptomycin (35500; Serva), 36 µg/ml pyruvate (P3662; Sigma), 1 mg/ml D-glucose (Carl Roth GmbH, Karlsruhe, Germany), 133 µg/ml calcium chloride dehydrate (Merck, Darmstadt, Germany) and 2 IU/ml heparin (24590; Serva) and BSA (fraction V, A9647; Sigma). Cumulus oocyte complexes were then collected into TCM containing Hepes (TCM 199 with Earle's salts, L-glutamine [G6392; Sigma] and 25 mM Hepes [M2520; Sigma] supplemented with 22 µg/ml pyruvate [P3662; Sigma], 350 µg/ml NaHCO<sub>3</sub> [31437; Riedel-deHaen], 50 µg/ml gentamycin [G3632; Sigma], and 0.1 % BSA-fatty acid free [A7030; Sigma]).

The maturation medium consisted of TCM 199 (M2520; Sigma) supplemented with 22 µg/ml pyruvate (P3662; Sigma), 2.2 mg/ml NaHCO<sub>3</sub> (31437; Riedel-deHaen), 50 µg/ml gentamycin (G3632; Sigma), 10 IU/ml of PMSG, 5 IU/ml of hCG (Suigonan,

Intervet, Tönisvorst, Germany), 0.1% BSA-fatty acid free (A7030; Sigma) and 100 µM cysteamine (M6500; Sigma). The COCs from each category of follicle size were matured in pools of 20 in separate 100 µl drops under silicone oil (35135; Silicone DC 200 fluid; Serva) in a humidified atmosphere composed of 5% CO<sub>2</sub> in air at 39°C for 24 h.

### *In vitro fertilization*

For fertilization in vitro, the COCs were washed twice in 100 µl drops of fertilization medium and groups of 10 were transferred to drops with the same medium under silicone oil (35135; Silicone DC 200 fluid; Serva). Fertilization medium consisted of Fert-TALP (Parrish et al., 1986, 1988) containing 10 µM hypotaurine (H1384; Sigma), 0.1 IU/ml heparin (24590; Serva), 1 µM epinephrine (E4250; Sigma), [HHE], and 6 mg/ml BSA (Fraction V; Sigma). Frozen semen from one bull with proven fertility for in vitro fertilization (IVF) was used throughout this experiment. For IVF, semen was thawed at 30°C during 1 min and 250 µl of thawed sperm was placed on top of 750 µl of 90% Percoll (P1644; Sigma) and centrifuged for 16 min at 380g. The supernatant was removed and the pellet (sperm) resuspended in 750 µl of fertilization medium and washed for 3 min at 380g. This procedure was repeated twice. Finally the supernatant was removed leaving a 50 µl pellet (sperm). Motility and concentration of sperm were then assessed. The final sperm concentration used per fertilization drop was adjusted to 1 × 10<sup>6</sup> sperm/ml. Fertilization was achieved by 18-h coincubation under the temperature and gas conditions described for maturation.

### *In vitro culture*

After complete removal of the adhering cumulus cells by vortexing for 4 min in TCM containing Hepes, denuded presumptive zygotes were washed twice in culture medium. Groups of 5 were transferred to 30 µl culture medium consisting of synthetic oviduct fluid (SOF) medium supplemented with 4mg/ml BSA-fatty acid free (Wrenzycki et al., 2001) under silicone oil (35135; Silicone DC 200 fluid; Serva). Culture conditions consisted of 5% O<sub>2</sub>, 90% N<sub>2</sub>, and 5% CO<sub>2</sub> (Air Products, Hattingen, Germany) using a modular incubation chamber (615300; ICN Biomedicals Inc., Aurora, OH) in a humidified atmosphere at 39°C. Cleavage rate and blastocyst formation rate were evaluated at days 2 and 8 (day 0 = IVF) of in vitro culture.

### *Evaluation of meiotic progression*

Random samples were taken from pools of maturing oocytes from each follicle category at different time points during in vitro maturation to determine the progression of meiosis. Oocytes were denuded using 0.1% hyaluronidase (H4272; Sigma) for 10 min at 37°C and vortexing for 4 min. Subsequently, the oocytes were washed twice in PBS containing 0.1% polyvinyl alcohol (PVA) (P8136; Sigma) and stained with Hoechst 33342 stain (B2261; Sigma) 1 µl/100µl PBS-PVA for 10-15 min. Stained oocytes were assessed under a fluorescence microscope at 400X magnification (BX60 F-3, Olympus Optical, Hamburg, Germany) employing a filter with 420 nm for excitation and 365 nm for emission. The percentages of GV, GVBD,

MI and MII were recorded at 0 h; 8 h and 9 h; 15 h, 15.5 h and 16 h; and 24 h in maturation medium, respectively. Random samples were taken from pools of maturing oocytes at these different time points for freezing and subsequent RNA isolation. According to the observed meiotic progression, the time points selected for freezing were GV-0 h; GVBD-8 h for oocytes from < 2 mm and 9 h for oocytes from 2 - 8 mm; MI-15 h; MII-24 h (Table 2.3). Groups of two or five oocytes were frozen in a minimum volume ( $\leq$ 5  $\mu$ l) of PBS-PVA medium in a 0.6 ml siliconized reaction tubes (no. 710136; Biozym Diagnostic GmbH, Hessisch Oldendorf, Germany) and stored at -80°C prior to analysis of mRNA expression by RT-PCR. Oocytes from both follicle sizes from at least twelve different IVM replicates were frozen for subsequent mRNA analysis.

***Determination of the relative abundance of developmentally important gene transcripts in bovine oocytes***

The relative abundances (RA) of transcripts for DYNLL1, DYNC1I1, DCTN1, PMSB1, PMSA4, PAP and Cx43 from bovine oocytes were assessed by RT-PCR analysis. Poly (A)<sup>+</sup> RNA was prepared from the pools of two or five oocytes as described previously (Wrenzycki et al., 2002). Reverse transcription (RT) was carried out in a total volume of 20  $\mu$ l for the positive reaction and 20  $\mu$ l for the negative control. Prior to RNA isolation, 2 pg of rabbit globin RNA (BRL, Gaithersburg, MD) was added as an internal standard. Half of the amount of RNA isolated was used for the positive reaction and half for the negative control, i.e. DNA contamination

control for which the reaction was performed without reverse transcriptase and RNAase inhibitor. The RT reaction was performed in 10x buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4; Invitrogen), 5 mM MgCl<sub>2</sub> (Invitrogen), 1 mM of each deoxynucleotide triphosphate (dNTP; Amersham, Biosciences Europe GmbH, Freiburg, Germany). Further ingredients such as 50 U murine leukemia virus reverse transcriptase, 20 U RNase inhibitor, and 2.5 μM random hexamers were supplied by Applied Biosystems (Foster City, CA). RT was carried out at 25°C for 10 min, followed by 1 h at 42°C, a denaturation step at 99°C for 5 min, and flash cooling at 4°C. Two different reaction mixtures were used, (i) 0.05 cDNA oocyte equivalents/μl (one oocyte in a 20-μl reaction volume) or (ii) 0.125 cDNA oocyte equivalents/μl (2.5 oocytes in a 20-μl reaction volume) each with 50 fg globin RNA/μl. PCR was performed using the cDNA equivalent of 0.05 oocyte (1 μl from the first cDNA oocyte equivalent concentration obtained) for DYNLL1, DYNC1I1, PMSB1 and PAP transcripts or 0.25 oocyte equivalent (2 μl from the second cDNA oocyte equivalent concentration) for DCTN1, PMSA4 and Cx43 transcripts, and 50 fg of globin cDNA (1 μl) in a final volume of 50 μl of 10x buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 1.5 mM MgCl<sub>2</sub>, 200 μM of each dNTP, 0.5 μM of each sequence-specific primer in a thermocycler (PTC- 200; MJ Research, Watertown, MA). To ensure specific amplification, a hot-start PCR protocol in which 1 U *Taq* DNA polymerase (Invitrogen) added at 72°C was employed. PCR primers were designed from the coding regions of each gene sequence using the OLIGO™ program. The sequences of the primers, the annealing temperatures, the fragment sizes, and sequence

references are summarized in Table 2.1. The PCR program employed an initial denaturation step of 97°C for 2 min followed by a step of 72°C for 2 min (during which time hot-start was performed) and cycles of 15 sec at 95°C for DNA denaturation, 15 sec at the primer-specific annealing temperature, and 15 sec at 72°C for primer extension. The last cycle was followed by a 5-min extension at 72°C and cooling to 4°C (for number of cycles, see Table 1). PCR reactions for the genes of interest and the globin internal standard were performed in separate tubes. The PCR products were subjected to electrophoresis on a 2% agarose gel in 1x TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) containing 0.2 µg/ml ethidium bromide, present in both gel and running buffer to stabilize the concentration of ethidium bromide for quantification. The fragments were visualized on a 312-nm UV transilluminator; the image of each gel was recorded using a CCD camera (Quantix, Photometrics, München, Germany) and the IPLab Spectrum program (Signal Analytics Corp., Vienna, VA). The intensity of each band was determined by densitometry using an image analysis program (IPLab Gel). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each meiotic stage by the intensity of the corresponding globin band. For each gene, twelve separate RT-PCR reactions were performed. The RNA recovery rate was estimated as the ratio between the intensity of the globin band with and without the RNA preparation procedure, starting with an equivalent of 50 fg in the PCR reaction. On average, 60% of poly (A)-tailed RNA was recovered using the Dynabead oligo d(T) mRNA isolation method, which is in agreement with previous results from our

laboratory (Yaseen et al., 2001; Wrenzycki et al., 1999; 2002). This semi-quantitative endpoint RT-PCR assay is highly sensitive and accurate and yields similar results as semi-quantitative real time RT-PCR techniques (Knijn et al., 2002; Gal et al., 2006).

Table 2.1. Primers used for PCR of bovine oocyte gene transcripts.

Genes	Primers sequences and positions	Annealing Temperature (°C), cycle number	Fragment size (bp)	Sequence references [EMBL accession no.]
Globin				
5' (241-260)	GCAGGCCACGGTGGCGAGTAT	60 × 27	257	X 04751
3' (555-657)	GTGGGACAGGAGCTTGAAAT			
DYNLL1				
5' (220-241)	CAGGAECTCGGTGGAGTGTGCTA	56 × 31	196	BT021030
3' (394-415)	GAATGGCCACTTGGCCCAGGTA			
DYNC1II				
5' (1445-1466)	GCAGCGAGGAGGGTACAGTCTA	59 × 36	397	BC114074
3' (1820-1841)	CCACCTTGAGCCCCAACGAACAC			
DCNT1				
5' (1289-1310)	GGGGCTGAGGAGATGGTAGA	58 × 33	321	XM865305
3' (1588-1609)	GGATGCTTCCTGCTGGTTTGTC			
PMSB1				
5' (542-563)	CCGGTAGGGTCCTACCAGAGAG	56 × 32	282	BC109494
3' (802-823)	ACAAGCGCGCAGAAGTCAGTCC			
PMSA4				
5' (137-158)	TCCAGGACCACTATATTCTC	56 × 34	352	BC102102
3' (467-488)	ACTGTGTATAAGCTTGTGAT			
PAP				
5' (886-915)	GTTCCTCGGTGGTGTTCCTGGCTATGC	56 × 33	252	X 63436
3' (1108-1137)	TGGAGTTCTGTTGTGGGTATGCTGGTGAA			
Cx43				
5' (231-252)	ATGGGTGACTGGAGTGCCTAG	58 × 33	339	BC105464
3' (548-569)	GAGTTCCCTCCTCTTGTTC			

Globin (Cheng et al., 1986)

### *Statistical Analysis*

The data were analyzed with SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) software package. After testing for normality (Kolmogorov-Smirnov test with Lilliefors correction) and equal variance (Levene Median test), a one-way ANOVA followed by multiple pair-wise comparisons using the Tukey test was employed to test effects of meiotic stages, blastocyst and cleavage rates between groups. Two-way ANOVA and one-way ANOVA followed by multiple pair-wise comparisons using the Tukey test were used for the RAs between stages and categories of follicle sizes and for the RAs within one follicle size, respectively. Differences of  $P < 0.05$  were considered significant.

## Results

Cleavage and blastocyst formation rates were significantly higher in embryos derived from oocytes isolated from follicles of the 2 - 8 mm size category (Table 2.2). No significant differences were found in the progression of meiosis in both groups of oocytes and the maturation rates were not statistically different between the two groups (Table 2.3). The RAs of DYNLL1 and DYNC1I1 were significantly higher in oocytes from follicle size 2 - 8 mm in size compared to oocytes from the smaller follicles (Fig. 2.1). No significant differences in the RAs of DCTN1, PMSB1, PMSA4, PAP and Cx43 transcripts were found between the two groups (Fig. 2.1). The dynamics of DYNLL1, DYNC1I1, and PMSB1 transcripts during maturation were significantly different between the two groups of oocytes. These transcripts decreased significantly during IVM in oocytes from follicles 2 - 8 mm in size. DYNLL1 decreased significantly during maturation in oocytes isolated from small follicles. DYNLL1 RA was significantly higher in GVBD and MI in oocytes from follicle size 2 - 8 mm compared to oocytes derived from small follicles. The expression profile of DYNLL1 in oocytes with higher developmental competence showed a marked decrease from MI to MII stage while no differences in oocytes recovered from small follicles with regard to the RA for DYNLL1 in MI and MII were determined.

Table 2.2. Cleavage and blastocyst formation rates in fertilized oocytes isolated from two different categories of follicles

Follicle size	Number of presumptive zygotes (n)	Cleavage % (n)	Blastocyst formation % (n)
2 – 8 mm	218	80.9 ± 4 <sup>a</sup> (174)	27.2 ± 3 <sup>a</sup> (57)
< 2 mm	126	65.8 ± 2 <sup>b</sup> (83)	3.1 ± 2 <sup>b</sup> (5)

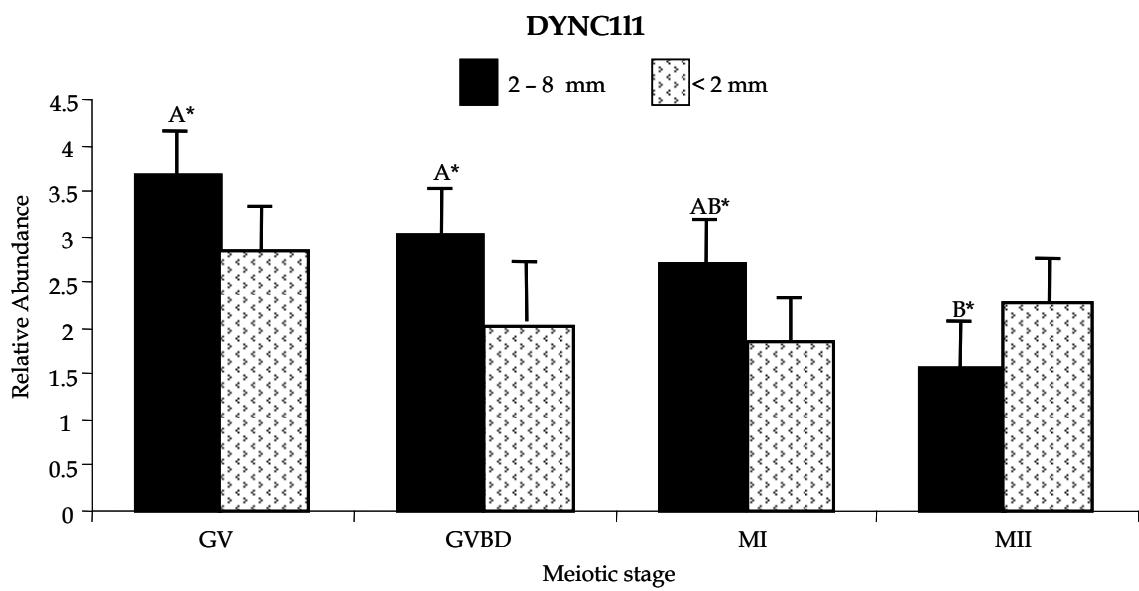
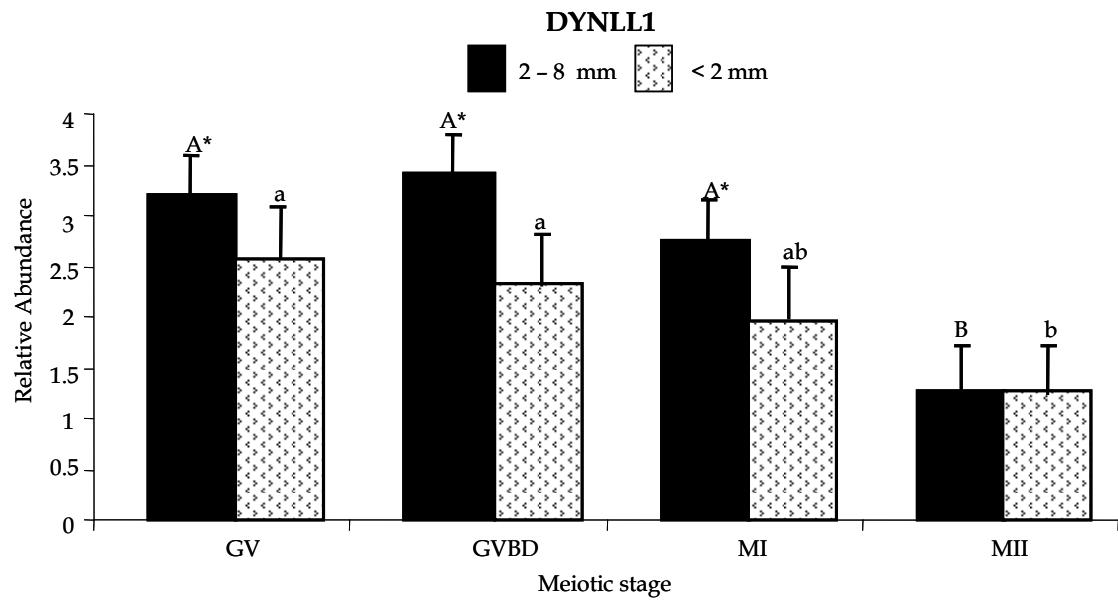
\*Values shown as mean ± S.E.M. Numbers with different superscripts within a column are significantly different (<sup>a, b</sup>  $P < 0.05$ ) ANOVA one way. Total numbers from five independent replicates.

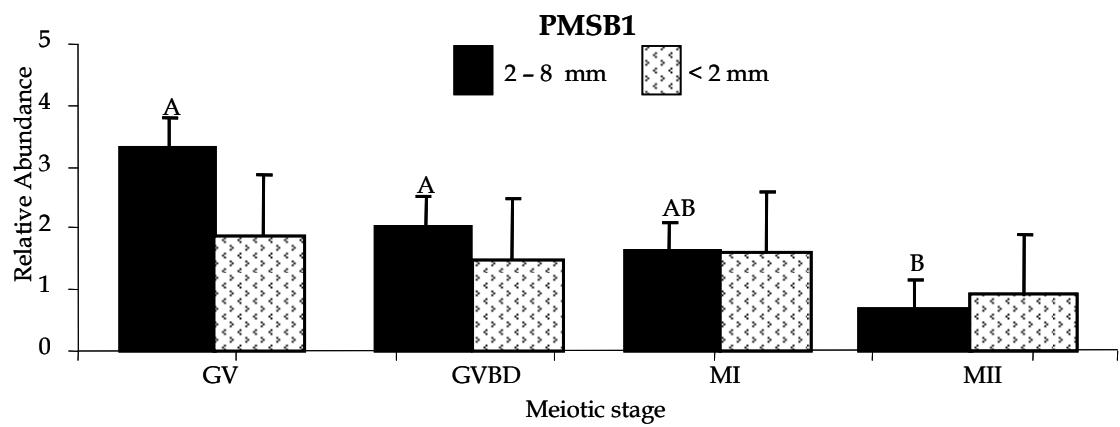
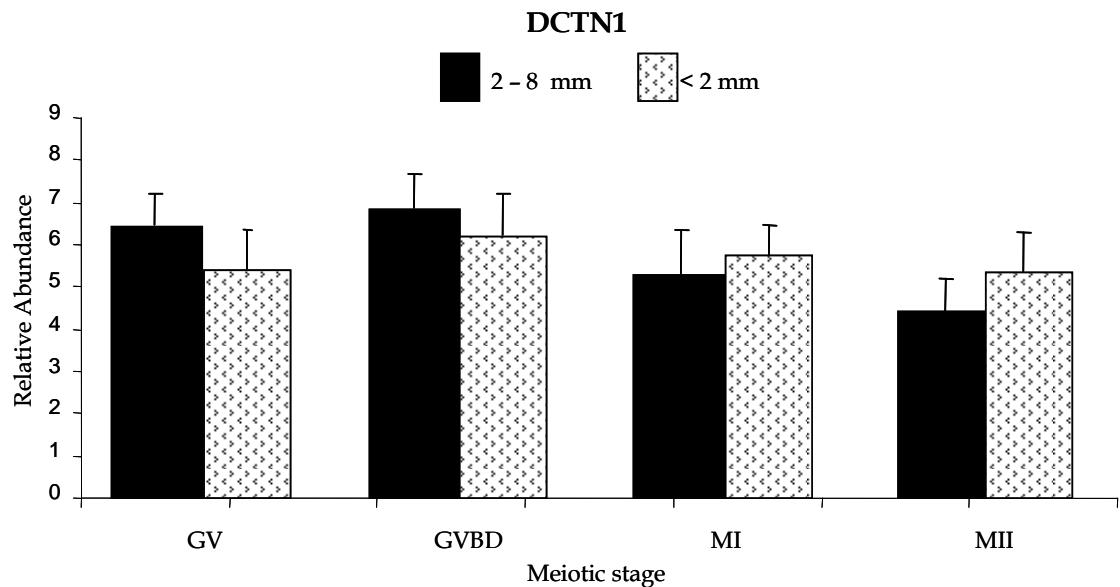
Table 2.3. Meiotic progression in bovine oocytes derived from two different sizes of follicles.

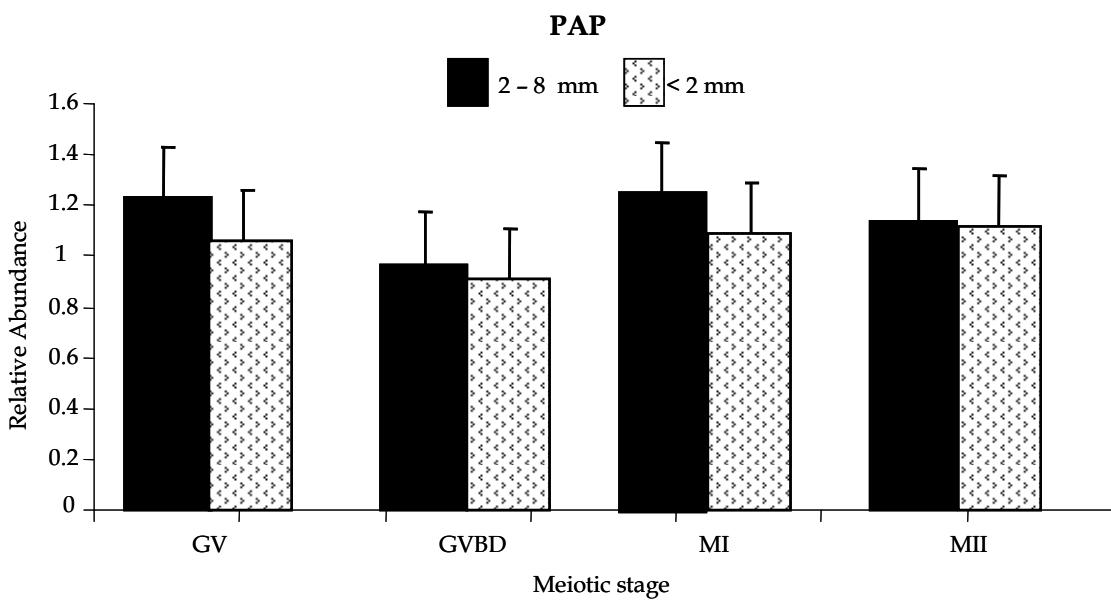
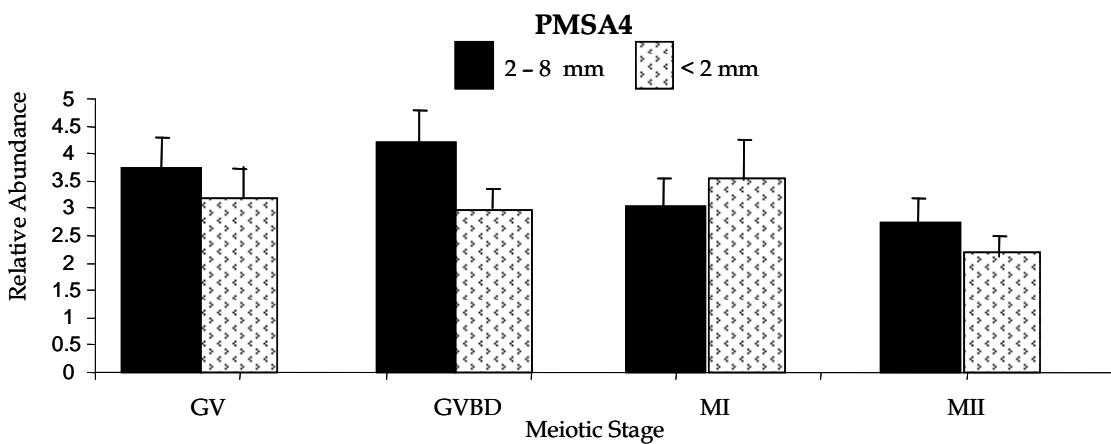
Follicle size	Total number of oocytes	Meiotic stage						
		GV		GVBD		MI		
		0 h	8 h	9 h	15 h	15.5 h	16 h	24 h
2 – 8 mm	307	94 ± 3 (30/32)	73 ± 9 (33/45)	83 ± 3 (25/30)	97 ± 3 (42/43)	84 ± 3 (26/31)	90 ± 4 (45/51)	85 ± 7 (69/75)
< 2 mm	236	95 ± 5 (24/25)	82 ± 2 (36/44)	84 ± 9 (25/31)	89 ± 1 (26/29)	75 ± 8 (22/29)	70 ± 7 (32/47)	89 ± 2 (28/31)

8

\*Values shown as mean ± S.E.M. No statistically significant differences were determined (ANOVA one way). Total numbers (n/N) from four independent replicates. The time selected for freezing oocytes before RNA isolation were: 0 h for GV in both groups of oocytes, 8 h for oocytes recovered from follicles < 2 mm and 9 h for oocytes recovered from follicles 2 – 8 mm for GVBD, 15 h for MI and 24 h for MII.







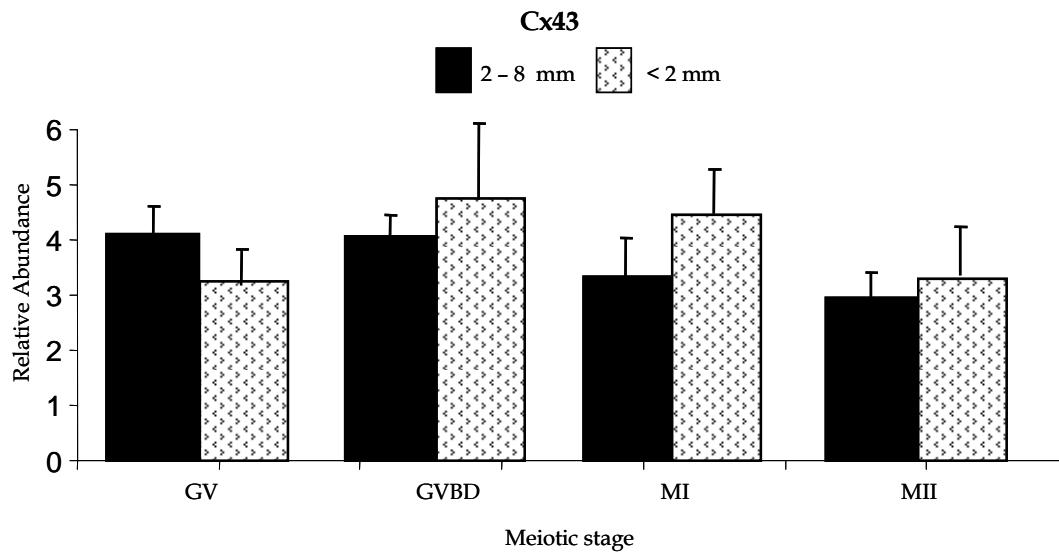
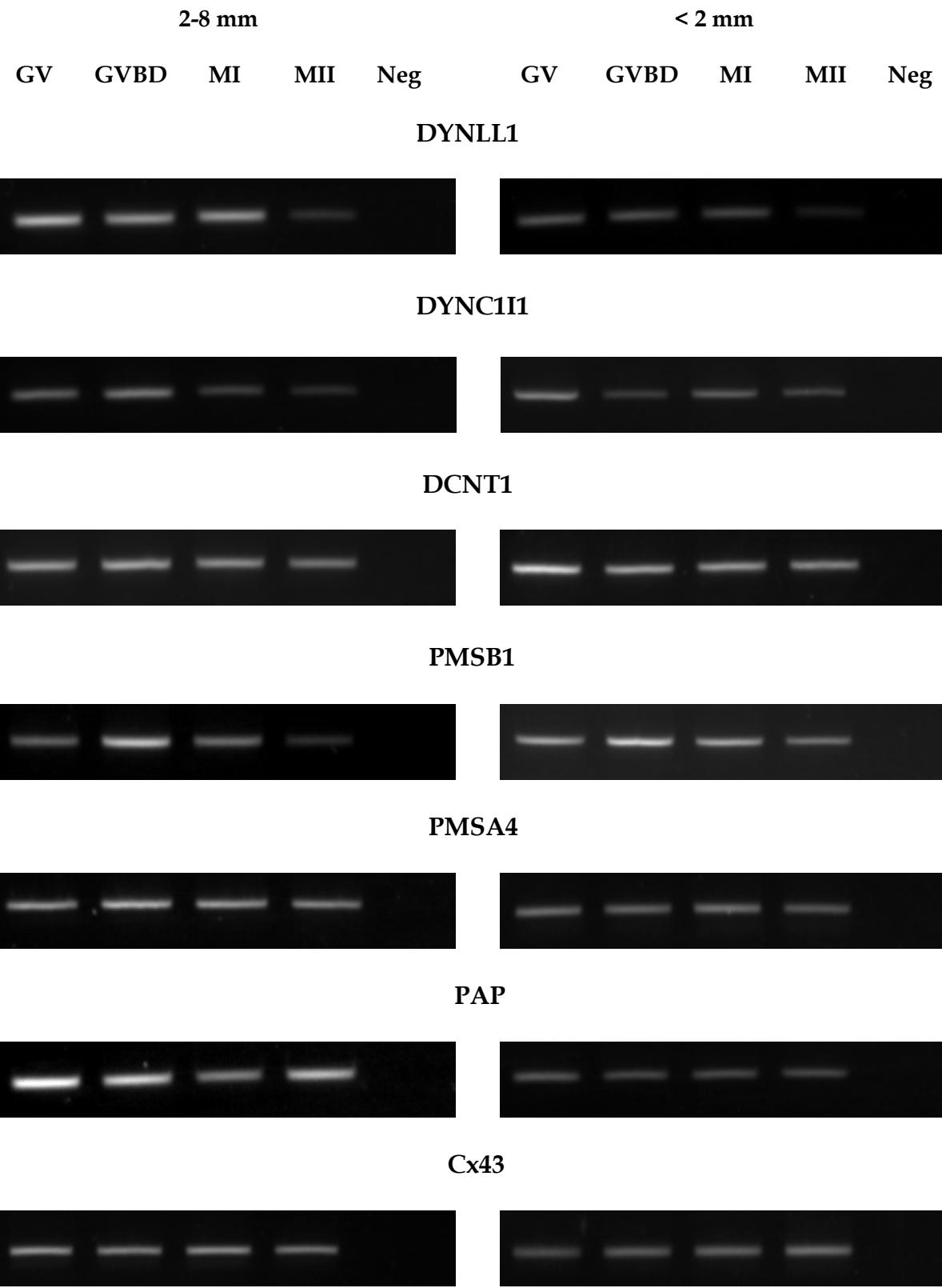


Fig. 2.1. Relative abundance of some important genes transcripts (values shown as mean  $\pm$  S.E.M.) in bovine oocytes recovered from follicles 2 - 8 mm size and follicles < 2 mm throughout in vitro maturation (GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I and MII, metaphase II). Significant differences between follicles sizes are indicated by (\*) ( $P < 0.05$ ) ANOVA two way, whereas (<sup>A, B</sup>) ( $P < 0.05$ ) indicates significant differences within bigger follicles through meiotic stages and (<sup>a, b</sup>) ( $P < 0.05$ ) indicates significant differences within smaller follicles through meiotic stages, ANOVA one way. Each experiment contained 12 replicates.



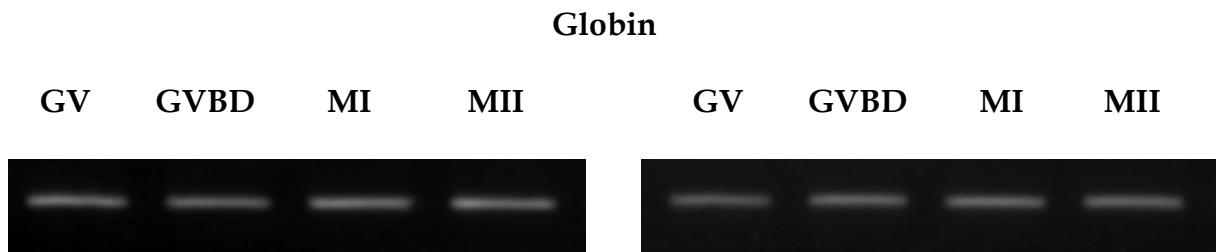


Fig. 2.2. Representative photograph of gels from semi-quantitative endpoint RT-PCR analysis of some important gene transcripts in bovine oocytes recovered from follicles 2 – 8 mm size and follicles < 2 mm throughout in vitro maturation (GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I and MII, metaphase II). Each lane represents the PCR product derived from poly (A)<sup>+</sup>RNA after RT-PCR from the equivalent of 0.05 oocyte for DYNLL1, DYNC1I1, PMSB1 and PAP, or 0.25 oocyte for DCNT1, PMSA4 and Cx43 and the standard rabbit *globin*. Each experiment contained 12 replicates.

## Discussion

Messenger RNA expression profiling has emerged as a useful tool to determine factors affecting the transcriptional level in oocytes and early embryos. Numerous studies have demonstrated a close relationship between oocyte/embryo quality and the mRNA abundance of panels of various genes (Brevini and Gandolfi, 1999; Robert et al., 2000; Robert et al., 2002a; Robert et al., 2002b; Brevini et al., 2002; Wrenzycki et al., 2003; Donnison and Pfeffer, 2004; Baran et al., 2004; Gutierrez-Adan et al., 2004; Fair et al., 2004; Lequarre et al., 2005; Nemcova et al., 2006; Mourot et al., 2006; Alves-Nunes-Dode et al., 2006) or the effects of culture conditions on mRNA abundance (for reviews see Lonergan et al., 2003b; 2006; Wrenzycki et al., 2005). The relationship between oocyte quality and embryo development has been assessed by different means, including the morphology of the ovaries (Brevini and Gandolfi, 1999; Nemcova et al., 2006), developmental timing of embryos (Brevini et al., 2002; Wrenzycki et al., 2003; Gutierrez-Adan et al., 2004; Fair et al., 2004; Alves-Nunes-Dode et al., 2006) or by isolating oocytes from follicles of different sizes (Robert et al., 2000; Robert et al., 2002a,b; Donnison and Pfeffer, 2004; Baran et al., 2004; Lequarre et al., 2005; Nemcova et al., 2006; Mourot et al., 2006). Here, we analyzed the relative abundance of a panel of genes thought to be critically involved in oocyte maturation. We included five novel genes that had not yet been studied previously (DYNLL1, DYNC1I1, DCTN1, PMSB1 and PMSA4), and the expression of DYNLL1, DYNC1I1 and PMSB1 was significantly affected by follicle size and

maturity stage. We report for the first time the dynamics of these transcripts during oocyte maturation. The decrease of DYNLL1 during maturation is in agreement with findings made on bovine oocytes in GV and MII recovered from 3 - 5 mm follicles (Dalbies-Tran and Mermilliod, 2003). DYNLL1 is a motor protein and functions during maturation as a subunit of the cytoplasmic dynein complex 1 for transport in the oocyte. DYNC1I1 protein is part of the cytoplasmic dynein complex 1. DYNC1I1 and PMSB1 mRNA expression decreased slightly during maturation in oocytes from large follicles, i.e. in oocytes with higher developmental competence. Previously, it was found that the relative abundance of PMSB1 transcripts decreased between GV and MII oocytes (Dalbies-Tran and Mermilliod, 2003). The PMSB1 protein is involved in the protein degradation machinery of the cell; the protein plays a critical role in the cell cycle. The decrease during oocyte maturation observed here could be explained by translation of the messenger and future analysis of protein expression could substantiate this hypothesis.

The RAs of DCTN1, PMSA4, PAP and Cx43 RAs were not affected by follicle size and their levels remained stable during maturation. In contrast, PMSA4 increased from GV to MII oocytes (Dalbies-Tran and Mermilliod, 2003). In the present study we did not find a significant increase of PMSA4 mRNA between GV and GVBD stages when RNAs can be synthesized de novo.

PAP and Cx43 transcripts have long been studied in association with developmental competence of bovine oocytes and embryos (for reviews see Lonergan et al., 2003;

2006; Wrenzycki et al., 2005). In the present study, we did not find differences in the RA of PAP and Cx43 either between groups of oocytes or between stages during in vitro maturation. Baran et al. (2004) analyzed oocytes from small, medium and large follicles and showed that the amount of Poly A transcripts significantly increase in oocytes from medium and large follicles compared to oocytes from small follicles. Wrenzycki et al. (1999) analyzed the RA of PAP and Cx43 in bovine GV and MII oocytes cultured in different media supplements, either with serum or PVA. In the presence of PVA, PAP mRNA was increased in MII oocytes, whereas Cx43 transcripts were not affected. In the present study, we did not detect differences between GV found relatives abundances of this transcript in both types of oocytes. In the present experiment using BSA as the supplement to the culture medium the population of oocytes isolated from small follicles was different from that of the previous study as here they were aspirated from the ovarian surface. These differences in the follicle population and culture conditions may explain the diverging findings related to mRNA expression of these specific genes.

Brevini-Gandolfi et al., (1997), suggested that the developmental capacity of bovine oocytes correlates with the length of the poly-A tail. A shorter poly-A tail for several transcripts, including PAP and Cx43 in oocytes was related to a lower oocyte quality (Brevini and Gandolfi, 1999). In the present study the method used for mRNA isolation does not allow to differentiate between short and long poly-A tails. The length of the poly-A tail was related to different timings of cleavage; the PAP poly-A

tail was longer in slowly dividing embryos, while Cx43 poly-A tail was shorter in slowly dividing embryos (Brevini et al., 2002). The mRNA steady state level, the correct temporal and local translation of the messenger and finally the proper protein activation are essential steps for the acquisition of regular biological function of the protein during oocyte maturation and early embryo development (Brevini et al. 2007). In the present study Dynabeads were added in excess and random primers were used, thus possible differences in polyadenylation did not affect the results. Our results provide a solid molecular approach towards a first characterization of genes critically involved in important cellular events during oocyte maturation.

The relationship between developmental competence and follicle size is well established (Pavlok et al., 1992; Lonergan et al., 1994; Blondin and Sirard, 1995; Hagemann et al., 1999; Lequarre et al., 2005). Bovine oocytes recovered from follicles > 2 mm have a greater developmental competence than oocytes recovered from follicles < 2 mm (Hendriksen et al., 2000; Lequarre et al., 2005). Results from this study significantly extend these data and provide new insight into the underlying transcriptional activities. In agreement with a previous study (Lequarre et al., 2005); we did not find significant differences between the two groups of oocytes with regard to the time necessary to reach each meiotic stage. With a few exceptions the time points identified in this study for the various meiotic stages coincided with previous results (Sirard et al., 1989; Wehrend and Meinecke, 2001; Lequarre et al., 2005). We observed that oocytes recovered from small follicles reached GVBD and

the transition from MI stage to anaphase I faster than their counterparts from large follicles (see Table 2.3) although no significant difference was found. Previously it was found that oocytes recovered from follicles 2 – 3 mm reached anaphase I faster compared to oocytes obtained from follicles > 4 mm (Torner et al. 2001). Usually bovine oocytes with signs of atresia resume meiosis faster than those without atretic symptoms (Mayes and Sirard, 2001). Some of the smaller follicles used in this study could have undergone early atresia. Previously, it was shown that the number of layers of granulosa cells surrounding the oocyte affected the kinetics of maturation as denuded oocytes resume meiosis faster (Fissore et al., 1996).

In conclusion, we have analyzed the mRNA expression profile of a panel of seven genes (novel genes: DYNLL1, DYNC1I1, DCTN1, PMSB1 and PMSA4) and show that DYNLL1, DYNC1I1 and PMSB1 were significantly affected by follicle size and/or the maturation stage. We demonstrate for the first time the dynamics of the genes analyzed in the present study during oocyte maturation. The differences found between groups of oocytes could serve as a marker to assess the developmental capacity of bovine oocytes.

## **Capítulo 3**

### **Dynamics of cytoplasmic events in bovine in vitro matured oocytes**

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## Summary

Oocyte maturation is a complex, well orchestrated process that finally results in the release of a metaphase II oocyte that has undergone proper nuclear and cytoplasmic maturation and is ready for successful fertilization and subsequent regular early embryo development. Microtubule- molecular motors such as DYNC1I1 (cytoplasmic dynein 1 intermediate chain), and its cofactor DCTN1 (dynactin p150<sup>Glued</sup>) are critically involved in transporting vesicles and the formation of the mitotic spindle. The ubiquitin-proteasome system controls the levels of crucial cell cycle proteins. The role of molecular motors and proteasomes during oocyte maturation is poorly understood. The goal of this study was to unravel the dynamics of selected proteins thought to be critically involved in oocyte maturation and their association with microtubules in oocytes at different stages of in vitro maturation (IVM), i.e. GV, GVBD, MI and MII. The oocytes were collected from follicles of two different size categories, i.e. < 2 mm and 2 - 8 mm. The distribution of cytoplasmic dynein, dynactin and 20S proteasomes was assessed by immunocytochemistry (ICC) and laser confocal microscopy. Sodium-orthovanadate (SOV), a specific phosphatase and ATPase activity inhibitor blocked meiosis indicating a crucial role of dynein-dependent transport in the progression of bovine oocyte maturation.

Immediately after chromatin condensation (GVBD), dynactin was in close association with the DNA and interacted with the MI and MII spindles in oocytes recovered from large follicles. Only weak staining was detected in MI and MII

spindles and no clear association with the DNA in GVBD was observed in oocytes obtained from small follicles. Dynein was homogeneously distributed in the cytoplasm of GV stage of both groups of oocytes while dynactin showed a perinuclear concentration in the same stage. Dynein was not associated with the DNA at the GVBD stage while it was found at the MI and MII spindle. The dynein signal was weak in MI microtubules of oocytes from small follicles. 20S proteasomes were found predominantly in the nucleus at the GV stage and were associated with the DNA up to MII stage in both groups of oocytes. Results show the distinct dynamics of molecular motors and proteasomes during bovine oocyte maturation and their possible relationship with developmental competence of the oocyte.

## **Introduction**

Oocyte developmental competence is acquired progressively during late follicular growth and is obtained during oocyte maturation (Hyttel et al., 1997). Acquisition of oocyte developmental competence is closely related to the size of the follicle. Fertilized bovine oocytes recovered from small follicles (< 2 mm) developed in vitro at a significantly lower frequency than their counterparts from bigger follicles (> 2 mm) (Pavlok et al., 1992; Lequarre et al., 2005). Oocyte maturation is a complex, well orchestrated process that culminates in the release of a full competent oocyte that can undergo fertilization and sustain early embryo development. Nuclear and cytoplasmic oocyte maturation is regulated by RNA and protein pools synthesized during oocyte growth phase and maturation. Recently the mRNA expression profiles of cytoplasmic dynein 1 intermediate chain, dynactin and two subunits ( $\alpha 4$  and  $\beta 1$ ) of the 20S proteasomes were analyzed during bovine in vitro maturation in relation with oocyte developmental competence defined by oocytes derived from small (< 2 mm) and large (> 2mm) follicles (Racedo et al., 2008). Here, we investigated the protein dynamics of cytoplasmic dynein 1 intermediate chain, dynactin and 20S proteasomes in the same biological system.

Cytoplasmic dynein 1 intermediate chain is a critical component of the cytoplasmic dynein 1 complex, which represents the most abundant transport system of the cell (Pfister et al., 2006; Höök and Vallee 2006). Prominent physiological mechanisms regulated by cytoplasmic dynein 1 complex include nuclear envelope breakdown (Beaudouin et al., 2002; Salina et al., 2002) and transport of molecules and organelles

towards the minus ends of microtubules in somatic cells (Karki and Holzbaur, 1999; Höök and Vallee 2006). Additionally, these molecular motors show a progressive bidirectional motion of dynein-dynactin in vitro (Ross et al., 2006). Dynactin is associated with the cytoplasmic dynein complex 1 (Karki and Holzbaur, 1995; 1999; Vaughan and Valle, 1995) and also involved in molecule transport. Chi-Ying et al. (1999) showed the importance of phosphorylation on dynein and dynactin during the progression of meiosis in *Xenopus* oocytes. Blocking studies using sodium-orthovanadate (SOV), a specific ATPase inhibitor with high selectivity for dyneins (Niclas et al., 1996), revealed that dyneins are crucially involved in the meiotic progression of mouse oocytes (Wang et al., 2004) as well as in the formation of murine spindle structure (Carabatsos et al., 2000). The role of these molecular motors in bovine development has been studied during fertilization (Payne et al., 2003) but their dynamics during oocyte in vitro maturation is unknown.

Twenty S proteasomes form the catalytic core of the 26S proteasome, the end point for the degradation of ubiquitinated proteins in all eukaryotic cells (DeMartino and Gilette, 2007). Proteasomes degrade the bulk of proteins in somatic cells as well as regulatory proteins involved the cell cycle (Goldberg 1995; Coux et al., 1996; Hershko 2005). Their role and localization during oocyte maturation is poorly explored. Here, we determined the dynamics of cytoplasmic dynein and dynactin as well as the distribution of 20S proteasomes at various time points during maturation of bovine oocytes in relation with the developmental competence of the bovine

female gamete and assessed the physiological function by applying the specific dynein-inhibitor-sodium-orthovanadate.

## **Materials and methods**

### ***Oocyte collection and in vitro maturation***

Bovine ovaries were collected at a local abattoir and transported to the laboratory at 25-30°C. Ovaries were washed three times in 0.9% NaCl (S5886; Sigma) supplemented with penicillin (PEN-NA, Sigma) and streptomycin (35500; Serva). Two populations of cumulus-oocyte complexes (COCs) were aspirated from follicles, either < 2 mm or 2 - 8 mm in size, using an 18-gauge needle attached to a 5-ml syringe. The COCs were selected under a stereomicroscope and only oocytes with at least three layers of compact cumulus cells and a homogeneous granulated cytoplasm were used for this study. The COCs were maintained in Dulbecco's PBS medium (D5773; Sigma) supplemented with 50 IU/ml penicillin (PEN-NA, Sigma), 50 µg/ml streptomycin (35500; Serva), 36 µg/ml pyruvate (P3662; Sigma), 1 mg/ml D-glucose (Carl Roth GmbH, Karlsruhe, Germany), 133 µg/ml calcium chloride dehydrate (Merck, Darmstadt, Germany), 2 IU/ml heparin (24590; Serva) and BSA (fraction V, A9647; Sigma). Cumulus oocyte complexes were then collected into TCM containing Hepes (TCM 199 with Earle's salts, L-glutamine [G6392; Sigma] and 25 mM Hepes [M2520; Sigma] supplemented with 22 µg/ml pyruvate [P3662; Sigma], 350 µg/ml NaHCO<sub>3</sub> [31437; Riedel-deHaen], 50 µg/ml gentamycin [G3632; Sigma], and 0.1 % BSA-fatty acid free [A7030; Sigma]).

The maturation medium consisted of TCM 199 (M2520; Sigma) supplemented with 22 µg/ml pyruvate (P3662; Sigma), 2.2 mg/ml NaHCO<sub>3</sub> (31437; Riedel-deHaen), 50 µg/ml gentamycin (G3632; Sigma), 10 IU/ml of PMSG, 5 IU/ml of hCG (Suigonan,

Intervet, Tönisvorst, Germany), 0.1% BSA-fatty acid free (A7030; Sigma) and 100 µM cysteamine (M6500; Sigma). The COCs from each group (small: < 2 mm, large: 2 - 8 mm) were matured in pools of 20 in separate 100 µl drops under silicone oil (35135; Silicone DC 200 fluid; Serva) in a humidified atmosphere composed of 5% CO<sub>2</sub> in air at 39°C for 24 h.

#### *Fixation at different meiotic stages*

The time points selected for fixation were as previously described; GV: 0 h; GVBD: 8-9 h; MI: 15 h and MII: 24 h (Racedo et al., 2008). Random samples were taken from pools of maturing oocytes at these specific time points for fixation and subsequent protein analysis. Oocytes from both follicle sizes from at least three different IVM replicates were fixated for each protein detection. Prior to fixation the attached cumulus cells and zona pellucidae of oocytes were gently removed with short incubations in TALP culture medium containing 1 mg/ml hyaluronidase (H4272; Sigma) and 2 mg/ml pronase (P6911; Sigma), respectively. The oocytes were then incubated in a fixation-permeabilization solution containing 2% formaldehyde (Sigma) and 0.1% Triton X-100 (Sigma) for 1h. Then samples were blocked for at least 1 h in 10 mM PBS + 0.3% BSA (fraction V, A9647; Sigma) + 1% fetal calf serum (ICC blocking solution) prior to incubation with the antibodies.

#### *Antibodies, immunocytochemistry and confocal microscopy*

Primary antibodies were applied over night at 4°C.

Dynactin p150<sup>Glued</sup> was identified by using mouse monoclonal antibody clone 1 (610474; BD Transduction Labs) at 1:50; dynein (cytoplasmic dynein 1 intermediate

chain) was detected by mouse monoclonal antibody MAB1618 (Chemicon) at 1:50. Tubulin was studied using a sheep polyclonal antibody (ATN02, Cytoskeleton) at 1:150 and 20S proteasomes were identified by using a rabbit polyclonal antibody reacting with both the  $\alpha$  and  $\beta$  subunits (PW8155; BIOMOL) at 1:200. Control experiments were performed with antibodies which had been pre-incubated with their corresponding antigens for 1 h.

Alexa-Fluor 488 goat anti mouse IgG (11001; Molecular Probes) was used as secondary antibody and applied for 1 h at 37°C in the dark and 400  $\mu$ g/ml RNase (R5500; Sigma) was added to reduce the background labeling of RNA. An additional control was performed using the secondary antibody in the absence of primary antibodies. Alexa-Fluor 568 donkey anti sheep (A21099; Molecular Probes) was used for labelling microtubules and 20S proteasomes as described above. DNA was labeled with 10  $\mu$ g/ml TOTO-3 (T3604; Molecular Probes) during 30 minutes in the dark, 200  $\mu$ g/ml RNase (R5500; Sigma) was added for further reduction of the background labeling of RNA. Oocytes were mounted in anti-fade medium (Vectashield H-1000; Vector Laboratories) to avoid photobleaching and were imaged with a Zeiss LSM 510 laser scanning microscope at 488-, 568-, and 633-nm wavelengths. Images were then processed by using Adobe Photoshop 7.0.

#### ***Dynein inhibition and evaluation of maturation rate***

Sodium-orthovanadate (SOV) (S6508; Sigma), which among different phosphatases and ATP-ases is highly selective to inhibit dynein ATPase activity, was used at 400

$\mu\text{M}$  (Wang et al., 2004) at different time points during bovine oocyte in vitro maturation for different exposure times.

COCs recovered from follicle size 2 – 8 mm were randomly divided at GV stage (0 h of IVM) and incubated for 0.5 h; 1 h or 2 h in maturation medium containing 400  $\mu\text{mol/l}$  SOV. Then the COCs were washed and transferred in SOV-free maturation medium to complete maturation (24 h total). A similar protocol was applied after 5 h of IVM and 9 h of IVM (GVBD) (Table 3.1). The effects of SOV on the maturation process (24 h) were also investigated.

To determine the maturation rate at the end of the 24 h maturation period, the COCs were denuded using 0.1% hyaluronidase (H4272; Sigma) for 10 min at 37°C and vortexed for 4 min. Subsequently, the denuded oocytes were washed twice in PBS containing 0.1% polyvinyl alcohol (PVA) (P8136; Sigma) and were then stained with Hoechst 33342 stain (B2261; Sigma) 1 ml/100 ml PBS-PVA for 10–15 min. Extrusion of the first polar body was determined under an inverted microscopy at 100X magnification. Stained oocytes were observed under a fluorescence microscope at 400X magnification (BX60 F-3, Olympus Optical, Hamburg, Germany) employing a filter with 420 nm for excitation and 365 nm for emission. The proportions of oocytes that reached MII stage were recorded at 24 h. Oocytes from at least three different IVM replicates were evaluated per time point.

### *Statistical Analysis*

The effects of SOV on the maturation rate were analyzed with SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) software package using Chi-square and Fisher exact tests. Differences of  $P < 0.05$  were considered significant.

## Results

Cytoplasmic dynein intermediate chain and dynactin p150<sup>Glued</sup> exhibit distinct spatial and temporal associations with microtubules during bovine in vitro maturation that are affected by follicle size

Dynactin was distributed in the cytoplasm of GV oocytes and showed a strong signal near the nucleus in oocytes obtained from both follicle sizes: large follicles, 92% of oocytes (57 of 62 total oocytes observed [57/62]) and small follicles, (90%; 64/71) (Fig. 3.1A and 3.1a). Dynactin protein was not co-localized with the microtubules (Fig. 3.1A and 3.1a insets). Immediately after chromatin condensation (GVBD), dynactin was closely associated with the DNA in oocytes recovered from large follicle (75%; 53/71) (Fig. 3.1B) while GVBD in oocytes obtained from small follicles no clear association with DNA was observed (72%; 43/60) (Fig. 3.1b). At metaphase I stage (MI), dynactin was enriched at the spindle, surrounding the chromosomes and co-localizing with tubulin in oocytes recovered from large follicles (100%; 40/40) (Fig. 3.1C). Dynactin staining was weak in MI spindles in oocytes recovered from small follicles (82.5%; 33/40) (Fig. 3.1c).

In metaphase II stage (MII), dynactin was interacting with the spindle, showing the same pattern as in the MI stage. Abundant dynactin localized close to the spindle apparatus in oocytes from large follicles (90%; 35/39) and weak staining was found in oocytes recovered from small ones (83%; 40/48) (Fig. 3.1D and 3.1d).

Dynein was rather evenly distributed in the cytoplasm of GV oocytes, both recovered from large (77%; 46/60) and small (71%; 47/66) follicles (Fig. 3.2A and 3.2a). No clear association with the microtubules was observed at this stage in both groups of oocytes (Fig. 3.2A and 3.2a insets). Dynein was not associated with the DNA at GVBD stage in oocytes from both large (80%; 55/69) and small follicles (79%; 46/58) (Fig. 3.2B and 3.2b). At MI stage, dynein interacted with the microtubules in oocytes recovered from large follicles (79%; 33/42) (Fig. 3.2C), weak staining was found in the oocytes recovered from small follicles (66%; 23/35) (Fig. 3.2c). At MII stage, dynein localized at the poles of the metaphase II in both groups of oocytes (82%; 27/33) and (87%; 33/38) recovered from large and small follicles, respectively (Fig. 3.2D and 3.2d).

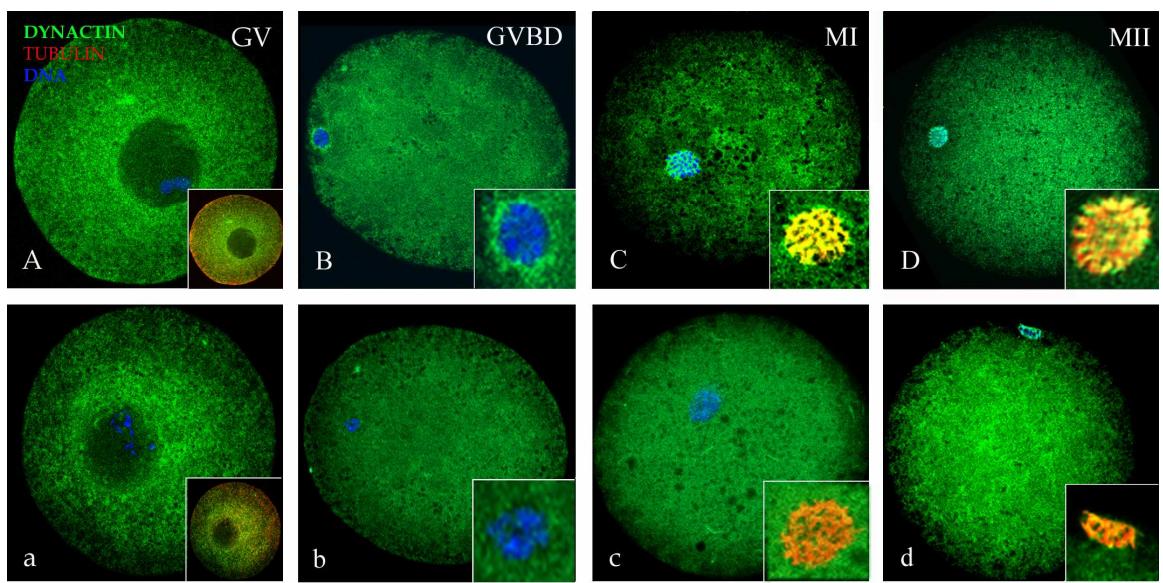


Fig. 3.1. Confocal microscopic images showing the dynamics of dynactin in relation to DNA and tubulin during bovine oocyte in vitro maturation. (A-D) oocytes recovered from follicles 2 – 8 mm at diameter in GV stage (A), GVBD (B), MI (C) and MII (D) and (a-d) oocytes recovered from follicles < 2 mm in GV stage (a), GVBD (b), MI (c) and MII (d). Green colour: dynactin, red: tubulin and blue: DNA. Yellow: merge of green and red showing co-localization. Dynactin was distributed in GVs with stronger signal in the periphery of the nucleus (A and a) and no colocalization with tubulin was found (A and a insets), it was associated with DNA at GVBD stage in oocytes recovered from large follicles (B and B inset), no clear association was found in the oocytes recovered from small follicles (b and b inset). At MI and MII dynactin interacted with the spindles in oocytes from large follicles (C and D) and was hardly found in the spindles of those collected from small follicles (c and d). Insets show the associations of dynactin with tubulin. Scale bars: 50 µm.

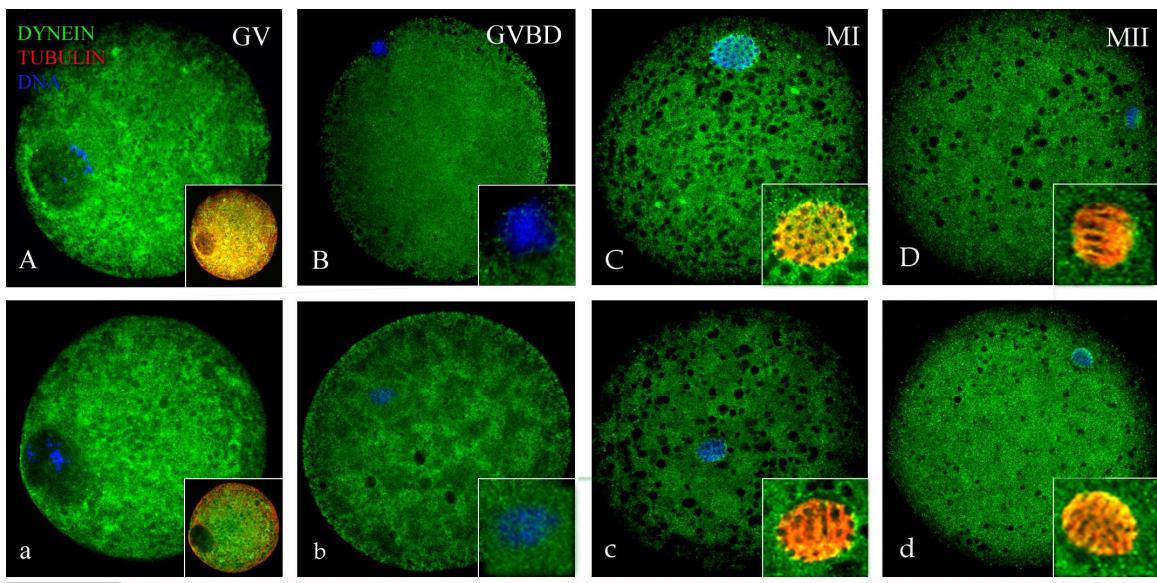


Fig. 3.2. Confocal microscopic images showing the dynamics of dynein in relation to DNA during bovine oocyte in vitro maturation. (A-D) oocytes recovered from follicles 2 – 8 mm in diameter in GV stage (A), GVBD (B), MI (C) and MII (D) and (a-d) oocytes recovered from follicles < 2 mm in GV stage (a), GVBD (b), MI (c) and MII (d). Green colour: dynein, red: tubulin and blue: DNA. Yellow: merge of green and red showing co-localization. Dynein was homogenously distributed in GVs (A and a) and no co-localization with tubulin was found (A and a insets). It was not associated with DNA at GVBD in oocytes recovered either from large follicles (B and B inset) or from small follicles (b and b inset). At MI dynein interacted with the spindles in oocytes from large follicles (C). It was hardly found at the MI spindle of those collected from small follicles (c). At MII stage the localization at the poles of the metaphase II was evident in both groups (D and d). Insets show the associations of dynein with tubulin. Scale bars: 50  $\mu$ m.

Inhibition of dynein by SOV indicates a crucial role of dynein-dependent transport during bovine oocyte maturation

We have used sodium orthovanadate (SOV), a phosphatase and ATPase activity inhibitor which allows to study dynein-dependent transport virtue of its high level of selectivity for dyneins over kinesins (Niclas et al., 1996). Previously, it has been used in mouse oocytes (Carabatsos et al., 2000; Wang et al., 2004; FitzHarris et al., 2007). To examine the role of dynein-dependent transport during bovine oocyte maturation, we used either a continuous treatment (24 h) starting at the GV stage or different time periods of SOV exposition (0.5; 1h or 2h) during oocyte in vitro maturation (Table 3.1). After 24 h of treatment, SOV completely blocked in vitro maturation and the chromatin configuration observed was similar to the GV stage; the DNA was localized in the periphery of the oocytes.

Oocyte maturation rates in groups treated with SOV for 1 h and 2 h were significantly decreased compared to the controls irrespective of the starting points of SOV treatments (Table 3.1). Maturation rates did not differ between 0.5 h SOV treatments and the control group. Oocytes blocked by SOV for 1 h or 2 h remained at different immature stages, i.e. GVBD in those groups exposed to SOV for 2 h and mainly MI in those groups exposed to SOV for 1 h.

Table 3.1. Effect of the dynein ATPase activity inhibitor sodium-orthovanadate on bovine oocyte maturation rate

Hours in SOV	Start-points of SOV treatment													
	Control IVM			0 h				5 h				9 h		
	%	%	%	(n/N)	(n/N)	(n/N)	(n/N)	(n/N)	(n/N)	(n/N)	(n/N)	(n/N)	(n/N)	
-	0.5 h	1 h	2 h	24 h				0.5 h	1 h	2 h	0.5 h	1 h	2 h	
<b>Maturation rate after 24 h IVM</b>														
%	83 $\pm$ 6	85 $\pm$ 4	22 $\pm$ 6	21 $\pm$ 4	9 $\pm$ 1			80 $\pm$ 4	26 $\pm$ 9	29 $\pm$ 4	78 $\pm$ 4	14 $\pm$ 3	11 $\pm$ 1	
(n/N)	(126/152)	(50/59)	(16/74)	(16/76)	(7/76)			(53/66)	(21/82)	(19/65)	(61/78)	(10/70)	(7/61)	

Numbers with asterisk were significantly different compared to the control ( $P < 0.05$ ); Chi-Square and Fisher exact tests. Total numbers, (n=MII oocytes and N=total number of oocytes observed [n/N]). Mean  $\pm$  SD from three independent replicates. 0 h = Germinal Vesicle stage (GV); 9 h = Germinal Vesicle Break Down (GVBD) recently established by Racedo et al. (2008).

Distribution of 20S proteasomes during bovine oocyte maturation is not affected by follicle size

The 20S proteasomes accumulated in the nucleus at the GV stage in both groups of oocytes, i.e. (100%; 43/43) (Fig. 3.3A) or (95%; 40/42) (Fig. 3.3a) from large and small follicles, respectively. The 20S proteasomes surrounded the DNA at the GVBD stage in oocytes collected from large (87%; 27/31) (Fig. 3.3B) and small follicles as well (83%; 25/30) (Fig. 3.3b). At MI, proteasomes were closely associated with the metaphase plate in oocytes from both size categories (91%; 32/35) (Fig. 3.3C) and (84%; 27/32) (Fig. 3.3c). Finally, at the MII stage, 20S proteasomes surrounded the metaphase plate in both types of oocytes (large follicles- 86%; 49/57) (Fig. 3.3D) (small follicles-82%; 31/38) (Fig. 3.3d).

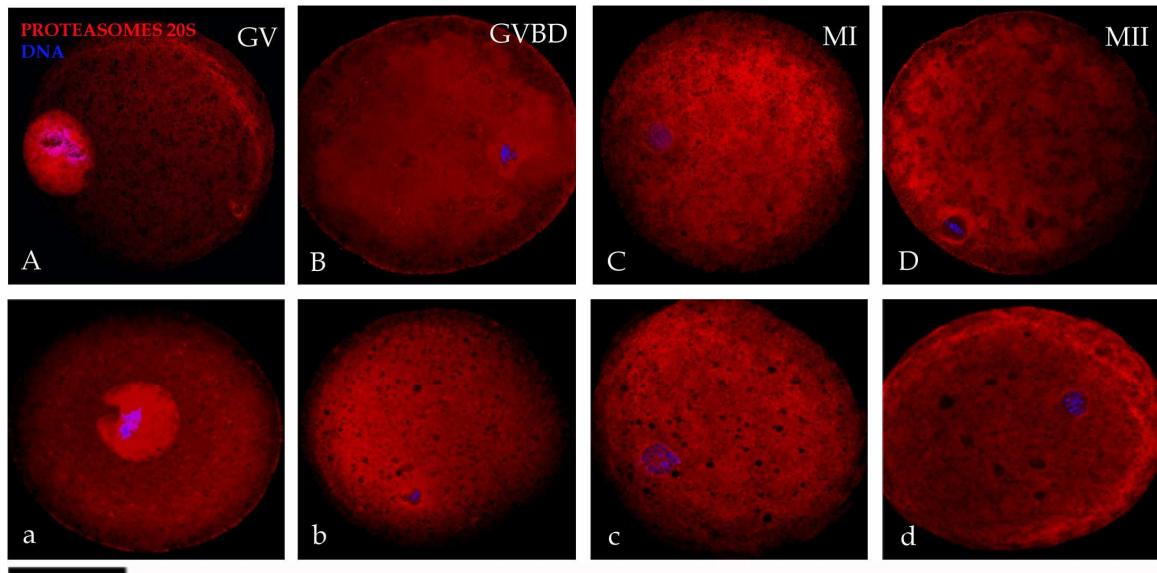


Fig. 3.3. Confocal microscopic images showing the dynamics of the 20S proteasomes ( $\alpha\beta$  subunits) in relation to DNA during bovine oocyte in vitro maturation. (A-D) oocytes recovered from follicles 2 – 8 mm in diameter in GV stage (A), GVBD (B), MI (C) and MII (D) and (a-d) oocytes recovered from follicles < 2 mm in GV stage (a), GVBD (b), MI (c) and MII (d). Red colour: 20S proteasomes and blue: DNA. The pattern of 20S proteasomes was not affected by the follicle size. 20S proteasomes were concentrated in the germinal vesicles (A and a), they were surrounding the DNA at GVBD (B and b), closely associated with the DNA at MI (C and c) and surrounding the metaphase II (D and d). Scale bars: 50  $\mu$ m.

## Discussion

In the present study, we showed that the ATPase activity of cytoplasmic dynein is essential for the progression of bovine oocyte maturation. Oocytes incubated with SOV were not able to complete meiosis, suggesting that inhibition of dynein-dependent transport correlates with cell cycle arrest. Recently, it was shown that cytoplasmic dynein is involved in meiotic checkpoint inactivation in mouse oocytes by transporting specific proteins from kinetochores to the spindle poles (Zhang et al., 2007) and controlling the endoplasmic reticulum structure (FitzHarris et al., 2007), suggesting that dyneins control both nuclear and cytoplasmic maturation. Previously, Carabatsos et al. (2000) had shown that the spindle structure was perturbed in mouse oocytes treated with SOV. Wang et al. (2004) demonstrated the effects of SOV on mouse oocyte maturation and the relationship with cyclin B1 mRNA expression level. Ectopic expression of this messenger was observed when oocytes where incubated with SOV and progression of meiosis was interrupted in a dose-time-dependent manner. Our results show that cellular events controlled by dynein ATP-ase activity play a crucial role for bovine meiotic progression similar to mouse oocytes (Wang et al., 2004). The movement of the nucleus from the central part of the oocyte to the periphery of the cell was not disturbed in oocytes incubated for 24 h with SOV, suggesting a microtubule independent mechanism for translocation which is in agreement with previous studies (Kim et al., 2000; Sun and Schatten 2006).

Specific proteins must be present in a proper spatial-temporal localization to carry out their functions allowing to assess the biological function of proteins by determining the localization. The dynein protein dynamics in bovine oocytes showed no specific localization until metaphase I as in mouse oocytes (Zhang et al., 2007), indicating similarities after GVBD across mono- and poly-ovulatory species. Obviously, dynein plays a critical role in the progression from meiosis I to meiosis II. Recently, we demonstrated that the relative abundance (RA) of dynein mRNA decreased during maturation comparing GV and GVBD to MII, in oocytes collected from large follicles and the RA was higher in the latter group (Racedo et al., 2008). Dynein protein was specifically localized to the spindle at metaphase I, in parallel with decreasing dynein mRNA abundance, suggesting that the decrease could be due to specific spatio-temporal translation of the dynein messenger in this oocyte population. On the contrary, in oocytes recovered from small follicles the staining of dynein protein in the spindle at metaphase I was weak, coinciding with stable and lower relative abundance of the dynein mRNA. Thus, apart from the lower abundance of the messenger in this group, the specific spatio-temporal translation of dynein messenger may be less efficient in this oocyte population.

In contrast to dynein, no effects of follicle size or maturational stage on the RA of dynactin mRNA was found in our previous study (Racedo et al., 2008). Nevertheless, dynactin protein dynamics was affected, showing the complexity of the mechanisms that regulate transcription and translation during oocyte maturation (Brevini et al., 2007). Dynactin was found to surround the condensed

chromatin in oocytes at GVBD stage. In a recent study in *Drosophila melanogaster* somatic cells, it was shown that dynactin binding to microtubules is required for organizing spindle microtubules arrays (Kim et al., 2007). We speculate that dynactin may play a role in the organization of microtubules for the progression of nuclear maturation during bovine oocyte maturation as foci of co-localization with tubulin were observed at this stage (data not shown). Dynactin was localized to the perinuclear region during bovine fertilization independently of microtubules, and dynein found exclusively around the migrating female pronucleus (Payne et al., 2003). Transport of the nucleus during fertilization and the transport of the GV from the center of the oocyte to the periphery involve various molecular mechanisms (Sun and Schatten 2006). We detected dynactin together with the spindles and dynein. It has been shown that dynactin associates with dynein (Karki and Holzbaur 1999), and both proteins would have the same localization in a specific stage of the cell cycle. Dynactin p150 functions independently of dynein as dynactin also has been shown to act as adaptor for at least two motors of the kinesin superfamily, i.e. heterotrimeric kinesin-2 (Deacon et al., 2003) and mitotic kinesin Eg-5 (Blangy et al., 1997).

In view of the variety of functions of dynein and dynactin, the differences found between meiotic stages and oocytes recovered from different size follicle indicate that dynein and dynactin are key factors in the regulation of cytoplasmic processes such as transport of organelles and molecules during oocyte cytoplasmic maturation that are closely related to the acquisition of oocyte developmental competence.

We did not find an effect of follicle size on the localization of the 20S proteasomes as detected by antibodies reacting with their  $\alpha$  and  $\beta$  subunits, which supports our recent finding for mRNAs of 20S proteasomes  $\beta 1$  and  $\alpha 4$  subunits (Racedo et al., 2007). The varying presence of proteasomes during different stages of bovine oocyte maturation, suggests that they have an important role for remodeling nuclear and cytoplasmic processes. Previously, an essential role of proteasomes for the decrease in MPF activity and completion of the first meiotic division could be demonstrated in rat oocytes, (Josefsberg et al., 2000), acting in parallel with the translocation of proteasomes to the spindle at MI. Here, we showed that the localization of proteasomes during bovine oocyte maturation and the association with the nucleus is similar to the distribution observed in porcine and human oocytes (Huo et al., 2004; Wójcik et al., 2000). Our results suggest that proteasomes might have a critical role during oocyte maturation, particularly in the progression from MI to MII, as during early meiosis I the proteasomes move to the periphery of the chromosomes to get in close contact at the metaphase I stage.

In conclusion, the present study describes for the first time the dynamics of dynein protein, dynactin p150 protein and 20S proteasomes during bovine oocyte maturation which significantly substantiate and expands our previous findings about mRNAs for these specific molecules. Results indicate a crucial role of dynein-dependent transport in bovine female meiosis and a possible relationship of dynein and dynactin proteins dynamics with the developmental competence of the bovine oocytes. Whether the present results obtained in *in vitro* matured oocytes reflect the

physiological situation remains to be investigated on in vivo derived oocytes. Significant differences with regard to oocytes and embryos derived from in vitro procedures and in vivo can be found with regard to mRNA expression for a variety of genes (Wrenzycki et al., 2007).

## **Capítulo 4**

### **Dynamics of reprogramming mRNA expression and histone modifications during bovine in vitro maturation**

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In preparation.

## Summary

Epigenetic mechanisms control eukaryotic development without changes in the DNA-stored information. A well-orchestrated gene expression pattern within the oocyte as well as proper epigenetic modifications should happen for a correct maternal embryonic transition (MET) and the subsequent embryo development. Developmental competence of in vitro produced embryos is affected for the origin of the oocyte and the culture conditions. The goal of this study was to investigate the mRNA expression profile of selected reprogramming genes and two histone modifications, the dimethylation status of histones H3 at position lysine K9 (H3-K9diMe) and the acetylation status of histones H4 at position lysine K12 (H4-K12 acetylation), in bovine oocytes. The RAs of G9A (HLA-B associated transcript 8), SUV39H1 (suppressor of variegation 3-9 homolog 1), DNMT1 (DNA methyltransferase 1), DNMT3b (DNA methyltransferase 3b) and ZAR1 (zygote arrest 1) were assessed by semiquantitative endpoint RT-PCR during in vitro maturation, i.e. GV, GVBD, MI and MII stages in oocytes recovered from follicles < 2 mm and 2 - 8 mm. H3-K9diMe and H4-K12 acetylation were assessed by immunocytochemistry. An effect of the follicle size on the RAs of G9A, SUV39H1 and DNMT1 was found, being higher in immature oocytes isolated from follicles 2 - 8 mm. We detected a dynamic expression of ZAR1 during the analyzed stages only in those oocytes isolated from the bigger follicles. No effect either of follicle size or meiotic stage on DNMT3b expression was found. When comparing size and stages,

G9A RA in GVBD and DNMT1 RA in GV were significantly higher in those oocytes isolated from follicle size 2 - 8 mm compared to the other group.

H3-K9diMe signal was present in GV stage and maintained up to the end of the maturation, a light decrease of the signal was seen after GVBD stage, no effect of the follicle size was found. The H4-K12 acetylation showed stronger signal in GV and GVBD and markedly decreased after GVBD compared to H3-K9diMe, this pattern was not affected by the follicle size. Results provide for the first time a detailed analysis of the expression of above genes involved in epigenetic modifications, the dynamics of H3-K9diMe and H4-K12 acetylation during bovine oocyte in vitro maturation and its relationship with oocyte developmental competence.

## **Introduction**

The capacity of the oocyte to sustain the maturation process, the oocyte activation after fertilization, and the early embryo development including the maternal embryonic transition (MET) is acquired progressively during late follicular growth and is fully acquired during oocyte maturation (Hyttel et al., 1997). A well-orchestrated expression of genes is required to ensure progression of mammalian oocytes through the meiotic stages to achieve full developmental competence. Bovine oocytes recovered from small follicles (< 2 mm) developed at a significantly lower frequency than their counterparts from bigger follicles (> 2 mm) after in vitro fertilization (Pavlok et al., 1992; Lequarre et al., 2005). Prior to maternal-embryonic transition (MET), i.e. 8- to 16-cell stage in the bovine, the oocyte is dependent on mRNA and proteins, which are accumulated during oogenesis (Memili and First, 2000). De novo synthesis of RNA has been observed up to the germinal vesicle stage (GV), and ceases after GV breakdown (GVBD) (Memili et al., 1998; Tomek et al., 2002). Thus, maturation and early embryo development are subject to regulation by the RNA and protein pools synthesized during the growth phase and the first period of maturation. Reprogramming genes and epigenetic modifications during oocyte maturation have been poorly explored, nevertheless, are play a crucial role in the regulation of the chromatin activation or repression.

Epigenetic modifications include methylation of DNA, histone modifications and noncoding RNAs that together regulate gene expression that is not directly related to the DNA sequence (Rutherford and Henikoff, 2003).

The genes selected for this study are indicative for critical steps during the early embryo development as proper epigenetic modifications should happen for a correct maternal embryonic transition (MET) and successful development (Wrenzycki et al., 2005). Here, we have analyzed the mRNA expression profiles of the histone methyltransferases (HTMs), G9A (HLA-B associated transcript 8) and SUV39H1 (suppressor of variegation 3-9 homolog 1), the DNA methyltrasferases (DNMTs), DNMT1 (DNA methyltransferase 1) and DNMT3b (DNA methyltransferase 3b) and the oocyte-specific maternal factor ZAR1 (zygote arrest 1) as well as the global dimethylation status of H3-K9 and acetylation of H12-K14 during maturation.

DNMT1 is a maintenance enzyme that methylates hemimethylated CpG dinucleotides after DNA replication (Bestor, 1992) while DNMT3b is required for de-novo methylation and for establishing new DNA-methylation patterns during development (Okano et al., 1999; Hsieh, 1999). Methylation of DNA is generally associated with repression of transcription (Bird and Wolffe, 1999) although it has been shown methylation of imprinted genes in the active allele (Reik and Walter, 2001).

G9A and SUV39H1 specifically methylate the histones H3 at position K9 (lysine 9) and this modification inactives the chromatin (Lachner and Jenuwein, 2002) with the final consequence of repression of gene expression. G9A has been shown to play a dominant role in histone H3-K9 methylation essential for early embryogenesis (Tachibana et al., 2002).

ZAR 1 has been shown critically involved in the maternal-zygotic transition of mouse embryos (Wu et al., 2003) and according to the molecular structure is thought to have got a role in transcription regulation.

Among the variety of histone tail modifications, we have chosen H3-K9diMe as it is the final effect of the product of the genes G9A and SUV39H1 above mentioned and H4-K12 acetylation thought to be a critical epigenetic marker for active genes (Kruhlak at el., 2001; Smith et al., 2002). H4-K12 acetylation has been studied during maturation in mouse oocytes (Kim et al., 2003; Akiyama et al., 2004); porcine oocytes (Endo et al., 2005) and recently studied in ovine oocyte (Tang et al., 2007) while H3-K9 methylation has been studied in porcine oocyte and embryos (Bui et al., 2007) and in bovine cloned embryos (Santos et al., 2003).

Here, we unrevealed the dynamics of the above genes during oocyte maturation, the dynamics of H3-K9diMe and H4-K12 acetylation as well as the possible relationship with oocyte developmental competence.

## **Materials and methods**

### ***Oocyte collection and in vitro maturation***

Bovine ovaries were collected at a local abattoir and transported to the laboratory at 25-30°C. Ovaries were washed three times in 0.9% NaCl supplemented with penicillin (PEN-NA, Sigma) and streptomycin (35500; Serva). Cumulus-oocyte complexes (COCs) were aspirated from follicles either < 2 mm or 2 - 8 mm in size using a 18-gauge needle attached to a 5-ml syringe. COCs were selected under a stereomicroscope and only oocytes with at least three layers of compact cumulus cells with a homogeneous granulated cytoplasm were used for this study. The COCs were maintained Dulbecco's PBS medium (D5773; Sigma) supplemented with 50 IU/ml penicillin, 50 µg/ml streptomycin (35500; Serva), 36 µg/ml pyruvate (P3662; Sigma), 1 mg/ml D-glucose (Carl Roth GmbH, Karlsruhe, Germany), 133 µg/ml calcium chloride dehydrate (Merck, Darmstadt, Germany) and 2 IU/ml heparin (24590; Serva) and BSA (fraction V, A9647; Sigma). Cumulus oocyte complexes were then collected into TCM-air (TCM 199 with Earle's salts, L-glutamine and 25 mM Hepes [M2520; Sigma] supplemented with 22 µg/ml pyruvate [P3662; Sigma], 350 µg/ml NaHCO<sub>3</sub> [31437; Riedel-deHaen], 50 µg/ml gentamycin [G3632; Sigma], and 0,1 % BSA-fatty acid free [A7030; Sigma]).

Maturation medium consisted of TCM 199 (M2520; Sigma) supplemented with 22 µg/ml pyruvate (P3662; Sigma), 2.2 mg/ml NaHCO<sub>3</sub> [31437; Riedel-deHaen], 50 µg/ml gentamycin (G3632; Sigma), 10 IU/ml of PMSG, 5 IU/ml of hCG (Suigonan, Intervet, Tönisvorst, Germany), 0.1% BSA-fatty acid free (A7030; Sigma) and 100 µM

cysteamine (M6500; Sigma). COCs from each follicle size category were matured in pools of 20 in separate 100 µl drops under silicone oil (35135; Silicone DC 200 fluid; Serva) in a humidified atmosphere composed of 5% CO<sub>2</sub> in air at 39°C for 24 h.

***Freezing at different meiotic stages***

Random samples were taken from the pool of maturing oocytes at different time points for freezing and subsequent RNA isolation. According to the observed meiotic progression the time points selected for freezing were GV-0 h; GVBD-8 h for oocytes from < 2 mm and 9 h for oocytes from 2 - 8 mm; MI-15 h; MII-24 h (Racedo et al., 2008). Groups of four oocytes or two oocytes were frozen in a minimum volume ( $\leq$ 5 µl) of PBS containing 0.1% polyvinyl alcohol (PVA) (P8136; Sigma) medium in a 600 µl siliconized Eppendorf tube (no. 710136; Biozym Diagnostic GmbH, Hess Oldendorf, Germany) and stored in a -80°C freezer prior to analysis of gene expression by RT-PCR. Oocytes from both categories of follicle sizes from at least ten different IVM were frozen.

***Determination of the relative abundance of developmentally important gene transcripts in bovine oocytes***

The relative abundances (RA) of transcripts for G9A, ZAR1, SUV39H1, DNMT3b and DNMT1 from bovine oocytes were assessed by RT-PCR analysis. Poly (A)<sup>+</sup> RNA was prepared from the pools of two or five oocytes as described previously (Wrenzycki et al., 2002). Reverse transcription (RT) was carried out in a total volume

of 20 µl. Prior to RNA isolation, 2 pg of rabbit globin RNA (BRL, Gaithersburg, MD) was added as an internal standard. The RT reaction was performed in 10x buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4; Invitrogen), 5 mM MgCl<sub>2</sub> (Invitrogen), 1 mM of each deoxynucleotide triphosphate (dNTP; Amersham, Biosciences Europe GmbH, Freiburg, Germany). Further ingredients such as 50 U murine leukemia virus reverse transcriptase, 20 U RNase inhibitor, and 2.5 µM random hexamers were supplied by Applied Biosystems (Foster City, CA). RT was carried out at 25°C for 10 min, followed by 1 h at 42°C, a denaturation step at 99°C for 5 min, and flash cooling at 4°C. Two different reaction mixture were used, (i) 0.1 cDNA oocyte equivalents/µl (2 oocytes in a 20-µl reaction volume) or (ii) 0.05 cDNA oocyte equivalents/µl (one oocyte in a 20-µl reaction volume) each with and 50 fg globin RNA/µl. PCR was performed using the cDNA equivalent of 0.1 oocyte (1 µl from the first cDNA oocyte equivalents concentration obtained) for DNMT3b, 0.3 oocyte (3 µl from the first cDNA oocyte equivalents concentration) for G9A, 0.5 oocytes (5 µl from the first cDNA oocyte equivalents concentration) for ZAR1, 0.6 oocytes (6 µl from the first cDNA oocyte equivalents concentration) for SUV39H1, 0.05 oocyte (1 µl from the second cDNA oocyte equivalents concentration obtained) for DNMT1 and 50 fg of globin cDNA (1 µl) in a final volume of 50 µl of 10x buffer (50 mM KCl, 20 mM Tris-HCl, pH 8.4), 1.5 mM MgCl<sub>2</sub>, 200 µM of each dNTP, 0.5 µM of each sequence-specific primer in a thermocycler (PTC- 200; MJ Research, Watertown, MA). For detection of G9A and SUV39H1, a special reaction mixture with 5 µl (10%) of DMSO (Merck, Darmstadt, Germany) was used to increase the primers specificity (Nowak et al.,

2007). To ensure specific amplification, a hot-start PCR protocol in which 1 U *Taq* DNA polymerase (Invitrogen) added at 72°C was employed. PCR primers were designed from the coding regions of each gene sequence using the OLIGO program. The sequences of the primers (*Globin* [Cheng et al., 1986]) the annealing temperatures, the fragment sizes, and sequence references are summarized in Table 4.1. The PCR program employed an initial denaturation step of 97°C for 2 min followed by a step of 72°C for 2 min (during which time hot-start was performed) and cycles of 15 sec at 95°C for DNA denaturation, 15 sec at the primer-specific annealing temperature, and 15 sec at 72°C for primer extension. The last cycle was followed by a 5-min extension at 72°C and cooling to 4°C (for number of cycles, see Table 1). PCR reactions for the genes of interest and the globin internal standard were performed in separate tubes. The PCR products were subjected to electrophoresis on a 2% agarose gel in 1x TBE buffer (90 mM Tris, 90 mM borate, 2 mM EDTA, pH 8.3) containing 0.2 µg/ml ethidium bromide, present in both gel and running buffer to stabilize the concentration of ethidium bromide for quantification. The fragments were visualized on a 312-nm UV transilluminator; the image of each gel was recorded using a CCD camera (Quantix, Photometrics, München, Germany) and the IPLab Spectrum program (Signal Analytics Corp., Vienna, VA). The intensity of each band was determined by densitometry using an image analysis program (IPLab Gel). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each meiotic stage by the intensity of the corresponding globin band. For each gene, ten separate RT-PCR reactions were

performed. The RNA recovery rate was estimated as the ratio between the intensity of the globin band with and without the RNA preparation procedure, starting with an equivalent of 50 fg in the PCR reaction. On average, 60% of poly (A)-tailed RNA was recovered using the Dynabead oligo d(T) mRNA isolation method, which is in agreement with previous results from our laboratory (Yaseen et al., 2001; Wrenzycki et al., 1999; 2002). This semi-quantitative endpoint RT-PCR assay is highly sensitive and accurate and yields similar results as real time RT-PCR techniques (Knijn et al., 2002; Gal et al., 2006).

Table 4.1. Primers used for PCR of bovine oocyte gene transcripts.

Genes	Primers sequences and positions	Annealing Temperature (°C), cycle number	Fragment size (bp)	Sequence references [EMBL accession no.]
Globin				
5' (241-260)	GCAGGCCACGGTGGCGAGTAT	60 × 27	257	X 04751
3' (555-657)	GTGGGACAGGAGCTTGAAAT			
G9A				
5' (523-548)	GAGACGTCCACCATGAACATTGACC	55 × 31	508	TC189846
3' (1006-1031)	GCATGAAGACTCGGACGGGGATGAT			
SUV39H1				
5' (259-284)	GGACTGAATCCTGCCGCAAATACC T	60 × 34	307	TC230460
3' (541-566)	GGCCATGAATCCCAACTGCAGAAAG			
DNMT1				
5' (3856-3879)	CGCATGGCTACCAGTGCACCTT	60 × 33	312	AY1730481
3' (4145-4168)	GGGCTCCCCTTGTATGAAATCT			
DNMT3b				
5' (1288-1310)	GACTCATTGGAGGACCAGCTGAAGC	61 × 33	555	AF331857
3' (1818-1840)	CAGCACCTCCAGGCCTCCACACAG			
ZAR1				
5' (951-973)	GCTGCGCTTCCAGTTCTTAGAG	55 × 32	255	NM_175619.1
3' (1184-1206)	TCTTGACGGTGGGGCCGTTAG			

Globin (Cheng et al., 1986)

### *Immunofluorescence staining*

The time points selected for fixation were the same used for freezing and mRNA analysis. Random samples were taken from pools of maturing oocytes at different time points for fixation and subsequent protein analysis. Oocytes from both follicle sizes from three different IVM replicates were fixated for ICC. The attached cumulus cells of the COCs were removed using 0.1% hyaluronidase (H4272; Sigma) for 10 min at 37°C and vortexing for 4 min and zona pellucidae of oocytes were gently removed with short incubations in TCM containing Hepes (TCM 199 with Earle's salts, L-glutamine [G6392; Sigma] and 25 mM Hepes [M2520; Sigma] supplemented with 22 µg/ml pyruvate [P3662; Sigma], 350 µg/ml NaHCO<sub>3</sub> [31437; Riedel-deHaen], 50 µg/ml gentamycin [G3632; Sigma], and 0.1 % BSA-fatty acid free [A7030; Sigma]) and 2 mg/ml pronase (P6911; Sigma). After the removal of zona pellucida, the oocytes were fixed for 30 min in 3.7% paraformaldehyde (Sigma) in PBS (D5773; Sigma), and permeabilized with 0.2% Triton X-100 (Sigma) in PBS for 10 min at room temperature. The fixed oocytes were blocked overnight at 4°C in 1% BSA, 0.1% Triton X-100 in PBS. After blocking, the oocytes were incubated in the same solution with anti PanHistones (mouse polyclonal, Roche); at 1:500 in all the stages, it recognizes the core histones and serves as positive control for the immunostaining procedure and anti dimethyl H3/K9 antibody (rabbit polyclonal, a gift from T. Jenuwein to J. Walter); at 1:500 or anti acetyl H4-K12 antibody (rabbit polyclonal, Upstate Biotechnology, Inc., Lake Placid, NY); at 1:250 overnight at 4°C,

followed by several washes and incubation for 1 hour with antimouse secondary antibodies coupled with fluorescein (Sigma-Aldrich), and anti-rabbit secondary antibodies coupled with Rhodamine Red-X (Jackson ImmunoResearch Laboratories Inc.). The secondary antibodies were applied at 1:100. After final washes the oocytes were placed on slides and mounted with a small drop of Vectashield (VectorLab) mounting medium containing 0.5 µg 4,6-diamino-2-phenylindole (DAPI). More than 20 oocytes have been analyzed for each stage of in vitro maturation in each category of follicle size and at least 90% of examined oocytes in each experimental group showed the same results.

#### *Immunofluorescence microscopy*

The slides were analyzed on Zeiss Axiovert 200 M inverted microscope equipped with the fluorescence module and B/W digital camera for imaging. The images were captured, pseudocoloured and merged using AxioVision software (Zeiss).

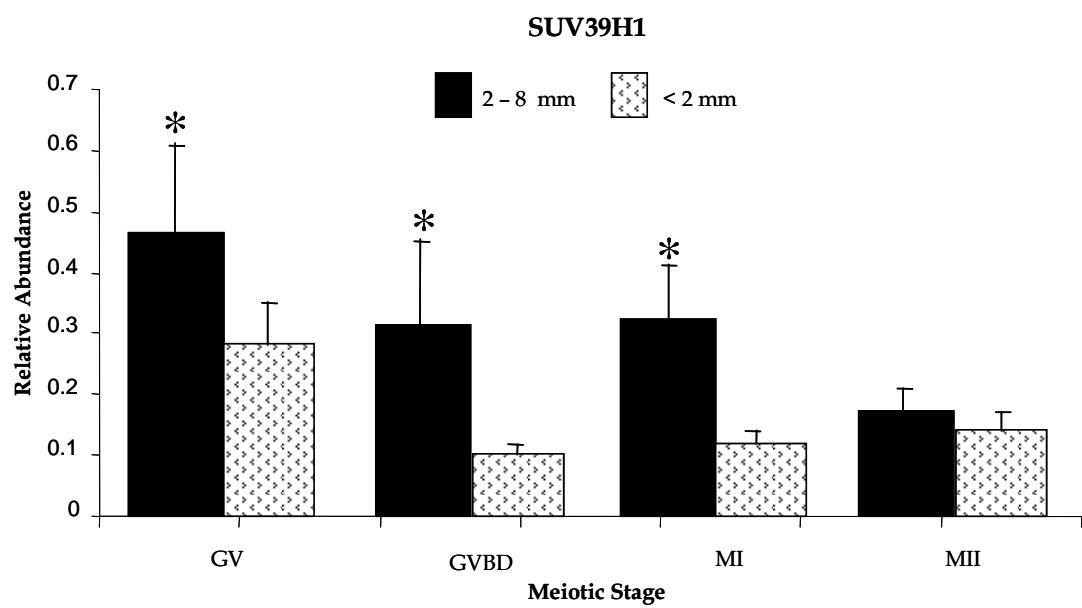
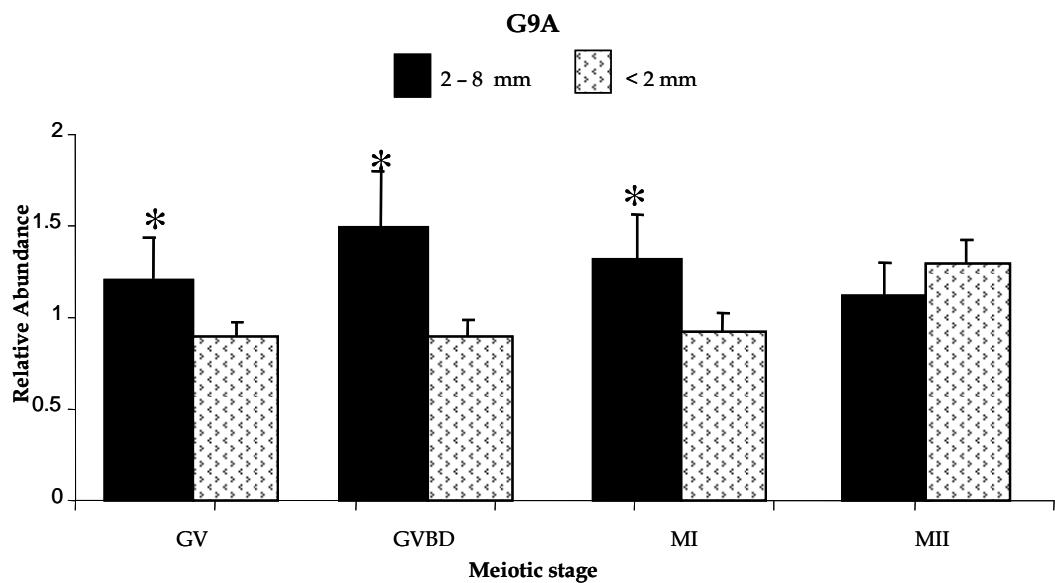
#### *Statistical analysis*

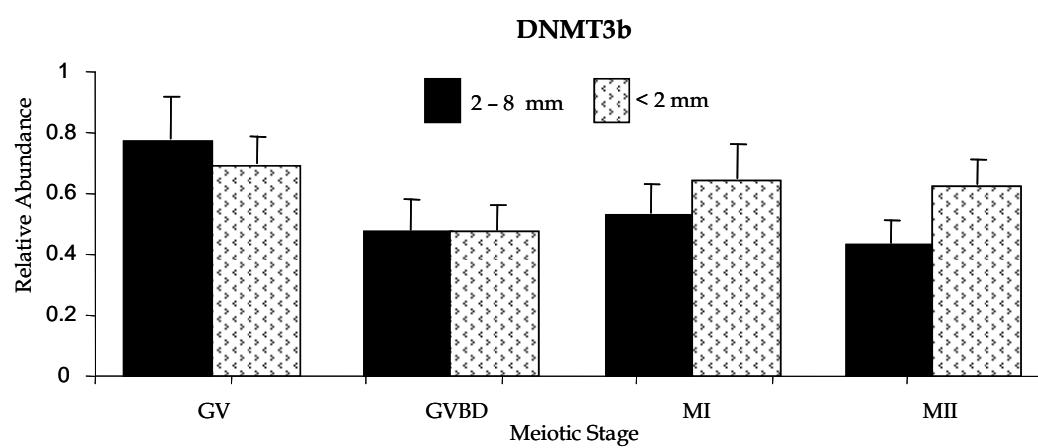
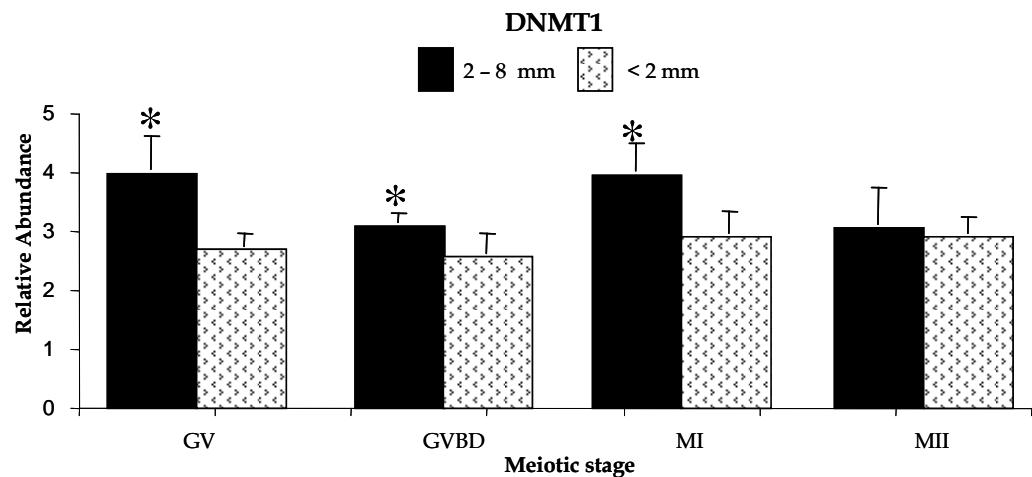
The data were analyzed using the SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) software package. After testing for normality (Kolmogorov-Smirnov test with Lilliefor correction) and equal variance (Levene Median test), a two-way ANOVA and one-way ANOVA followed by multiple pair-wise comparisons using the Tukey test were used for the RAs between stages and categories of follicle sizes and for the RAs within one follicle size, respectively. Differences of  $P < 0.05$  were considered significant.

## Results

### Relative abundances of reprogramming gene transcripts during bovine in vitro maturation

The RA of G9A, SUV39H1 and DNMT1 were significantly higher in oocytes from follicle size 2 - 8 mm compared to oocytes from the smaller follicles (Fig. 1). No significant differences in the RAs of DNMT3b and ZAR1 were found between the two groups (Fig. 4.1). Nevertheless, the dynamics of ZAR1 transcript during maturation is significantly different between the two groups. ZAR1 mRNA decreased significantly during IVM in oocytes from follicles 2 - 8 mm whereas it remained stable in the oocytes isolated from the smaller follicles. When comparing size and stages, RA of G9A was significantly higher in GVBD in the oocytes from follicle size 2 - 8 mm compared to the same stage in the other group, the same for DNMT1 in GV.





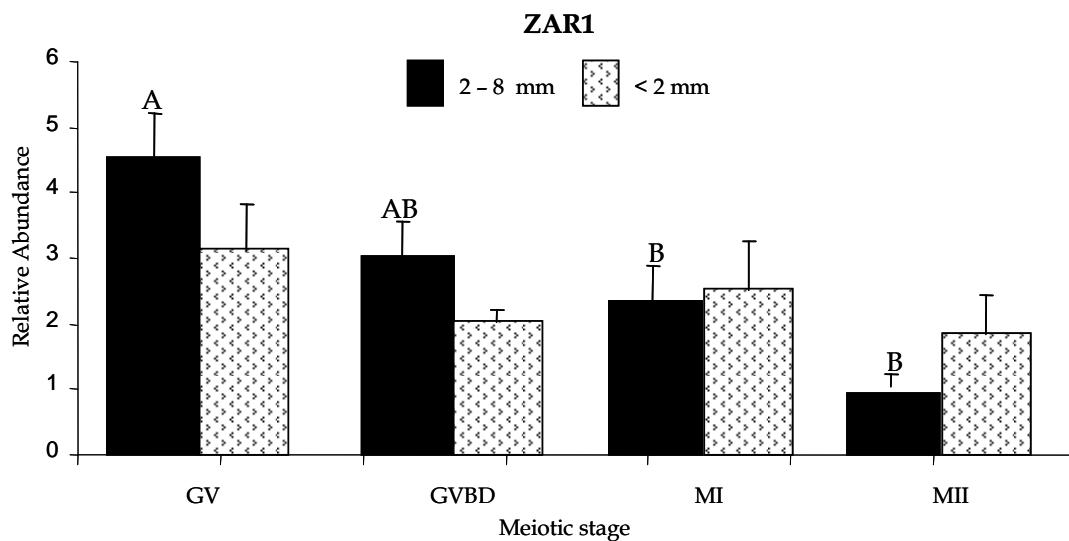


Fig. 4.1. Relative abundance of some important genes transcripts (values shown as mean  $\pm$  S.E.M.) in bovine oocytes recovered from follicles 2 – 8 mm size and follicles < 2 mm throughout in vitro maturation (GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I and MII, metaphase II). Significant differences between follicles sizes are indicated by (\*) ( $P < 0.05$ ) ANOVA two way, whereas (A, B) ( $P < 0.05$ ) indicates significant differences within bigger follicles through meiotic stages, ANOVA one way.

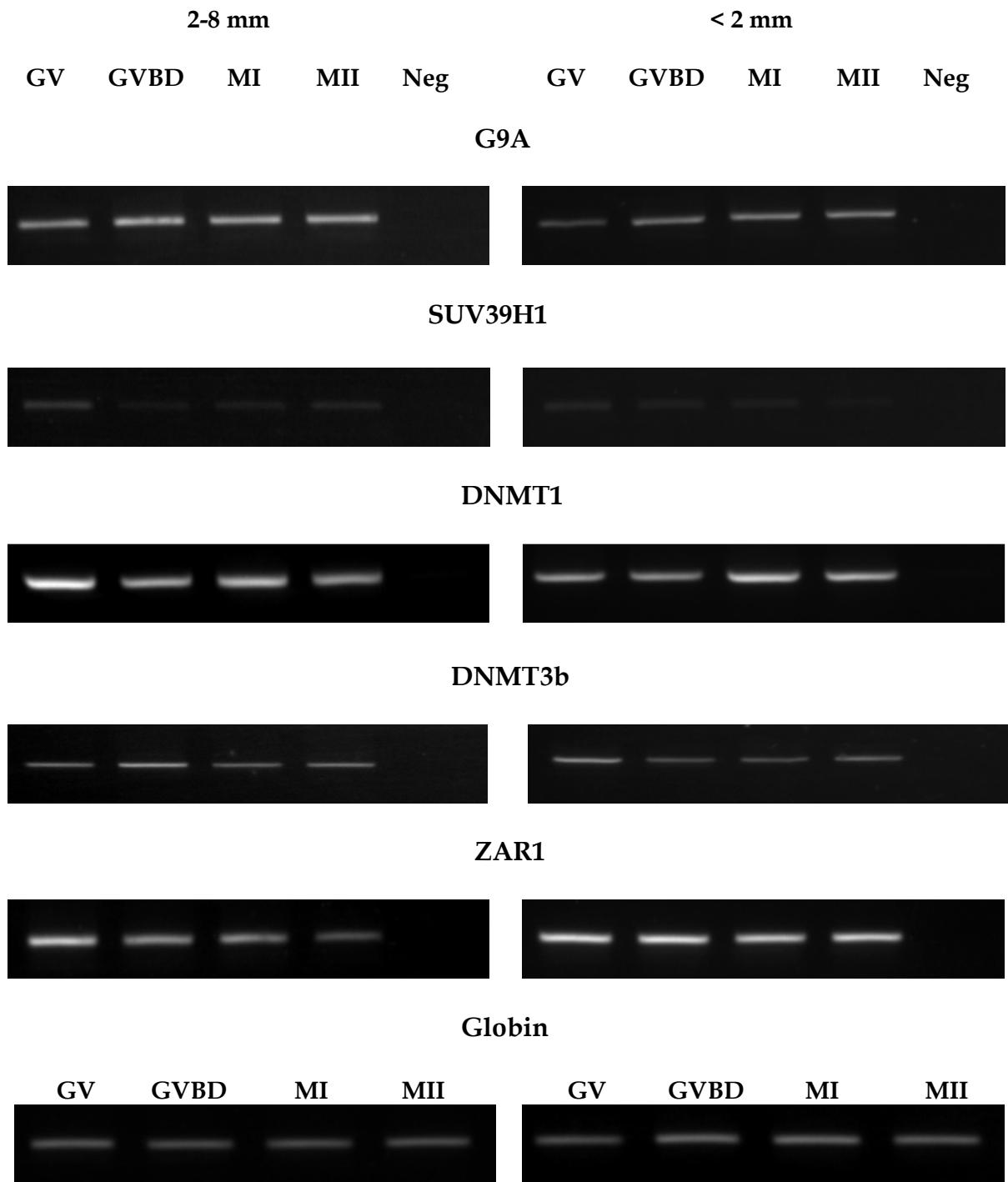


Fig. 4.2. Representative photograph of gels from semi-quantitative endpoint RT-PCR analysis of some important gene transcripts in bovine oocytes recovered from follicles 2 – 8 mm size and follicles < 2 mm throughout in vitro maturation (GV, MI, MII).

germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I and MII, metaphase II). Each lane represents the PCR product derived from poly (A)<sup>+</sup>RNA after RT-PCR from the equivalent of 0.3 oocyte for G9A and DNMT3b, 0.6 oocyte for SUV39H1, 0.05 oocyte for DNMT1 and 0.5 oocyte for ZAR1 and the standard rabbit *globin*. Each reaction was assessed ten times.

## Dynamics of H3-K9 dimethylation and H4-K12 acetylation during bovine oocyte maturation

The dimethylation of histones 3 at position K9 showed signal during complete oocyte maturation. The signal was stronger at GV and GVBD stages; a light decrease of the signal was observed in MI and MII, nevertheless, this modification is maintained up to the end of the maturation (Fig. 4.3A). A similar pattern and dynamics was found in both types of oocytes indicating no effect of the follicle size on this epigenetic modification (Fig. 4.3A and 4.3B).

The acetylation of histones 4 at position K12 showed an enhanced dynamics during oocyte maturation evidenced by a strong signal in GV and GVBD and markedly decreased in MI and MII, being almost undetectable at these stages (Fig. 4.4A). The same pattern and dynamics was observed in both populations of oocytes indicating no effect of the follicle size on this epigenetic modification (Fig. 4.4A and 4.4B). H4-K12 acetylation showed a stronger signal at GV and GVBD compared to the H3-K9diMe signal in the same stages.

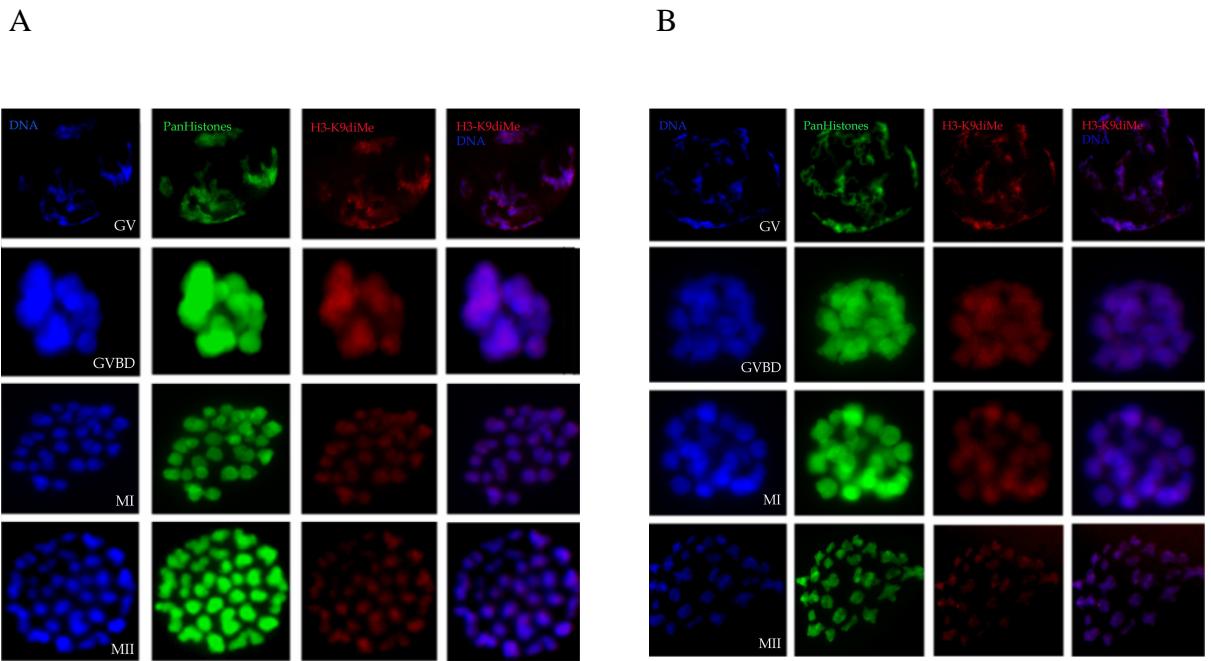
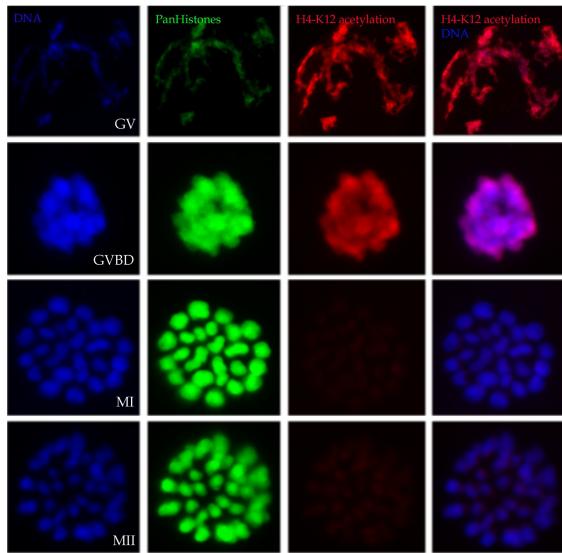


Fig. 4.3. Dynamic changes of H3-K9 dimethylation during bovine oocyte in vitro maturation. (A) oocytes recovered from follicles 2 – 8 mm in diameter in GV stage, GVBD, MI and MII and (B) oocytes recovered from follicles < 2 mm in GV stage, GVBD, MI and MII. DNA is visualized by DAPI (blue colour) staining. Mouse monoclonal anti PanHistones antibodies were detected by fluorescein conjugated anti-mouse secondary antibodies (green colour). H3/K9diMe was detected by Rhodamine Red-X conjugated anti-rabbit secondary antibody (red colour).

A



B

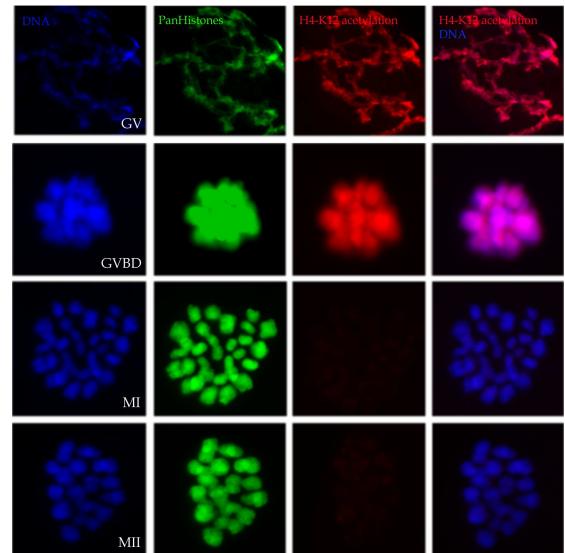


Fig. 4.4. Dynamic changes of H4-K12 acetylation during bovine oocyte in vitro maturation. (A) oocytes recovered from follicles 2 – 8 mm in diameter in GV stage, GVBD, MI and MII and (B) oocytes recovered from follicles < 2 mm in GV stage, GVBD, MI and MII. DNA is visualized by DAPI (blue colour) staining. Mouse monoclonal anti PanHistones antibodies were detected by fluorescein conjugated anti-mouse secondary antibodies (green colour). H4/K12 acetylation was detected by Rhodamine Red-X conjugated anti-rabbit secondary antibody (red colour).

## Discussion

The expression profiling of oocytes and embryos has emerged as a useful tool to determine factors affecting the transcriptional level during early development. Numerous studies have demonstrated a close relationship between oocyte/embryo viability and the mRNA abundance of panels of various genes (Wrenzycki et al., 2007). Isolating oocytes from follicles of different sizes has been assessed in an effort to elucidate molecular markers involved in the acquisition of developmental competence of the oocytes (Robert et al., 2000; Robert et al., 2002a,b; Donnison and Pfeffer, 2004; Baran et al., 2004; Lequarre et al., 2005; Nemcova et al., 2006; Mourot et al., 2006; Pfeffer et al., 2007; Racedo et al., 2007). Here, we analyzed the relative abundance of a panel of genes thought to be critically involved in epigenetic modifications, G9A, SUV39H1, DNMT1, DNMT3b and ZAR1. The expression of G9A, SUV39H1 and DNMT1 was significantly affected by follicle size and the expression of ZAR1 was affected by the maturation stage. Previous studies have shown a significant decrease in the relative abundance of SUV39H1 between GV and MII in bovine oocytes (Nowak et al., 2007) and downregulation in murine matured oocytes (Oliveri et al., 2007); here SUV39H1 showed a decrease during maturation but it was not statistically different between the stages analyzed. SUV39H1 mRNA was significant higher expressed in oocytes recovered from big follicles.

G9A transcripts were found absent in immature and matured murine oocytes (Oliveri et al., 2007) and downregulated in bovine matured oocytes (Nowak et al.,

2007), our results show a stable expression during bovine maturation and significant higher expressed in oocytes recovered from large follicles. The relative abundance of DNMT3b did not change during maturation and the same pattern was observed in murine oocytes (Vassena et al., 2005; Oliveri et al., 2007). DNMT1 mRNA was first detected in mouse oocytes in GV and MII stages although the protein was not present in any oocyte stage (Ratnam et al., 2002); suggesting that this enzyme does not have a role during maturation. In bovine oocytes we found a stable expression of the DNMT1 messenger and an expression significant higher in oocytes recovered from big follicles. ZAR1 mRNA has been detected for the first time in bovine oocytes (Pennetier et al., 2004; Brevini et al., 2004); it was markedly present in oocytes recovered from antral follicles (Pennetier et al., 2004). We found a decrease of the relative abundance of ZAR1 during maturation, which is in agreement with findings made on bovine oocytes comparing GV and MII stages (Uzbekova et al., 2006; Nowak et al., 2007) and ovine oocytes in the same stages (Ariu et al., 2007). In the present study we observed a decrease of ZAR1 mRNA already in MI stage compared to GV oocytes, suggesting a processing of the mRNA during meiosis I. This dynamics of ZAR1 mRNA was observed only in oocytes collected from bigger follicles while the relative abundance of this messenger remained stable in the population recovered from small follicles. Taking together previous studies and the roles described for the products of the genes above mentioned, we hypothesize that G9A and ZAR1 maybe closely associated with the acquisition of oocyte developmental competence that produce higher cleavage and blastocyst formation

rates after fertilization. To test if the higher expression of mRNAs of the histone methyltransferases, G9A and SUV39H1, found between oocyte populations makes a difference in the pattern of methylation of histones 3 at position lysine 9, we studied H3-K9diMe pattern in the same biological system using immunofluoresce. H3-K9 methylation, which is mainly associated with inactivation of genes (Lachner and Jenuwein, 2002), has been studied in detail during porcine oocyte maturation (Endo et al., 2005; Bui et al., 2007). Endo et al. (2005), described the pattern of H3-K9triMe as a constant epigenetic modification throughout the porcine oocyte maturation. H3-K9Me was studied by Bui et al. (2007), it was described the presence of this epigenetic modification in oocyte recovered from early antral follicles with absence of signal in earlier follicular stages. Our results in bovine oocytes show H3-K9diMe as a epigenetic modification that persist during maturation, only a light decrease of the signal was observed after GVBD stage. The signal in all the stages was similar in both populations of oocytes, suggesting that at least during maturation the higher expression of SUV39H1 and G9A mRNAs found in oocytes from big follicles was not enough to produce a difference in the methylation pattern. Further studies are necessary for describing the dynamics of the enzymes and their activities in each stage. The signal was present in GV stages of oocytes recovered from small antral follicles and large antral follicles, suggesting that this modification is already established in small antral follicles. The persistence of H3-K9diMe during maturation that we observed in bovine oocytes and the previous observations in pig oocytes (Endo et al., 2005; Bui et al., 2007) could be involved in maintaining a

defined expression profile during the final phase of oocyte growth and the maturation process.

Because of the important role of histone acetylation in the regulation of chromatin structure and gene expression, we have studied the pattern of H4-K12 acetylation during maturation. H4 are hyperacetylated in active genes (Grunstein, 1997), opposite to H3-K9diMe that regulates by repression. Previous studies have shown the dynamics of H4-K12 acetylation during maturation of mouse oocytes (Kim et al., 2003; Akiyama et al., 2004), pig oocytes (Endo et al., 2005) and recently sheep oocytes (Tang et al., 2007). Mouse oocytes showed a strong signal at GV stage and it decreased drastically at GVBD stage, it was undetectable from GVBD to MII stage (Kim et al., 2003) except in anphase I (AI)/ telophase I (TI) and in the first polar body (Akiyama et al., 2004) that a stronger signal was found. In pig oocytes the dynamics differed from mouse oocytes, as in the pig, H4-K12 acetylation signal decreased in MI and MII stages otherwise it was still detectable in these stages (Endo et al., 2005). The signal was higher in AI/TI as it was described for mouse oocytes. Sheep oocytes showed the same pattern as pig oocytes but in sheep oocytes the signal was strong in MII stage (Tang et al., 2007), what differs from mouse and pig oocytes. In bovine oocytes we found a strong signal in GV stages and GVBD, a markedly decrease in MI and MII that made the signal almost undetectable. The dynamics of H4-K12 acetylation in the bovine is close to the pig in GV and GVBD and quite similar to the mouse in MI and MII. We did not find differences between oocytes recovered from

the two categories of follicle size studied, what suggests that in addition to H3-K9diMe, this epigenetic modification is also established in small antral follicles as GV stages from both types of oocytes showed the same results. According to the role of histone acetylation in gene expression activation, it can be expected to decrease during oocyte maturation as transcription is not possible after chromatin condensation (GVBD) otherwise it has been observed signal in MII stage of sheep oocytes and the intensity was comparable to that observed in GV stage (Tang et al., 2007). Thus, each specie seems to be under its own regulation mechanisms of gene expression in spite of some similarities that can be found.

The signal of H4-K12 acetylation was strong in GV and GVBD when transcription is still possible during oocyte maturation; and it was stronger compared to H3-K9diMe in the same stages. We hypothesized an important role of H4-K12 acetylation during oocyte maturation.

In conclusion, we have analyzed the mRNA expression profile of a panel of five genes (G9A, SUV39H1, DNMT1, DNMT3b and ZAR1) and show that it was significantly affected by follicle size (G9A, SUV39H1 and DNMT1) and the maturation stage (ZAR1). We demonstrate for the first time the dynamics of the genes analyzed in the present study during bovine oocyte maturation as well as the dynamics of H3-K9diMe and H4-K12 acetylation.

## **Capítulo 5**

### **Resumen y consideraciones finales**

La maduración de los ovocitos es un proceso fundamental en la preparación de las gametas para la fecundación y el desarrollo embrionario temprano. Incluye numerosos eventos altamente regulados por factores extraovocitarios, así como también, factores propios del ovocito. Dada la importancia económica del bovino se lo ha utilizado como modelo para ampliar el conocimiento en este tema. Se han estudiado las abundancias relativas de ARNm y la distribución de proteínas que aún no habían sido descriptos durante la maduración ovocitaria bovina y su asociación con la capacidad de desarrollo. En coincidencia con estudios previos, los ovocitos bovinos han sido recuperados de folículos de dos tamaños distintos (< 2 mm y 2 - 8 mm) que mostraron diferente capacidad de desarrollo *in vitro* luego de la fecundación.

La maduración ovocitaria implica la maduración nuclear, esto es, alcanzar el estadio de metafase II, lo que resulta relativamente fácil de conseguir en ovocitos que se maduran *in vitro*. Sin embargo, numerosos estudios y este trabajo han demostrado que los ovocitos bovinos recuperados de folículos pequeños alcanzan el final de la maduración nuclear pero desarrollan con dificultad luego de la fecundación. Entonces, resulta necesario considerar la maduración citoplasmática que incluiría la síntesis y localización de ciertos ARNm y proteínas, la fosforilación de factores

específicos, así como también, la redistribución de organelas y el citoesqueleto, todos los cuales son regulados espacio-temporalmente. Esta maduración se trata de una sumatoria de eventos que requieren de una alta regulación y coordinación para que sea llevada a cabo exitosamente. En esta tesis se han estudiado aspectos celulares y moleculares de la maduración de ovocitos bovinos provenientes de folículos de diferente tamaño y su relación con la capacidad de desarrollo ovocitaria. A continuación se resumen los resultados obtenidos en esta tesis:

I) Efectos del tamaño folicular y el estadio meiótico sobre la expresión de ARNm en ovocitos bovinos madurados in vitro.

En una primera serie de experimentos se han semi-cuantificado los ARNms de siete genes, tres motores moleculares (DYNLL1, DYNC1I1 y DCNT1), dos subunidades de los 20S proteasomas (PMSB1 y PMSB4), la enzima responsable del proceso de poliadenilación de ARNm (PAP) y una proteína conectora intercelular (Cx43), durante la maduración de ovocitos bovinos. Los ARNms de DYNLL1, DYNC1I1, DCNT1, PMSB1 y PMSA4 han sido semi-cuantificados por primera vez en ovocitos a través del diseño de primers y la obtención de las condiciones específicas de detección de estos transcriptos.

Demostramos la expresión dinámica de los genes DYNLL1, DYNC1I1 y PMSB1 durante la maduración ovocitaria bovina y el efecto del tamaño folicular. Mientras que, la expresión de los transcriptos de los genes DCNT1, PMSB4, PAP y Cx43

permanecieron constantes durante la maduración ovocitaria y no se observó un efecto del tamaño folicular.

Los resultados obtenidos nos llevaron a concluir que DYNLL1, DYNC1I1 y PMSB1 son posibles marcadores de calidad ovocitaria entre los ovocitos recuperados de los dos tamaños foliculares estudiados. Ya que, los niveles de expresión de los ARNm de DYNLL1 y DYNC1I1 fue mayor en ovocitos provenientes de folículos de 2 - 8 mm. Además, difirieron las dinámicas de los tres transcriptos entre las poblaciones de ovocitos.

## II) Eventos citoplasmáticos en ovocitos bovinos madurados in vitro.

Se han detectado en ovocitos bovinos la distribución de las proteínas motoras dynein (DYNC1I1) y su cofactor dynactin (DCNT1), así como también de los 20S proteasomas. Las proteínas motoras, dynein y dynactin se distribuyen de manera diferente durante las primeras etapas de la maduración ovocitaria en ovocitos provenientes de ambos tamaños foliculares. La distribución de dynactin en GVBD se vió afectada por el tamaño folicular. Hacia el final de la maduración estas proteínas colocalizan con el huso meiótico tanto en metafase I como en metafase II en ambas poblaciones de ovocitos, con diferencias en la intensidad de la señal de dynein en MI y de dynactin en MI y MII, siendo menor en los ovocitos provenientes de folículos pequeños comparado con los recuperados de folículos grandes.

El transporte dependiente de dyneins resulta fundamental para la progresión de la meiosis en ovocitos bovinos, lo cual se demostró mediante el uso del inhibidor

específico de la actividad ATPásica de estas proteínas, ortovanadato de sodio, bloqueándose la maduración con el uso del mismo.

Los 20S proteasomas están presentes en el núcleo de los ovocitos inmaduros y cercanos al ADN durante la maduración ovocitaria, sugiriendo su rol en la progresión de la maduración. A pesar de esto, no hemos encontrado un efecto del tamaño folicular.

El análisis de distribución protéica de dynein y dynactin en ovocitos recuperados de folículos pequeños y grandes demuestra que estas proteínas motoras son posiblemente importantes reguladores de eventos que suceden durante la maduración ovocitaria y que están relacionados a la adquisición final de la capacidad de desarrollo de ovocitos bovinos.

### III) Expresión de ARNm de genes de reprogramación y modificaciones de histonas durante la maduración in vitro de ovocitos bovinos.

Finalmente, se ha descripto el patrón de expresión de los ARNm de cinco genes responsables de modificaciones epigenéticas, dos histonas-metiltransferasas (G9A y SUV39H1), dos ADN metiltransferasas (DNMT1 y DNMT3b) y el factor de reprogramación ZAR1, durante la maduración ovocitaria. Hemos demostrado diferencias en las abundancias relativas de los ARNm de G9A, SUV39H1 y DNMT1, siendo mayor en ovocitos provenientes de folículos de 2 - 8 mm y diferencias en la dinámica de la expresión de ZAR1 entre las poblaciones de ovocitos en estudio. La expresión del ARNm de DNMT3b resultó estable durante la

maduración en ambas poblaciones de ovocitos y su abundancia relativa no se vió afectada por el tamaño folicular.

Como la expresión de los ARNm de G9A y SUV39H1 fue mayor en ovocitos recuperados de folículos grandes, complementamos este estudio con la detección del efecto final de los productos de los genes G9A y SUV39H1, esto es, la dimetilación de lisinas 9 de las histonas H3. Los resultados mostraron que las diferencias encontradas en los ARNm de G9A y SUV39H1 no se vieron reflejadas en una diferencia en el patrón de metilación de las histonas H3 durante la maduración de ovocitos provenientes de ambos tamaños foliculares.

Teniendo en cuenta que los productos de genes de regulación epigenética tienen un rol fundamental durante el desarrollo embrionario temprano, no se puede descartar que las diferencias halladas en los ARNm de este grupo de genes estudiados no vayan a influenciar el desarrollo embrionario temprano luego de la fecundación. Especialmente los productos de los genes G9A y ZAR1, cuyas funciones se han descripto como fundamentales para la progresión del desarrollo embrionario temprano.

La dimetilación de H3-K9 en general está asociada a la represión de la expresión génica, en tanto que la acetilación de H4-K12 está asociada a la activación de la expresión génica. Durante la maduración de los ovocitos bovinos se observó que la dimetilación de H3-K9 disminuyó levemente luego de GVBD y se mantuvo hasta el final de la maduración ovocitaria en ambas poblaciones de ovocitos. Contrariamente, en la acetilación de las H4-K12 demostramos una fuerte reducción

de esta modificación luego de la primera condensación de la cromatina (GVBD), en ambas poblaciones de ovocitos. Estas modificaciones acompañan los cambios que se dan en la represión y/o activación de la expresión génica durante la meiosis. Los mismos patrones fueron observados en el estadio de GV en ambos tipos de ovocitos, lo cual demostró que estas modificaciones están presentes ya en folículos antrales pequeños.

En resumen, de la hipótesis planteada confirmamos que existen diferencias a nivel molecular y celular entre los ovocitos bovinos provenientes de folículos pequeños (baja capacidad de desarrollo) comparado con los provenientes de folículos grandes (alta capacidad de desarrollo) en cuanto a:

- Niveles de los ARNm de los motores moleculares (DYNLL1 y DYNC1I1), una subunidad de los 20S proteasomas (PMSB1), las histonas-metiltransferasas (G9A y SUV39H1), una ADN metiltransferasa (DNMT1) y el factor de reprogramación ZAR1.
- Las distribuciones de las proteínas motoras dynein y dynactin.

Estas diferencias encontradas entre los dos tipos de ovocitos muestran la asociación de los factores ooplasmáticos estudiados con la adquisición de la capacidad de desarrollo ovocitaria bovina. No se observaron diferencias en las modificaciones epigenéticas de las histonas estudiadas.

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